

Compendium of Transgenic Crop Plants

Volume



Transgenic Cereals and Forage Grasses



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Rice

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Rice is Life

—Theme of UN International Year of Rice, 2004

*Cutting stalks at noon time Perspiration drips to the earth
Know you that your bowl of rice Each grain from hardship comes?*

—Cheng Chan-Pao, Chinese Philosopher

1. INTRODUCTION

Rice serves as the principal source of nourishment for over half of the global population and vies with wheat and maize as the most important cereal crop. As a symbol of life, prosperity, fertility, and self-sufficiency, it is deeply embedded in the cultural heritage of many societies. The global importance of this crop reached its pinnacle on the occasion of the 57th session of the United Nations General Assembly, when the Food and Agricultural Organization declared the year 2004 as the International year of Rice (FAO, 2004). This unprecedented step in United Nation's history of devoting a year to a commodity was based on the need to heighten awareness of the role of rice in alleviating global poverty and malnutrition. The declaration was also aimed at "the need to focus world attention on the role that rice can play in providing food security and eradicating poverty in the attainment of the internationally agreed development goals" (UN Declaration, 57th Session, 2004).

Rice accounts for more than one-fifth of the calories consumed by human beings in their global diets (Smith, 1998). In Asia, rice and its derivatives account for 60–70% of energy intake for over 2 billion people (FAO, 2004). As an important commercial crop, it is the most rapidly growing food source in Africa and is of significant importance to food security in an increasing number of low-income food-deficit countries. Rice-based production systems and their associated postharvest operations employ nearly 1 billion people in rural areas of developing countries, and about four-fifths of the world's rice is grown by small-scale farmers in low-income countries (FAO, 2004). The production of 700 million metric tons (FAO data for 2005) makes rice the world's largest seed crop, closely followed by maize and wheat.

1.1 History, Origin, and Distribution

The word *rice* is believed to be of Indo–Iranian origin, the term being derived from the Tamil word

Arisi and the Arabic *Ar-ruzz*. The English origins are attributed to Greek *Ōryza*, via Latin *Oriza*, Italian *Riso*, and the Old French *Ris* (Wikipedia, 2007).

Cultivation of rice is thought to have originated along the Yangtze Delta, one of the cradles of East Asian civilization in China. Paleobotanical hypothesis credits the Chinese of the Late Pleistocene era with collecting wild rice, leading to its eventual domestication about 6400 BC (Zhao, 1998). Early Neolithic groups are also known to have cultivated rice, possibly as early as 9000 BC with radiocarbon dating evidence going back to at least 7000 BC (Crawford and Shen, 1998; Diamond, 1999; Zohary and Hopf, 2000).

The oldest scriptures in India mention rice (the term used was *Dhanya*), rice dishes, and aspects of rice cultivation (Nene, 2005). Farmers practiced mixed farming techniques planting the rice crop in integrated fields during the Indus Valley Civilization (Kahn, 2005). Wild rice appeared as early as 5440 BC in the Belan and the Ganges valleys of northern India. Along with barley, meat, dairy products, and fish, rice was a dietary staple of ancient Dravidian society (Taylor, 2004).

Dryland rice was introduced to Japan and Korea between 3500 and 1200 BC (Crawford and Lee, 2003). Wetland cultivation techniques migrated to Indonesia around 1500 BC and then to Japan by 100 BC. The Niger River delta extending to Senegal was the rice farming region in Africa during 1500–800 BC. The Moor invasion of the Iberian Peninsula in 700 AD introduced rice to Spain, from whence it spread during the 15th century through Italy and France to all continents during the great age of European exploration (Wikipedia, 2007). Rice arrived in North America in 1694, from Madagascar to South Carolina. Plantation owners in Georgetown, Charlestown, and Savannah learnt techniques of rice culture such as dyking of marshes and periodical flooding of fields from African slaves, who also brought with them rice mills made of wooden paddles and winnows made of sweetgrass baskets. Subsequent improvements in rice production can be attributed to the invention of rice mill and the addition of waterpower to these mills. The predominant strains of rice in Carolina were christened “Carolina Gold” (Wikipedia, 2007).

Plants of the genus *Oryza* are known to thrive in desert, hot, humid, flooded, dry, and cool

conditions, and grow in saline, alkaline, and acidic soils. There are 24 species of *Oryza*. Domesticated or cultivated rice comprise two main species, the Asian rice *Oryza sativa*, and the African rice *Oryza glaberrima*. *O. sativa* was domesticated from wild Asian rice, *Oryza rufipogon*, originating in the foothills of the Himalayan mountain ranges, with *O. sativa* var. *Indica* of India and *O. sativa* var. *Japonica* from China and Japan (Londo *et al.*, 2006). *O. sativa* cultivars consist of three groups: the short-grained “*Japonica*” or “*Sinica*” (e.g., Japanese rice); long-grained “*Indica*” varieties (e.g., *Basmati* rice); and the broad-grained “*Javanica*” rice (e.g., Jefferson rice) (Zohary and Hopf, 2000).

Originating from its Asian homeland, rice shows a diverse distribution and is cultivated in 113 countries and 6 continents (none in Antarctica!). Rice is grown under a wide range of soil moisture regimes, from deep flood to dry land, and in various soil conditions. Rice-based production systems span from 53°N, in the Heilongjiang Province of China, to 35°S in New South Wales of Australia; from the tropical rain forest climate of the Congo to the continental temperate climate in Krasnodar of Russia; from the arid desert climate found in Egypt’s Nile Delta, to the sea-level regions in Guinea-Bissau, to 2700m above sea level as in the Himalayan mountain chains in Nepal and India (FAO, 2004).

1.2 Botanical Description

1.2.1 Scientific classification

Kingdom: Plantae
 Division: Magnoliophyta
 Class: Liliopsida
 Order: Poales
 Family: Poaceae
 Genus: *Oryza*
 Species: *O. glaberrima* and *O. sativa*

1.2.2 The plant

Rice is a semiaquatic, monocarpic annual grass plant, its height varying from 0.6 to 1.8 m (2–6 ft.) tall. The plants tiller, i.e., develop multiple shoots, depending on the variety, spacing, and soil fertility.

The grass has long, slender leaves 50–100 cm long and 2–2.5 cm broad. The small wind-pollinated flowers are produced on a branched arching to pendulous inflorescence 30–50 cm long. The inflorescence is an open panicle. Flowers are distinct in having six anthers as opposed to the commonly seen three anthers in other grasses. Spikelets have a single floret, lemma, and palea enclosing a grain (caryopsis) 5–12 mm long and 2–3 mm thick that can be yellow, red, brown, or black. The lemmas may be awnless, partly or fully awned. The rice kernel has four primary components: the hull or the husk, the seed coat or bran, the embryo or germ, and the endosperm. Rice milling procedures yield a variety of products depending on the extent of outer layer removal. If just the inedible husk is removed, it results in brown rice where, the nutritious high-fiber bran is a source of 8% protein, iron, calcium, and B vitamins. Removal of the bran and germ results in white or polished rice, which is greatly diminished in nutrients.

1.2.3 Habit and habitat

The ideal climate for rice growth is 75 °F (24 °C). Its growth cycle is 3–6 months. In nonindustrialized nations, rice fields are typically prepared by ploughing (by cattle-drawn ploughs or a tractor), fertilizing (traditionally with dung or sewage), and smoothing (a process of dragging a log across the field). Seedlings are prepared in seedling beds, and after a month, are transplanted manually to the fields, which have been flooded by rain or river water. Dike-controlled canals or manual watering maintains irrigation during the entire growth period. Before harvesting the crop, the fields are allowed to drain.

1.2.4 Genome

Evolutionary lineages of major flowering plants, monocots, and dicots, as illustrated by rice and *Arabidopsis*, were shown to have diverged about 200 million years ago. The genomes of these two plants do not share extensive synteny, but similarities exist amongst many encoded proteins. Rice genes diverge from *Arabidopsis* in guanine-

cytosine (GC) content, codon usage, and amino acid usage. There is also a gradient in the GC content of rice genes that is not seen in *Arabidopsis* genes, with the 5' end being up to 25% richer in GC content than the 3' end.

The draft genome sequences of both *Indica* rice (Yu *et al.*, 2002) and *Japonica* rice (Goff *et al.*, 2002) were published in the same year. These analyses gave an average gene size of 4.5 kb with some 513 ribosomal RNA gene repeats and 688 centromeric repeats. The average rice genome is a compact 430 Mb, one-sixth the size of the human and maize genomes (Leach *et al.*, 2002). It is the smallest of all genomes amongst grasses, commonly grown as crops. The smaller genome is reflected in a higher gene density relative to other cereals, with an average of one gene every 15 kb (Goff, 1999). However, the overall organization of genes is preserved in such a way that rice genomic information can be used as a useful guide in deciphering the larger genomes of maize and barley. The genomic similarity makes it possible to approximate the genomes of these grain species in a concentric circle, and use the smaller rice genome as a guiding point to find related genes of interest in the larger genomes (Figure 1).

The characteristics of rice genome are summarized in Table 1 (Goff *et al.*, 2002; Yu *et al.*, 2002).

Figure not available

Figure 1 Genome colinearity among cereals [Reproduced from Rice Genome Poster (2002)]

Table 1 Salient features of the rice genome^(a)

Rice strain	Indica	Japonica
Genome size (Mb)	466	420
Number of genes	46–56 K	32–50 K
Duplicated genes (%)	74	77
Number of transposable elements	>24.9%	4220
Number of single sequence repeats	1.7%	46 666

^(a)Reproduced from the Rice Genome Poster (2002)

1.2.5 Cytological features

O. sativa L. has a chromosome number of $2n = 24$ (Kuwada, 1910). The DNA content was determined to be 0.87–0.96 pg/2C (Martinez *et al.*, 1993). In contrast to the rapid progress in molecular analysis, progress in cytological characterization of the rice genome has had limited success. Early studies using fluorescent *in situ* hybridization techniques to map DNA sequences on chromosomes (Fukui *et al.*, 1994; Jiang *et al.*, 1995; Ohmido *et al.*, 1998) involved mapping on mitotic metaphase chromosomes. This approach provided limited details due to low mapping resolution. Cheng *et al.* (2001a) successfully used pachytene chromosome karyotyping by hybridizing 24 bacterial artificial chromosome (BAC) clones and a rice centromere DNA-specific probe with all 24 chromosomal arms. The arm-specific BAC clones unambiguously identified rice chromosomes in both mitotic and meiotic cells at $2n = 24$. The longest was chromosome 1 (61.12 μm) and the shortest was chromosome 10 (24.74 μm), the descending order of chromosome length being 1, 3, 2, 6, 4, 5, 7, 8, 11, 9 (not including the ribosomal DNA (rDNA)), 12, and 10. Heterochromatic regions that have a high AT content relative to euchromatic regions were preferentially stained by DAPI (4', 6-diamidino-2-phenylindole). Chromosomes 4 and 10 have the most distinct heterochromatic patterns, with one-third of the chromosome, including the entire short arm and part of the long arm, being highly heterochromatinized. Other heterochromatic regions were the pericentric region of chromosome 5, the distal region of the long arm of chromosome 11, and the short arm and pericentric region on the long arm of chromosome 10. The three longest chromosomes (1, 2, and 3) are more euchromatic than the others.

1.3 Economic Importance

1.3.1 Production and trade

Rice has a key role in the food security of the world. In 2005, world rice production reached a record level of 621 million tons (FAO, 2005). Much of the expansion was concentrated in Asia, with mainland China boosting production by 6 million tons compared to the previous year. Large increases were also reported from Bangladesh, India, Myanmar, Pakistan, Sri Lanka, and Thailand. Developing countries account for 95% of the total world rice production, 83% of exports, and 85% of imports, with China and India accounting for more than 50%. According to FAO fact sheets, global trade in rice grew at 7% a year throughout the 1990s.

The top 10 rice-producing countries in terms of metric tons are as in Table 2 (FAO, 2004).

1.3.2 Nutrition

Rice provides 20% of the world's dietary energy supply, and is followed by wheat (19%) and maize (5%) (FAO, 2004). Rice is a good source of thiamine, riboflavin and niacin. Brown rice is a good source of dietary fiber. The overall amino acid profile for rice seed shows high values for glutamic and aspartic acid, with lysine as the limiting acid. Rice has a rich genetic diversity with over 1000 varieties and its natural colors include brown, red, purple, and black. Nutrient contents of these varieties are summarized in Table 3.

Rice is an integral part to the culinary traditions of many cultures with personal preferences regarding texture, taste, color, and stickiness. Dry

Table 2 Top 10 rice producers^(a)

Country	Metric tons
1. China	166.0 $\times 10^6$
2. India	133.5 $\times 10^6$
3. Indonesia	51.8 $\times 10^6$
4. Bangladesh	38.0 $\times 10^6$
5. Viet Nam	34.6 $\times 10^6$
6. Thailand	27.0 $\times 10^6$
7. Myanmar	21.9 $\times 10^6$
8. Philippines	13.2 $\times 10^6$
9. Brazil	10.2 $\times 10^6$

^(a)Reproduced from FAO (2004)

Table 3 Nutrient contents of rice varieties^(a)

Type of rice	Protein (g/100 g)	Iron (mg/100 g)	Zinc (mg/100 g)	Fiber (g/100 g)
White	6.8	1.2	0.5	0.6
Brown	7.9	2.2	0.5	2.8
Red	7.0	5.5	3.3	2.0
Purple	8.3	3.9	2.2	1.4
Black	8.5	3.5	—	4.9

^(a)Reproduced from FAO Rice Fact Sheet: Rice and Human Nutrition (2004)

flaky rice is preferred in South Asia and the Middle East; moist sticky rice in Japan, Taiwan, Korea, Egypt, and northern China; red rice and long-grained scented rice in India. Many countries have signature recipes such as *sushi* (Japan), fried rice, *pulav*, and *biryani* (India), *paella*, *risotto*, and *pancīt* (Italy).

1.4 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

The science of plant breeding has been developed mainly in the 20th century. However, as a practice it goes back thousands of years when art and science as we know them now, were a unified learning process for enhanced survival. Archaeological finds in India and China indicate the beginning of rice cultivation from earlier than 10 000 years ago (Chang, 2000). Starting from India and China, the dispersal and selection of ancient varieties of *O. sativa* in South-East Asia, Austronesia, and Africa date back to 5000 years. At present, rice is the staple food of nearly half of the world population. Nearly 90% of all rice is grown in Asia with China and India together accounting for nearly one-third of the total global production of 618 million tons in 2005 (International Rice Research Institute, 2006). Only a little more than 5% of the global rice production is traded in international markets indicating its importance in feeding the domestic population. Since after the Second World War, there has been an exponential growth in population and in the number of people below poverty line in Asia and Africa. Hence, through major international research initiatives, rice breeding efforts have targeted the increase of rice yield. The Rice Development Program of FAO recommends “increases in rice production in all

ecologies—from high altitude to coastal areas and from temperate to tropical climates.” Various rice breeding efforts globally have directly or indirectly aimed at increasing the rice yield to support the unprecedented growth in population.

1.4.1 Breeding objectives

1.4.1.1 Abiotic stress resistance

This includes drought, salinity, waterlogging, cold and frost, soil pH, mineral deficiency, and toxicity.

1.4.1.2 Biotic stress resistance

This includes the following:

- (a) Major pests such as yellow stem borer (*Scirpophaga incertula*), gall-midge (*Orseolia oryzae*), brown planthopper (*Nilaparvata lugens*), white-backed planthopper (*Sogatella furcifera*), green leafhopper (*Nephotettix*), striped stem borer (*Chilo suppressalis*), rice leaf folder (*Cnaphalocrocis medinalis* and/or *Marasmia exigua*).
- (b) Major pathogens such as bacterial blight (*Xanthomonas oryzae*), sheath blight (*Rhizoctonia solani*), fungal blast (*Magnaporthe grisea*), fungal stem rot (*Sclerotium oryzae*), root-knot nematode (*Meloidogyne*), rice dwarf virus, rice grassy stunt virus (RGSV), rice hoja blanca virus (RHBV), rice yellow mottle virus (RYMV), and rice tungro virus (RTD).

1.4.1.3 Other agronomic traits

Other major agronomic traits include: lifecycle time, weed competitiveness, nitrogen (and other

macronutrient) uptake and utilization, plant height, number of tillers and panicles, photoperiod sensitivity, flowering time, wide compatibility, male sterility and/or self-incompatibility, fertility restoration, grain number, weight, size, shape, fragrance, composition, and nutritional quality including starch, proteins, macro- and micronutrients.

1.4.2 Breeding tools and strategies

According to Khush and Brar (2002) the tools and strategies used to achieve the above objectives can be divided into two phases, the evolution phase or broadening the gene pool of rice cultivars, whereby rice populations are created that can serve as selection stocks and the evaluation phase or increasing selection efficiency, whereby superior genotypes are selected.

1.4.2.1 Evolution phase

The following approaches have been used to expand the genetic pool of rice:

- (a) *Wide hybridization* refers to creating hybrids between the cultivated species of *O. sativa* containing the AA genome and any one of the wild variety represented by 24 species containing any one of the 10 possible genome compositions (AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ and HHKK). The strategy of embryo rescue has been successfully used to transfer a number of genes for resistance to insect pests and pathogens from the wild species into the cultivated species creating nuclear genome diversity (Jena and Khush, 1990; Khush *et al.*, 1990; Brar and Khush, 1997). Additionally, Lin and Yuan (1980) created cytoplasmic diversity when they successfully used the nucleus of the cultivated *O. sativa* and cytoplasm of the wild *O. sativa* L. f. *spontanea*. This hybrid has been the source of cytoplasmic male sterility in the highly successful Chinese hybrid rice programs.
- (b) *Somaclonal variation* refers to genomic changes created by dedifferentiation and

regeneration cycle of the tissue culture protocols. It serves, as a method for natural selection of mutations since *in vitro*-regenerated plants must retain genome functionality to give rise to a largely normal plant. The technique was successfully used to create a variety with superior cooking qualities and increased resistance to rice blast (Araujo and Prabhu, 2002).

1.4.2.2 Evaluation phase

The following methods are used for efficiently selecting for desired characteristics:

- (a) *Field-based evaluation* on hybrids or variants generated through embryo rescue or tissue culture, respectively was the main route of selection of desired phenotype in the conventional breeding methods. However, the process of evaluating and selecting the desired phenotype also now starts in the laboratory, for example, anther culture can be used to generate doubled haploid (DH) plants that serve as true breeding lines thus shortening the time toward generating a new variety (Khush and Brar, 2002). Selection efficiency of DH lines is higher if dominance variation as evidenced through a number of new varieties developed using DH lines (Khush and Brar, 2002).
- (b) *Molecular genetics based evaluation* is now the norm since DNA markers have highly facilitated the tracking of characters through crosses and generations. Although the methods have not been extensively used on wide hybrids or somaclonal variants, they have been very useful in tagging and tracking rice fungal blast and bacterial blight resistance quantitative trait loci (QTLs) through various crosses (Young, 1996). Various molecular markers are now available for use such as restriction fragment length polymorphism, amplified fragment length polymorphism, random amplified polymorphic DNA, simple sequence repeats, single nucleotide polymorphisms, etc. Semagn *et al.* (2006) provide a good review of the different molecular markers used in plant breeding, the

principles on which these are based, limited information on laboratory protocols and the advantages of each. Additionally, Khush and Brar (2002) provide a detailed account and examples of how rice breeding efforts have been positively affected by efficient selection using molecular marker technology, molecular maps, synteny relationships, marker-aided selection, QTL mapping, gene tagging and gene pyramiding, map-based cloning, and functional genomics.

Laboratory based techniques, such as the wide hybridization and embryo rescue, anther culture, somaclonal variation, and molecular markers may have indeed hastened the process of selection and reduced the time to generating a new variety, yet substantial advances and achievements were made using classical breeding systems of pedigree and bulk methods, three-way remote crosses, recurrent parent, and rapid generation advancement. The chromosomal deletion lines, recombinant inbred lines, near isogenic lines, and pure lines were made available through sheer diligence and perseverance in the fields. These methods were facilitated then by ethylmethane sulfonate (EMS)- and radiation-mediated mutation. Creation of semidwarf varieties, resistance to certain pests and pathogens, and maintaining cytoplasmic and genic male sterility were all achieved through classical breeding. By mid 1980s the rice production was double that of mid 1950s. Nearly half that improvement was due to additional land being cultivated but the other half was due to high-yielding varieties developed through conventional breeding methods. The achievements of conventional plant breeding have been reviewed at regular intervals (Bingham, 1981; Duvick, 1986; Reeves and Cassaday, 2002).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

A major constraint of conventional breeding approaches is the nonavailability of desirable genes within the gene pool, for example in rice, there is limited variability for resistance to diseases such as sheath blight, stem borer, and other insect pests, bacteria and fungi contain genes,

such as the *Bacillus thuringiensis* crystal proteins (*Bt Cry*) and chitinase, respectively, which can be useful against pests and pathogens. Transferring genes from one kingdom to another invokes for transgenic approaches. A variety of *Bt* genes have been transferred to rice using electroporation, biolistic or *Agrobacterium*-mediated transformation methods (Fujimoto *et al.*, 1993; Wunn *et al.*, 1996; Ghareyazie *et al.*, 1997; Nayak *et al.*, 1997; Cheng *et al.*, 1998; Maqbool *et al.*, 1998, 2001; Tu *et al.*, 2000). In cases when genes are available within the gene pool of rice for transfer to cultivated varieties, conventional breeding methods suffer from drawbacks such as hybridization barriers, low selection efficiency, and long breeding cycles that take a long time to generate a new variety. Transgenic approaches along with methods such as anther culture and marker-assisted breeding can overcome these limitations. Additionally, the genes available within the gene pool may not be expressed at desirable levels or may not have the desirable expression patterns, thus needing modification of regulatory elements. Such modifications and the downstream incorporation of the gene into the genome are possible only through transgenic approaches. For example, the endogenous rice chitinase gene was modified to constitutively express at higher than endogenous levels under the CaMV 35S promoter, conferring enhanced resistance to sheath blight (Lin *et al.*, 1995).

Introducing designer characteristics in modifying the nutritional quality and quantity of rice grains or having genes expressed at specific time and place of the plant life cycle also calls for transgenic approaches. For example, in the case of “Golden Rice”, enriching the rice grains with β -carotene was possible by expressing three genes responsible for provitamin A synthesis (Ye *et al.*, 2000). Rice anther specific expression of a gene isolated from anther complementary DNA (cDNA) was confirmed through transgenic plants. The gene is responsible for male fertility and hence, important in hybrid rice programs (Luo *et al.*, 2006).

Molecular markers have fast-tracked plant breeding. Similarly, molecular approaches to creating mutant lines using transgenic approaches with transposons or transfer DNA (T-DNA) have fast-tracked mutation detection. Either a known

gene of interest is examined for function and/or expression patterns or novel genes are identified through this approach. The added advantage of these approaches is that a genome saturated insertion-mutation library can be created and stored. Such a library represents a population of seeds with insertion mutation in every single gene, ideally one gene mutation per seed, so that any changes in the plant arising from such a seed can be tracked to changes in that one mutated gene. Such libraries have been created for rice using an endogenous retrotransposon (Hirochika, 2001; Miyao *et al.*, 2007), heterologous maize *Ac* or *Ac/Ds* transposon (Enoki *et al.*, 1999; Kohli *et al.*, 2001, 2004; Kolesnik *et al.*, 2004; Upadhyaya *et al.*, 2006), and the T-DNA (Jeon *et al.*, 2000; Chen *et al.*, 2003; Sallaud *et al.*, 2003; An *et al.*, 2005a, b; Fu *et al.*, 2006; Liang *et al.*, 2006). A number of novel genes have been identified using insertional mutations. Recently, a dwarf mutant gene was identified from the T-DNA tagged population (Zhou *et al.*, 2006). In a comprehensive review on advances in rice biotechnology, Kathuria *et al.* (2007) list other genes identified and isolated through molecular tagging approaches. These include genes involved in gibberellic acid biosynthesis (Margis-Pinheiro *et al.*, 2005), blast resistance (Kim *et al.*, 2003), tapetum development (Jung *et al.*, 2005), and pollen development (Han *et al.*, 2006).

2. DEVELOPMENT OF TRANSGENIC RICE

In the 1960s, doubling of the world's population to more than 6.4 billion, together with a drastic decrease in cultivated land led to a global food crisis. However, a Nobel Peace Prize winning effort by Norman E. Borlaug, now christened "the green revolution", averted a large-scale famine in the semi-arid tropics of South Asia, specifically in India, Pakistan, the Philippines, and Mexico. The introductions of high-yielding semidwarf varieties of wheat and rice, in combination with applications of large amounts of nitrogen fertilizer to increase grain yields and promote leaf and stem elongation, was crucial for the success of the first green revolution (Sakamoto and Matsuoka, 2004). The situation now comes full circle as the world population, estimated to

touch 8 billion by 2025, again looks out for strategies to increase grain production. The second green revolution calls for genetic engineering of food crops (Sakamoto and Matsuoka, 2004), and advances in transgenic rice production would be extremely beneficial toward achieving the needed goals. Parameters identified to be associated with increases in yield potential are plant height, tiller number, photosynthesis, photoperiod insensitivity, and disease and insect resistance (Khush, 1999). The green revolution genes, namely the wheat green revolution gene *Reduced height1* (*Rht1*) and the rice green revolution gene *semidwarf1* (*sd1*) have been identified and have been found to encode mutant gibberellin response modulators (Peng *et al.*, 1999). Genetic manipulations of gibberellin (GA) levels thus would prove to be a promising first step to generate semidwarf varieties that, in combination with artificial control of tillering, would lead to a dramatic increase in the yield potential of rice (Sakamoto and Matsuoka, 2004). Commercial use of transgenic rice is far less than that of transgenic maize that is now planted on more than 15 million hectares around the world (James, 2003). The ambitious 16-year-long program of the Rockefeller Foundation for rice biotechnology was a key factor in positioning rice biotechnology to be advanced to the domains of public and federal funding (O'Toole *et al.*, 2001). Sequencing of the rice genome provides added impetus to advances in transgenic rice technology, and also cements the "model monocot" status of rice. The rapid advances in rice biotechnology, starting nearly two decades ago with the production of the first transgenic rice plants (Toriyama *et al.*, 1988; Zhang and Wu, 1988) have resulted in the development of high-throughput, reproducible transformation protocols in the crop. Transgenic rice plants with improved tolerance to biotic/abiotic stresses, improved insect/pest/disease tolerance, and increased nutritional values have been developed (Upadhyaya *et al.*, 2000a; Bajaj and Mohanty, 2005; Kathuria *et al.*, 2007). Rice has also served as a model to understand the process of plant transformation in general, both in terms of transgene integration and modulation of gene expression (Kohli *et al.*, 1998, 1999a, b; Iyer *et al.*, 2000; Cheng *et al.*, 2001b; Kloti *et al.*, 2002; Vain *et al.*, 2002; Kathuria *et al.*, 2007).

2.1 Donor Genes

The need for transgenic technologies to improve the qualitative and quantitative yields and other agronomic characteristics of rice cannot be overstated. Lack of desirable genes within the rice gene pool and a consequent search for these genes in other organisms pushed the frontiers in locating, isolating and cloning heterologous genes, and designing transgene constructs for rice transformation.

The donor genes for rice transformation over the years have been isolated from different kingdoms. Examples abound for multiple genes from each kingdom that have been introduced in rice. Specific examples of a gene or a class of genes introduced in rice from each kingdom, highlighting an important agronomic or technical advance are mentioned in Table 4. The cited references for each of these genes point either to recent research or review papers or to one of the first reports in literature. Additional genes and regulatory sequences introduced in rice and their source organisms are mentioned in different sections of this chapter as directly related to the aim of generating the transgenic plants.

The donor gene sources ranging from viruses to humans, and the aims of transforming rice with these genes can be categorized as in Table 4.

2.1.1 Plant transformation

Increasingly efficient transformation, selection, and screening tools techniques and genes are a prerequisite for generating transgenic rice with improved agronomic characteristics. New selectable marker and reporter genes are continually being explored for their use in efficient selection of transformed cells/tissues or reporting transgene expression status. Increasingly these genes are chosen and designed to be neutral in terms of their substrates and products affecting the plant *per se* or the consumer. In most cases however, the effort now is to generate marker-free transgenic plants. The *hpt* and the *pat* genes have been the most frequently used selectable markers for rice transformations while the *gus* and the *gfp* genes have been the most frequently used reporter genes. Recently Lee *et al.* (2007) have

used the *Myxococcus xanthus* protoporphyrinogen oxidase (MxPPO) as an efficient selectable marker using the peroxidising herbicide butafenacil. Although the MxPPO is only an addition to the herbicide resistance class of selectable markers for rice transformation, it supplies an example of another experimentally tested system in rice. Latest comprehensive reviews on transgenic rice by Bajaj and Mohanty (2005) and Kathuria *et al.* (2007) illustrate the novel selectable markers and reporter genes used. For example, some novel and neutral selectable markers include the phosphomannose isomerase gene (Lucca *et al.*, 2001b), the α -subunit of anthranilate synthase (Yamada *et al.*, 2004); conditional negative selection with cytosine deaminase (Dai *et al.*, 2001a), indole acetic acid hydrolase (IAAH/tms2; Upadhyaya *et al.*, 2000b), aminoglycoside 3-adenyltransferase (*aadA*; Oreifig *et al.*, 2004), and D-amino acid oxidase from yeast (*dao1*; Erikson *et al.*, 2004). Modified *gus*, *gfp*, *luc*, and synthetic *sialidase* and *xylanase* in combination with *gus* used as the latest reporter genes have also been described (reviewed in Bajaj and Mohanty, 2005).

Generating marker-free transgenic rice plants has been accomplished through different strategies. For example, simple molecular analyses of the segregating population for the lack of selectable marker when it integrates separate from the gene of interest. With *Agrobacterium*-mediated transformation, the use of independent constructs to carry the marker gene and the gene of interest increases the frequency of independent integration and hence facilitates segregation in the following generations. Cleverly designed “twin T-DNA” vectors also accomplish the same (Komari *et al.*, 1996; Lu *et al.*, 2001), as does the dual binary vector system of pGreen/pSoup (Vain *et al.*, 2003). Alternatively, molecular excision of the selectable marker was achieved through site-specific recombination using *cre/loxP* (Gleave *et al.*, 1999; Moore and Srivastava, 2006), flipase recombination enzyme (FLP)/flipase recombination target (FRT) sites (Srivastava and Ow, 2004) and/or *Ac/Ds* transposon system (Cotsaftis *et al.*, 2002). Endo *et al.* (2002) used the multiauto transformation (MAT) vector containing the *Agrobacterium* isopentyl transferase (*ipt*) gene as a marker in combination with the *R/RS* site-specific recombination system to generate

Table 4 Donor genes for generating transgenic rice

Kingdom	Source	Gene	Functional category	Reference
Prokaryota	Viruses:	Class of viral genes	Viral disease resistance	Hayakawa <i>et al.</i> (1992); Sivamani <i>et al.</i> (1999); Pinto <i>et al.</i> (1999); Kouassi <i>et al.</i> (2006)
	Rice stripe			
	Rice tungro			
	Rice yellow mottle			
	Baculoviruses and Entomopoxviruses	EPV-fusolin	Insect disease resistance	Hukuhara <i>et al.</i> (1999)
	Eubacteria:	Class of <i>Bt Cry</i> genes	Insect disease resistance	Khush and Brar (2002); Bajaj and Mohanty (2005); Roh <i>et al.</i> (2007)
Protista	<i>Bacillus thuringiensis</i>			
	<i>Escherichia coli</i>	β -glucuronidase	Screenable marker	Kathuria <i>et al.</i> (2007); Terada and Shimamoto (1990)
		Trehalose biosynthetic genes	Abiotic stress resistance	Garg <i>et al.</i> (2002)
		<i>Phosphomannose isomerase</i>	Selectable marker	Lucca <i>et al.</i> (2001b)
	<i>Erwinia uredovora</i>	<i>Phytoene desaturase</i>	β -Carotene synthesis	Ye <i>et al.</i> (2000)
	—	—	—	—
Fungi	<i>Streptomyces</i>	<i>Phosphinothrycin N-acetyltransferase</i>	Selectable marker and herbicide tolerance	Christou <i>et al.</i> (1991)
		<i>Hygromycin phosphotransferase</i>	Selectable marker	Meijer <i>et al.</i> (1991)
Plantae	<i>Aspergillus niger</i>	Chitinase	Fungal disease resistance	Itoh <i>et al.</i> (2003)
		Phytase	Nutritional enhancement	Drakakaki <i>et al.</i> (2006)
	Rice	Class of <i>Xa21</i> genes	Bacterial blight resistance	Song <i>et al.</i> (1995)
		Oryzacystatin	Nematode resistance	Vain <i>et al.</i> (1998)
		<i>Ca₂₊ dependent protein kinase</i>	Cold/salt/drought tolerance	Saijo <i>et al.</i> (2000)
		<i>Pyruvate decarboxylase</i>	Submergence tolerance	Quimio <i>et al.</i> (2000)
		<i>NAC</i> transcription factors	Drought tolerance	Hu <i>et al.</i> (2006)
		<i>Gibberellic acid 2-oxidase</i>	Dwarfism and yield	Sakamoto <i>et al.</i> (2003)
	Cowpea	<i>Cowpea trypsin inhibitor</i>	Insect resistance	Xu <i>et al.</i> (1996)
	Soybean	<i>Soybean trypsin inhibitor</i>	Insect resistance	Lee <i>et al.</i> (1999)
	Barley	<i>Barley trypsin inhibitor</i>	Insect resistance	Alfonso-Rubi <i>et al.</i> (2003)
	Snowdrop	<i>Galanthus nivalis</i>	Insect resistance	Rao <i>et al.</i> (1998); Sudhakar <i>et al.</i> (1998)
	Daffodil	<i>Agglutinin</i>		
		<i>Phytoene synthase</i> and <i>Lycopene B cyclase</i>	β -Carotene (provitamin A) synthesis	Ye <i>et al.</i> (2000)
	Maize	<i>Phytoene desaturase</i>	Higher β -carotene synthesis	Paine <i>et al.</i> (2005)
		Anthocyanin genes	Fungal blast resistance	Gandikota <i>et al.</i> (2001)
		<i>Ribosome inactivating protein</i>	Sheath blight resistance	Kim <i>et al.</i> (2003)
	<i>Arabidopsis</i>	<i>Phytochrome A</i>	Grain Yield	Garg <i>et al.</i> (2006)
		<i>LEAFY</i>	Plant architecture and early flowering	He <i>et al.</i> (2000)
		CBF3/DREB1A/ABF3	Abiotic stress tolerance	Oh <i>et al.</i> (2005)
		<i>Glycerol-3-phosphate acetyltransferase</i>	Chilling tolerance	Yokoi <i>et al.</i> (1998)
		<i>Heat shock protein 101</i>	Heat tolerance	Agarwal <i>et al.</i> (2003)
		Gibberellin insensitive	Dwarfism	Peng <i>et al.</i> (1999)
		H ⁺ /Ca ²⁺ antiporter	Seed calcium content	Kim <i>et al.</i> (2005a)
Animalia	<i>Phaseolus vulgaris</i>	Ferritin	Increase seed iron content	Lucca <i>et al.</i> (2001b)
	Wheat	DREB/CBF	Abiotic stress tolerance	Shen <i>et al.</i> (2003)
	Human	Cytochrome P450 mono oxygenase genes	Herbicide tolerance	Inui <i>et al.</i> (2001)
	Jellyfish	Lactoferrin	Iron binding supplement	Nandi <i>et al.</i> (2002)
		Lysozyme	Valuable industrial enzyme	Hennegan <i>et al.</i> (2005)
		Human granulocyte colony stimulating factor	Valuable industrial factor	Hong <i>et al.</i> (2006)
		Green fluorescent protein	Selecting/screening marker	Vain <i>et al.</i> (2003)

marker-free plants. All of the strategies mentioned have been reviewed and extensively referenced by Bajaj and Mohanty (2005) and Kathuria *et al.* (2007). A further advancement and simplification on the use of *cre/loxP* system was achieved by circumventing the need to express *cre* under spatiotemporal control. A fusion of the membrane translocation sequence from the Kaposi fibroblast growth factor (FGF-4) with CRE was used as cell permeable recombinase to deliver the latter into plant cells and effect marker excision (Cao *et al.*, 2006). An additional strategy to generate transgenic plants devoid of undesirable sequences such as the vector DNA sequences and marker genes is to biolistically co-transform plants with minimal cassettes of the marker gene and the gene of interest (Fu *et al.*, 2000a; Breitler *et al.*, 2002; Loc *et al.*, 2002; Agrawal *et al.*, 2005). This strategy led to higher frequency of single copy integration events as also a higher frequency of independent integration of the marker and the gene of interest, which can be analyzed for segregation in the next generation.

2.1.2 Agronomic trait enhancement

The desirable characteristics in any crop plant are a healthy life cycle leading to copious yields. In almost 20 years since the first transgenic rice plants were generated (Toriyama *et al.*, 1988; Zhang and Wu, 1988), the state of the art now allows facile examination and assessment of the role of heterologous and endogenous genes for agronomic trait improvement. For improvement of rice through transgenic approaches these traits translate into several agronomically desirable characters as detailed below.

2.1.2.1 Virus resistance

Viruses affecting rice crop include the RYMV, rice tungro bacilliform or spherical virus (RTBV/RTSV), rice stripe virus (RSV), rice ragged stunt virus (RRSV), RGSV, and RHBV. Transgenic approach to generate virus-resistant rice was first reported by Hayakawa *et al.* (1992) against RSV using the coat protein (CP)-mediated resistance. Other examples include CP against RHBV (Lentini *et al.*, 2003), RTSV (Sivamani *et al.*, 1999),

and RYMV (Kouassi *et al.*, 2006). Additional strategies used expression of ribozyme against dwarf virus (Han *et al.*, 2000), spike protein against RRSV (Shao *et al.*, 2003), and replicase against RTSV (Huet *et al.*, 1999). Silencing of the viral RNA dependent RNA polymerase was used to generate resistance to RYMV (Pinto *et al.*, 1999). Modification of host factors involved in virus replication against RTBV (Dai *et al.*, 2006) or even translation initiation factor 4G against RYMV (Albar *et al.*, 2006) has also been used. Partial to complete resistance to viral infection was demonstrated but no field studies have been carried out.

2.1.2.2 Bacterial resistance

Bacterial blight in rice is caused by *X. oryzae* pv. *oryzae* (*Xoo*). Resistance to bacterial blight is conferred to a limited extent by a 30-member family of rice endogenous genes called *Xa1* to *Xa29* (Xiang *et al.*, 2006). However, the expression patterns of these do not restrict the natural variation and evolution of resistance in the bacterial strains thus necessitating transgenic approaches using the endogenous genes with heterologous promoters for a powerful and enduring resistance response. Using *Xa21*, the most promising gene of the family (Song *et al.*, 1995) and *Xa26*, transgenic rice resistant to blight in field tests was created (Tu *et al.*, 1998; Sun *et al.*, 2004; Zhai *et al.*, 2004). Additionally, overexpression of endogenous transcription factor *OSWRKY71* implicated in defense signaling pathway was used to obtain resistance to blight (Liu *et al.*, 2006). Recently overexpression of the *OSWRKY13* led to resistance against bacterial blight and fungal blast with evidence for links through activation of salicylic acid (SA)-dependent and suppression of jasmonic acid (JA)-dependent signaling pathways (Qiu *et al.*, 2007). Other endogenous rice genes used against bacterial diseases are the *OSYK1* against bacterial stripe (Hayashi *et al.*, 2005), the *OSRAC1* (Ono *et al.*, 2001), and *OSRACB* (Jung *et al.*, 2006) against bacterial blight. Heterologous genes used against bacterial diseases in rice are the antibacterial peptide cecropin from *Bombyx mori* (Sharma *et al.*, 2000), the oat *ASTH1* gene (Iwai *et al.*, 2002), the sweet pepper ferredoxin gene (Tang *et al.*, 2001) and the *Arabidopsis*

NPR1 gene (Chern *et al.*, 2001, 2005). Yuan *et al.* (2007) confirmed that the rice *NPR1* is an ortholog of the *Arabidopsis NPR1* and its overexpression also confers resistance against bacterial blight and fungal blast perhaps through mediating antagonistic crosstalk between the SA- and the JA-dependent pathways was shown to confer resistance to bacterial blight.

2.1.2.3 Fungal resistance

M. grisea is the causal organism for rice blast and *R. solani* is the causal organism for rice sheath blight disease. Transgenic rice overexpressing the endogenous resistance genes (*R*, *Pi-ta*, *Nbs2-Pi9*, *Pid2*) against fungal blast under altered expression regimes was shown to confer resistance to *M. grisea* (Bryan *et al.*, 2000; Chen *et al.*, 2004; Qu *et al.*, 2006). Chitinase genes from rice or other fungi have been used to generate transgenic rice resistant against *Magnaporthe* and *Rhizoctonia* (Lin *et al.*, 1995; Datta *et al.*, 2001; Sridevi *et al.*, 2003). Pathogenesis-related protein *PR2* was used by Nishizawa *et al.* (2003) and a defense-related gene *RIR1b* was used by Schaffrath *et al.* (2000) for rice resistance against *Magnaporthe*. Wang *et al.* (2006) demonstrated that the thiamine biosynthesis gene *OSDR8* that acts in the signal transduction pathway could confer blast resistance, while Uchimiya *et al.* (2002) demonstrated the similar use of *OSKY1*, a maize *HMI* homolog. The *OSWRKY13* gene, which confers blight resistance, also confers blast resistance through the involvement of salicylate- and jasmonate-dependent signaling (Qiu *et al.*, 2007). The *B. mori* antibacterial peptides cecropins were also used as antifungal peptides (Coca *et al.*, 2006). Recently, Zhu *et al.* (2007) introduced four antifungal genes, two basic chitinases *RCH10* and *RAC22*, and the β -glucanase and *B-RIP* (ribosome inactivating protein) in super hybrid rice and demonstrated high resistance against not just blast but also against false smut and kernel smut caused by *Ustilaginoides virens* and *Tilletia barclayana*, respectively. Manipulating the endogenous levels of JA in transgenic rice through pathogen inducible regulation of allene oxide synthase—a key enzyme in JA biosynthesis—led to enhanced activation of pathogenesis related (PR) genes and blast resistance (Mei *et al.*, 2006).

Examples of glucanases, ribozyme inactivating proteins (RIPs), and other genes used to develop blast-resistant rice are illustrated in Kathuria *et al.* (2007).

2.1.2.4 Nematode resistance

The endogenous gene oryzacystatin was altered and used to generate transgenic rice (Vain *et al.*, 1998), which resulted in 55% reduction in egg production by *Meloidogyne incognita* or the root-knot nematode.

2.1.2.5 Insect resistance

The major insect pests affecting rice crop include *Nephotettix virescens* (green leafhopper), *N. lugens* (brown planthopper), *O. oryzae* (gall-midge) *S. incertulas* (yellow/white stem borer), *C. suppressalis* (striped stem borer). Insects act as primary pest and as vectors for viral infections of rice. Transgenic strategies using baculovirus through the insect's enhanced susceptibility by expressing the virus-enhancing factor (Hukuhara *et al.*, 1999) did not become popular due to the slow mode of action of the virus. Expressing protease inhibitors targeted against the insect proteases has been a popular strategy. Protease inhibitors such as the *SBTI*, *CPTI*, *PINII*, and *ITRI* have been used with limited success (Duan *et al.*, 1996; Xu *et al.*, 1996; Lee *et al.*, 1999; Alfonso-Rubi *et al.*, 2003). However, by far the most popular approach has been the use of *Bt* *Cry* genes. Since the first transgenic rice expressing the *cry1Ab* generated by Fujimoto *et al.* (1993), various rice varieties have been transformed with different *cry* proteins in their original, modified, or fully synthetic versions (extensively reviewed in Tyagi *et al.*, 1999; Khush and Brar, 2002; Bajaj and Mohanty, 2005; Kathuria *et al.*, 2007). Countering the capacity of target insects to develop concomitant resistance to *cry* proteins has been the subject of extensive research in deploying the transgenic *Bt* *cry* protein strategy, which holds promise due to its initial desirable effects on controlling the insect infestation and crop damage. Gene pyramiding of different *cry* proteins is seen as one viable strategy (Maqbool *et al.*, 2001;

Loc *et al.*, 2002; Bashir *et al.*, 2005). Fusion between cry proteins and carbohydrate-binding moieties of lectins is another strategy that was shown to increase the range of target insects (Mehlo *et al.*, 2005). Field tests of rice expressing cry proteins have shown limited success (Tu *et al.*, 2000; Ye *et al.*, 2001, 2003; Wu *et al.*, 2002; Bashir *et al.*, 2004; Breitler *et al.*, 2004). The fusion proteins, gene pyramiding not just with cry proteins but also with other insecticidal proteins, and agronomic deployment strategies as part of the integrated pest management has been suggested as the route to creating insect-resistant plants (Christou *et al.*, 2006; Ferry *et al.*, 2006). The cry proteins are not effective against the hemipteran insects. Plant lectins such as the galanthus nivalis agglutinin (GNA) have been shown to be effective against insects such as brown planthopper (BPH), green leafhopper (GLH), and white-backed planthopper (Rao *et al.*, 1998; Sudhakar *et al.*, 1998; Sun *et al.*, 2002; Nagadhara *et al.*, 2003, 2004). The *Allium sativum* leaf lectin was shown by Saha *et al.* (2006) to be effective against BPH and GLH. The GNA in combination with the cry proteins (Maqbool *et al.*, 2001; Ramesh *et al.*, 2004) or in combination with *SBTI* (Li *et al.*, 2005) extended the range of target insects to which the transgenic plants were tolerant. Yet other molecules such as the avidin (Yoza *et al.*, 2005) and the spider insecticidal protein (Qiu *et al.*, 2001) have been tested in transgenic rice.

2.1.2.6 Drought, salt, submergence, and temperature stress tolerance

Although rice is a sturdy grass crop that grows in varied climates and weather conditions, the gap in yield potential and yield harvest as affected by abiotic stresses cannot be afforded under the present demand regimes. However, transgenic approaches to address a particular stress often favorably impact the other due to amelioration of a common stress response pathway. One approach that simultaneously affects favorably more than one abiotic stress is overproduction of cell solutes such as glycine betaine, trehalose, proline, polyamines and late embryogenesis abundant proteins. The genes used to achieve solute overproduction in transgenic rice are reviewed in Kathuria *et al.* (2007). A recent example of using osmolyte accumulation

against salt and drought tolerance is the overproduction of mannitol in rice using the *Escherichia coli* mannitol-1-phosphodehydrogenase (Pujni *et al.*, 2007). Ion channel and water channel proteins that facilitate modification of the cell's and the vacuole's osmotic balance have been used to develop drought-tolerant rice. For example, overexpression of the aquaporin *RWC3* from rice (Lian *et al.*, 2004) and that of the Na^+/H^+ antiporter from rice, *Suaeda salsa*, *Artiplex* or *E. coli* (Ohta *et al.*, 2002; Fukuda *et al.*, 2004; Zhao *et al.*, 2006) increased salinity tolerance in transgenic rice. The usefulness of the strategy to increase salt tolerance in transgenic rice through a rice Na^+/H^+ antiporter gene (*OSNHX1*) was reinforced recently by Chen *et al.* (2007). Production of free radicals and reactive oxygen species mostly accompanies different abiotic stresses (Mittler *et al.*, 2004) and the common signaling pathways for the perception and response to oxidative stress indicate that strategies targeting oxidative stress may ameliorate multiple stresses. The presence of oxidative stress response *cis* elements in abiotic stress response genes such as *OSISAP1* (Mukhopadhyay *et al.*, 2004; Tsukamoto *et al.*, 2005) supports this strategy. Hoshida *et al.* (2000) obtained salt-tolerant transgenic rice by overexpressing glutathione synthase, a reactive oxygen species metabolism enzyme. Similarly, constitutive overproduction of pea superoxide dismutase (Wang *et al.*, 2005) and protoporphyrinogen oxidase (Jung and Back, 2005) led to salt-tolerant rice. An additional common reaction to stress is the Ca^{2+} -mediated signaling to activate response pathways (Knight and Knight, 2001). Proteins involved in Ca^{2+} -mediated signaling such as calcineurin A (Ma *et al.*, 2005), calcium-dependent protein kinases (*OSCDPK7*; Saijo *et al.*, 2000), and MAP kinases (*OSMAPK5*; Xiong and Yang, 2003) were shown to generate salt-, drought-, and cold-tolerant rice.

Recently the *DREB/CBF* regulatory genes have also been shown to be common to cold, dehydration, and salt stress (Dubouzet *et al.*, 2003). Overexpressing endogenous and heterologous (wheat and *Arabidopsis*) *DREB/CBF* class of genes in rice has led to a better understanding of the signaling pathways and generated multiple-stress-tolerant rice (Oh *et al.*, 2005). Overexpression of other regulatory genes such as the transcription factor *SNAC1* has also

been shown to generate drought- and salt-tolerant rice (Hu *et al.*, 2006).

As opposed to salt and drought tolerance, submergence tolerance is important in certain areas. Quimio *et al.* (2000) generated the first rice transgenics exhibiting submergence tolerance by overexpressing the *pyruvate decarboxylase* gene (*PDC1*). Xu *et al.* (2006) demonstrated submergence tolerance in transgenic rice by expressing an ethylene responsive-factor-like gene (*SUB1A*), which affects the expression levels of other genes of the same family (*SUB1C*) and the *alcohol dehydrogenase* (*ADH1*) gene expression.

Temperature stress tolerance has been engineered through the use of heat shock proteins for high temperature from rice itself (SPL7; Yamanouchi *et al.*, 2002) or from *Arabidopsis* (HSP101; Agarwal *et al.*, 2003; SHSP17.7; Murakami *et al.*, 2004). Low-temperature stress tolerance was engineered through overexpression of genes affecting the level of unsaturation of the fatty acids in the chloroplast phosphatidylglycerol (Ariizumi *et al.*, 2002; Takesawa *et al.*, 2002).

2.1.2.7 Mineral stress tolerance

Plant stress associated with excess or lack of mineral nutrients can adversely affect yields to a large degree. Soil pH dictates the availability of nutrients such as iron, phosphorus, aluminum, etc. High pH soils limit the availability of iron, which was ameliorated by overexpressing nicotianamine synthase, because it is functional in the biosynthetic pathway of iron chelating phytosiderophores. Barley phytosiderophore synthesizing genes (*NAAT-A* and *NAAT-B*) were also used to show enhanced survival in high pH, low iron availability soils (Takahashi *et al.*, 2001). Phosphate starvation in pH imbalanced soils was relieved through the expression of a rice transcription factor *OSPTF1* (Yi *et al.*, 2005). Begum *et al.* (2005) demonstrated that transgenic rice expressing the maize *PEPC* led to exudation of oxalate thus enhancing the plant's capacity to adapt to high pH, low phosphorus soil conditions. Alternately, low pH soils pose the problem of toxic aluminum ions that inhibit root elongation. Aluminum ion complexing organic acids such as citrate and malate led to transforming rice with a

wheat malate transporter (*ALMT-1*; Sasaki *et al.*, 2002) to generate aluminum-resistant plants but the approach was not very successful.

2.1.2.8 Herbicide tolerance

The trait of herbicide tolerance was one of the first to be introduced in rice, tested in the fields, and used commercially. Transgenic rice has been developed expressing the herbicide tolerance gene *bar* from streptomyces (Christou *et al.*, 1991; Datta *et al.*, 1992), Cytochrome P450 monooxygenases from humans (Inui *et al.*, 2001; Kawahigashi *et al.*, 2005, 2006) or pigs (Kawahigashi *et al.*, 2005) and the rice glutathione S-transferases (Deng *et al.*, 2003). Tolerance to a number of herbicides was demonstrated through these studies. Lee *et al.* (2000) and Jung and Back (2005) also generated herbicide tolerance in rice through expressing the *Bacillus subtilis* *protax* gene.

2.1.2.9 Increased grain yield

With the increasing population, increasing crop yield through contribution of the transgenic technologies in the breeding programs is almost a prerequisite now. Although addressing biotic and abiotic stress resistance is an indirect route to yield increase, a more direct route is to increase grain number, size, and weight. Enhanced activity of adenosine diphosphate-glucose pyrophosphorylase led to increase in seed weight per plant (Smidansky *et al.*, 2003). Manipulating complex traits such as light signal transduction and photosynthetic efficiencies through efforts to convert the rice C₃ plant into C₄ plant is also underway. Kong *et al.* (2004) demonstrated an increase in seed yield in transgenic rice expressing the *Arabidopsis* phytochrome A gene. Plant hormones influence grain yield. Sakamoto *et al.* (2003) overexpressed the gibberellin 2-oxidase, generating a semidwarf, high-yielding transgenic after a connection was established between dwarfism and gibberellin response (Peng *et al.*, 1999; Sasaki *et al.*, 2002). Lack of C-22 hydrolase, a brassinosteroid biosynthetic enzyme leads to erect leaves and increased grain yield (Sakamoto *et al.*, 2006). Transgenic rice expressing

the brassinosteroid receptor gene *OSBRI* and exhibiting brassinosteroid insensitivity showed similar changes in morphology and increased yield (Morinaka *et al.*, 2006). Similarly, expressing the defective allele of *OSCKX2*—a cytokinin oxidase/dehydrogenase showed enhanced cytokinin and increased grain yield (Ashikari *et al.*, 2005). Flower development is directly related to grain yield capacities. Manipulation of the transgenic expression of different rice MADS box transcription factors was shown to affect flower morphology indicating genes necessary for normal flower development (Prasad *et al.*, 2001, 2005; Kyoizuka and Shimamoto, 2002; Sentoku *et al.*, 2005; Chen *et al.*, 2006a; Yamaguchi *et al.*, 2006). These studies helped to map the rice flower development on the known ABC model of *Arabidopsis* flower development. Genes other than the MADS box transcription factors that affect the flower morphology have also been elucidated (Jang *et al.*, 2003, 2004). Other rice genes studied through transgenic rice for their role in flowering time, shoot-to-flower transition time, panicle number, and density and other growth and development patterns have been reviewed in Kathuria *et al.* (2007) including the genes concerned with phytohormone biosynthesis mentioned above and the additional ethylene biosynthesis and response genes that affect plant morphology (Mao *et al.*, 2006). Regulatory genes apart from the transcription factors, such as those concerned with giving rise to further regulatory moieties of micro-RNA also provide an insight into plant architecture (Liu *et al.*, 2005). Flower development and viability also feeds into the important aspect of generating hybrid rice through male sterility. Generating cytoplasmic or genic male sterility or restoration of the same has been demonstrated through transgenic approaches (Komori *et al.*, 2004; Wang *et al.*, 2006). These studies help in understanding factors that can be used to develop an optimal plant architecture and life cycle that further helps increase grain yields. Interestingly, roots that sustain the entire plant and whose normal growth and development dictates that genetic growth patterns of the aerial parts are adhered to through supply of water and nutrients, have not been investigated to the same extent as perhaps shoot branching patterns and flower development. Recently, transgenic studies

have elaborated the role of some rice genes in root development showing relationships to auxin levels (Ge *et al.*, 2004; Nakamura *et al.*, 2006).

2.1.2.10 Nutritionally enhanced seeds

Since rice is the staple food of a large population in economically underdeveloped, and developing countries, which cannot afford expensive fruits and vegetables, enhancement of the nutritional quality of the rice seeds can directly contribute to the health of this population. The first target in this regard was increasing the content of carotenoids to result in enhancement of vitamin A in the seeds. Over the last decade, rice has been engineered with carotenoid biosynthesis genes from rice, maize, daffodil, and bacteria sequentially improving the expression levels by approaches such as codon optimization and tissue-specific expression (Burkhardt *et al.*, 1997; Ye *et al.*, 2000; Datta *et al.*, 2003, 2006 Hoa *et al.*, 2003; Paine *et al.*, 2005). The second target is to improve the rice seeds for essential amino acid content. To increase the content of tryptophan the biosynthetic enzyme anthranilate synthase gene was used (Morino *et al.*, 2005; Wakasa *et al.*, 2006). Similarly, the seed methionine and cysteine content has been improved by expressing the sesame albumin protein (Lee *et al.*, 2003). Increasing the levels of polyunsaturated fatty acid α -linolenic acid in rice bran has also been achieved (Anai *et al.*, 2003). Seed iron content was increased by transforming rice with human lactoferrin gene (Nandi *et al.*, 2002), the *Phaseolus vulgaris* ferritin gene (Lucca *et al.*, 2001a), the soybean ferritin gene (Goto *et al.*, 1999; Vasconcelos *et al.*, 2003) or the *Aspergillus fumigatus* phytase gene (Lucca *et al.*, 2001a). In another breakthrough, the seed calcium content was manipulated to lower the incidence of osteoporosis, an incidental problem occurring through rice consumption amongst the economically poorer communities. A H^+/Ca^+ transporter gene was integrated to achieve higher calcium content (Kim *et al.*, 2005a). However, any nutritional improvement of rice seed can most easily be affected by changes in starch content. Overexpression of one allele of the starch synthase gene resulted in the increase of seed starch (Hirano *et al.*, 1998). A change in the amylose to

amylopectin ratio of the rice seed was targeted by transforming rice either with isoamylase 1 silencing construct or with the *E. coli* glycogen-branching enzyme (Fujita *et al.*, 2003; Kim *et al.*, 2005b) to change the physiological properties of starch. A postharvest value-added modification for the use of rice starch was engineered by introducing the wheat puroindoline genes *PINA* and *PINB* that rendered the grains much softer than the control. The “Soft-Rice” has numerous applications in the food industry.

2.1.2.11 Variation in transgene constructs

Most transgenic plants are primarily developed using strong constitutive promoters. For rice, the most commonly used promoters are the CaMV 35S, rice *ACT1* and the maize *UBI1*. The CaMV 35S promoter was shown to contain a recombination hotspot (Kohli *et al.*, 1999a), readily leading to transgene rearrangements. Other viral promoters have been used in rice such as the one from cestrum yellow leaf curling virus (CYLF) (Stavolone *et al.*, 2003) and milk vetch dwarf virus (MVDV) (Shirasawa-Seo *et al.*, 2005). Among the nonviral constitutive promoters that have been used, the rice cytochrome c gene promoter was highly active in different parts of the plant including the embryo and the calli (Jang *et al.*, 2002). Using introns further enhanced expression of the constitutive promoters. Tanaka *et al.* (1990) used the first intron of castor bean catalase to enhance *gus* expression driven by the CaMV 35S promoter in rice. The maize *ADH1* first intron became quite popular in plant transformation constructs and was first used in rice by Kyoizuka *et al.* (1991). Simultaneously, McElroy *et al.* (1991) used the first intron of the *ACT1* gene in combination with the *ACT1* promoter. However, the most popular intron was the first intron of the maize *UBI1* gene in combination with the *UBI1* gene promoter itself (Toki *et al.*, 1992; Cornejo *et al.*, 1993). The *ADH1* intron 1 was, however, used downstream of the CaMV 35S promoter (Taylor *et al.*, 1993). Other DNA sequences tested for their functionality in regulating the level of expression of a transgene were the matrix attachment region (MAR) sequences (Vain *et al.*, 2002). Xue *et al.*

(2005) used the tobacco TM2 MARs to enhance expression from both constitutive and inducible promoters. Novel regulatory sequences such as the *REB* activator fused to the *GLB* promoter increased the transgene activity (Yang *et al.*, 2001) while the *SRS* element fused to the *ACT* promoter also achieved enhanced expression levels (Lu *et al.*, 1998). Nguyen *et al.* (2004) increased constitutive expression up to fivefold, by using the T7RNA polymerase-directed expression system in rice. However, constitutive promoters are not always desirable since transgene expression levels may need to be spatiotemporally controlled and coordinated. This can be achieved either through tissue/stage-specific or inducible promoters *per se* (Ito *et al.*, 2006) or through coupling regulatory, inducible/responsive *cis* elements to workhorse constitutive promoters (Su *et al.*, 1998). Further examples of both categories have been extensively reviewed in Bajaj and Mohanty (2005) and Kathuria *et al.* (2007). Promoters from diverse sources have been tested in rice suggesting its suitability for heterologous functional analysis or complementation of promoter elements of other organisms (Kathuria *et al.*, 2007).

2.2 Methods of Genetic Transformation

2.2.1 Protoplast technology

The first transgenic rice plants were obtained with rice protoplasts using electroporation (Toriyama *et al.*, 1988) and polyethylene glycol (PEG)-mediated methods (Zhang and Wu, 1988). Dividing rice protoplasts from which fertile plants can be regenerated are usually generated from embryogenic cell cultures normally derived from immature embryos (Vasil, 1988). Toriyama *et al.* (1988) used protoplasts isolated from anther-derived cell suspension cultures of *O. sativa* L. cv. Yamahoushi to electroporate two plasmids containing CaMV 35S promoter-driven aminoglycoside phosphotransferase II and β -glucuronidase genes. Shimamoto *et al.* (1989) were the first to recover fertile transgenic *Japonica* rice using the electroporation method. Datta *et al.* (1990) were the first to recover fertile transgenic *Indica* rice (*O. sativa* var. Chinsurah Boro II) from protoplasts derived from immature

pollen grains (microspores) with a hygromycin phosphotransferase (*hph*) gene under control of the CaMV 35S promoter using the PEG-mediated technique.

Protoplast-mediated transformation has many limitations. These include difficulties in regeneration of elite *Japonica* and *Indica* varieties (Ayres and Park, 1994), the production of sterile and phenotypically abnormal regenerated plants (Datta *et al.*, 1992), the integration of multiple copies of genes into genomes (Tada *et al.*, 1990), combined with the fragmentation and rearrangement of transgenes (Xu *et al.*, 1995) and their occasional non-Mendelian inheritance (Peng *et al.*, 1995).

2.2.2 Particle bombardment

Continuous and directed research efforts to use *Agrobacterium*-mediated transformation for cereals have recently made the technology routine in many laboratories around the world. However, earlier difficulties in transforming cereals with *Agrobacterium* led to a search for alternative strategies. Wang *et al.* (1998) first used the biolistics technology of bombarding DNA-coated gold particles accelerated under vacuum to achieve transient expression of transgenes in rice. Christou *et al.* (1991) reported the first stable transformation using a modification of the same technology whereby the particles were accelerated by an electric arc. This method of transformation became the method of choice for nearly a decade since it is genotype independent and less labor intensive. A number of genes were introduced in rice using the biolistics technology (reviewed in Giri and Laxmi, 2000; Bajaj and Mohanty, 2005; Kathuria *et al.*, 2007). An *Indica* rice population was created with *Ac/Ds* transposon for saturation mutagenesis and functional genomics using the biolistics method (Kohli *et al.*, 2001). Initial observations of transgene rearrangements and multicopy insertion events in transgenic rice lines (Kohli *et al.*, 1998, 1999a) were ascribed as reasons for transgene silencing (Kumapatla and Hall, 1998a, b; Dai *et al.*, 2001b). Transgene rearrangements were indeed shown to be responsible for lack of transgene expression but integration of multicopy transgenes does not always lead to silencing

(Gahakwa *et al.*, 2000; Kohli *et al.*, 1999b). In fact, fragmentation and rearrangement of transgene(s) were shown to be responsible for silencing (Yang *et al.*, 2005), especially when multiple copies are present. However, reducing the chances of rearrangements by removing the vector backbone sequences from the transforming constructs led to independent integration of intact multicopies, which exhibited high and stable expression patterns (Fu *et al.*, 2000a; Agrawal *et al.*, 2005). This approach also allayed the concerns on antibiotic selectable marker and other vector backbone sequence integration in the transgenic plants. This approach of “clean DNA” transformation using the biolistics method is gaining popularity for transformation of rice (Breitler *et al.*, 2002; Loc *et al.*, 2002), other cereals such as wheat (Yao *et al.*, 2006), and yet other plants such as potato (Romano *et al.*, 2003) and grapevine (Vidal *et al.*, 2006).

2.2.3 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a phytopathogenic soil bacterium, sometimes named as a “natural genetic engineer” due to its ability to transform plant cells with a part of its tumor-inducing plasmid (Ti plasmid) known as the T-DNA segment (Chilton, 2001). Wounding of plants allows entry of bacteria and provides phenolic compounds that activate the DNA transfer machinery of *A. tumefaciens* in which a series of molecular events results in transfer of T-DNA from the bacterial cell to a plant cell and subsequently into the plant genome (Gelvin, 2000, 2003; Zhu *et al.*, 2000; Zupan *et al.*, 2000). Diverse arrays of sophisticated plant transformation vectors have been derived to exploit this naturally occurring gene transfer mechanism in the genetic engineering of plants (Bevan, 1984; Tzifira and Citovsky, 2006). This mode of gene delivery is both convenient and efficient. Among its list of major advantages is the transfer of a small number of intact DNA fragments (Hamilton *et al.*, 1996; Dai *et al.*, 2001b) that exhibit a normal Mendelian gene transmission to progeny (Budar *et al.*, 1986).

2.2.3.1 Transformation of dicots

The earliest reports of successful *Agrobacterium*-mediated transformation were for several dicotyledonous plants. Murai *et al.* (1983) demonstrated that a part of a bean phaseolin seed protein gene was transcribed in transformed sunflower cells. Horsch *et al.* (1985) combined gene transfer, plant regeneration, and an effective kanamycin-based selection for transformants to generate transgenic petunia, tobacco, and tomato by means of a novel leaf disk transformation-regeneration method. Subsequently, *Agrobacterium*-mediated transformation was extended to other dicots such as soybean (Hinchey *et al.*, 1988) and walnut (McGranahan *et al.*, 1988) culminating in the *in planta* *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants (Bechtold *et al.*, 1993).

2.2.3.2 Transformation of monocots

De Cleene and De Ley (1976) showed that members of the Liliales and Arales are susceptible to *Agrobacterium*, and early reports exist for *Agrobacterium*-mediated transformation of both *Asparagus officinalis* (Hernalsteens *et al.*, 1984) and *Dioscorea bulbifera* (Schafer *et al.*, 1987). Grimsley *et al.* (1986, 1987) demonstrated transfer of T-DNA in maize seedlings infected with *A. tumefaciens* containing the maize streak virus (MSV) in the T-DNA (a process known as agroinfection). Nevertheless, monocots, especially the cereals were generally considered outside the host range of *A. tumefaciens* (Potrykus, 1990; Smith and Hood, 1995). As a consequence, protoplast electroporation and particle bombardment techniques were extensively used for transformation of cereals until Hiei *et al.* (1994) provided rigorous evidence for the production of many fertile rice plants using a well-defined *Agrobacterium*-mediated transformation procedure.

This built upon the first successful demonstration of *Agrobacterium*-mediated transformation of rice by Raineri *et al.* (1990) who used mature embryos from wounded germinated seeds of *Japonica* cultivars Nipponbare and Fujisaka5 to deliver *Agrobacterium* strain LBA4404 with *nos-nptII* and CaMV 35S-*gus*. They obtained kanamycin-resistant calli, and showed T-DNA

integration by Southern analysis, but failed to regenerate transgenic plants.

Chan *et al.* (1992) obtained G418-resistant calli from root explants of the *Indica* variety Taichung native I and showed T-DNA integration by Southern analysis. Later (Chan *et al.*, 1993) successfully regenerated four transgenic rice plants, only one of which produced progeny. To achieve successful infection, they added extracts from a potato suspension culture to the co-cultivation medium, as these are rich in phenolic compounds that aid in susceptibility to *Agrobacterium*.

In the landmark paper of Hiei *et al.* (1994), *Agrobacterium* strains LBA 4404 and EHA101 harboring pTOK233, a “superbinary” vector, were employed. In these strains, the plasmid containing the T-DNA bears a fragment of the S-Vir region that includes additional *virB* and *virG* genes derived from pTiBo542. Transgenic plants were successfully obtained for *Japonica* varieties Tsukinohikari, Asanohikari, and Koshihikari. T-DNA regions in the transformed plants were detected by Southern analysis; their boundaries sequenced, and Mendelian transmission of transgenes to the R₂ generation was demonstrated. This achievement was followed by the first reports of transgenic *Indica* rice plants, namely, Basmati 370 and Basmati 385 (Rashid *et al.*, 1996), and of *Javanica* rice, Gulfmont and Jefferson (Dong *et al.*, 1996). All these studies used calli derived from scutellar tissue, and included acetosyringone, a phenolic compound, in the co-cultivation media. Dong *et al.* (1996) also conducted sequence analysis of right border fragments of one of their transgenic lines, confirming that insertion was into a coding region of rice nuclear DNA. This analysis also revealed the presence of relatively short regions of permuted T-DNA border sequences, similar to those found after *Agrobacterium*-mediated transformation of dicots. Figure 2 details the production of fertile transgenic rice plants by *Agrobacterium*-mediated transformation of scutellum-derived calli.

Broadening the range of tissues competent for *Agrobacterium*-mediated transformation was another significant step in rice biotechnology. Immature embryos from both *Japonica* (Radon) and *Indica* (TCS10 and IR72) varieties (Aldemita and Hodges, 1996) and isolated shoot apices from tropical *Japonica* (Maybelle) varieties (Park *et al.*,

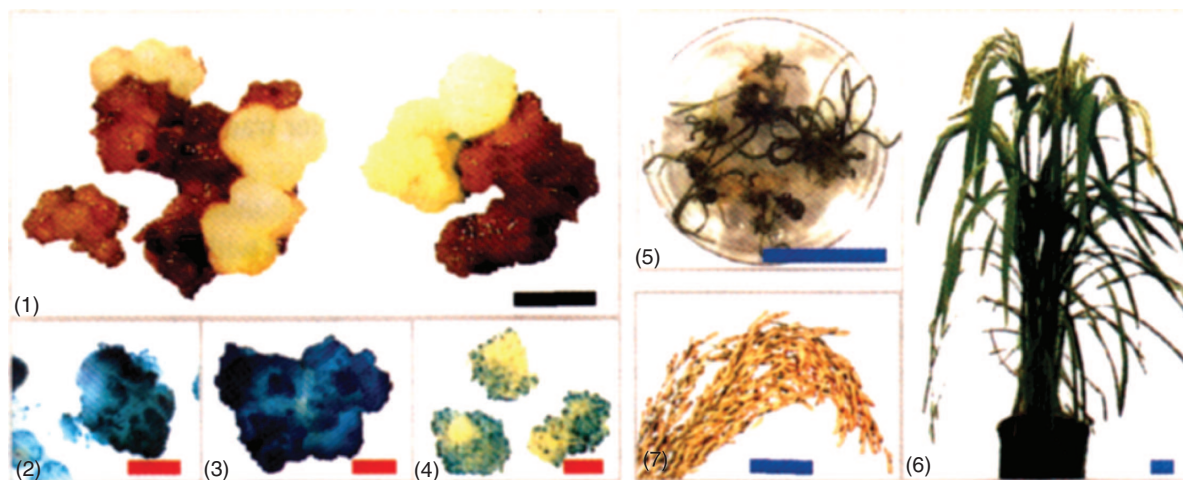


Figure 2 Production of fertile transgenic rice plants by *Agrobacterium*-mediated transformation of scutellum-derived calli. (1) Calli on selection medium (50 mg l^{-1} hygromycin); (2, 3, 4) GUS expression on different hygromycin-resistant calli; (5) plantlets after 3–4 weeks on regeneration medium; (6) fertile plant; (7) detail of panicle

1996) were used successfully. Dong *et al.* (2001) used inflorescence explants from the *Japonica* variety Taipei 309 and regenerated transgenic rice plants at frequencies as high as 80% from inflorescences when the individual floral organs were 1–2 mm long. This provided a simpler and more rapid alternative to the scutellar-based approach, by minimizing the tissue culture steps and reducing somaclonal variation, and also enhanced the potential for transformation of genotypes recalcitrant to tissue culture and regeneration. Another *in planta* method by Supartana *et al.* (2005) employed 2 days' presoaked seeds of *Japonica* variety Koshihikari to obtain transgenic plants by piercing a site of the husk overlying the embryonic apical meristem with a needle that had been dipped in an *Agrobacterium* inoculum. The inoculated seeds were then grown to maturity and allowed to pollinate naturally to set seeds. Transformation efficiency was estimated to be 40% by polymerase chain reaction (PCR) and 43% by β -glucuronidase histochemical assay. In other high-throughput methods, Terada *et al.* (2004) employed seed-derived calli to obtain 1000 stable transformants from as few as 150 explants in *Japonica* rice; Toki *et al.* (2006) successfully regenerated transgenic rice plants within a month (as opposed to the standard three months in all related techniques), from scutella of 1-day precultured mature seeds, thus reducing the risk

of somaclonal variation attributable to prolonged tissue culture.

A more recent method of efficient *Agrobacterium*-mediated transformation in rice used microspore-derived haploid callus cells (Y. Jiang and T.C. Hall, personal communication). Compared to methods using diploid cells or tissues as starting materials for *Agrobacterium*-mediated transformation, rice transformation of haploid cells such as microspore-derived callus has several advantages. Chromosome doubling of a transgenic haploid cell produces DH transgenic homozygous cells that provide a method for rapid genetic fixation of the gene of interest that is transferred. Homozygous plants are valuable for plant breeding because almost all of the final products or cultivars released from plant breeding are homozygous. Traditional methods to obtain homozygous breeding lines following hybridization or mutagenesis often require years to succeed. Another advantage of haploids for genetic analysis is that dominance and recessivity are less likely to obscure gene expression, and the phenotype is a direct manifestation of the genotype since there is only one chromosome set in the haploid. Although *Agrobacterium*-mediated transformation of microspore-derived embryos was reported some time ago for *Datura innoxia* and *Nicotiana tabacum* (Sangwan *et al.*, 1993), limited success for rice was reported only

recently (Chen *et al.*, 2006a, b). The protocol for transgenic rice extends the methods for a highly efficient production of transgenic haploids and DH procedure using microspore-derived haploid callus as a target for *Agrobacterium*-mediated transformation.

2.2.4 Miscellaneous methods

Reports of several novel methods for successful transformation exist in the literature. Luo and Wu (1989) described a method to transform rice through a pollen tube pathway method. Guo *et al.* (1995) reported an effective system for introducing exogenous DNA into cells of embryonic calli of *Japonica* rice by first dehydrating the cells in a hypertonic buffer, then placing the cells in a medium of less negative osmotic potential containing the exogenous DNA, and employing a laser beam to puncture holes in cell wall and membrane to enable uptake of DNA expressing the fluorescent calcein. Yoo and Jung (1995) reported the uptake of *gusA* and *hpt* genes by imbibition of dry and viable rice embryos from a DNA solution. Matsushita *et al.* (1999) used scutellar tissues of rice embryos obtained from mature seeds and vortexed them in liquid medium containing plasmid DNA and silicon carbide whiskers to obtain transgenic plants expressing *gusA* and bialaphos resistance.

2.3 Selection Methods for Transgenic Plants

There are numerous critical factors that are of paramount importance in obtaining transgenic rice plants, and the relative efficiency, economy, experimental time, and reproducibility must be taken into consideration to adopt a particular technology whether it is particle bombardment or *Agrobacterium*-mediated transformation. The ultimate aim is to select an easy, efficient, and inexpensive method suited to the introduction of a single gene or multiple genes or a study, which requires functional genomics to identify the function of numerous genes in a high-throughput fashion.

2.3.1 Bacterial strains and vectors

Successful *Agrobacterium*-mediated rice transformation procedures are mostly based on the use

of supervirulent strains and superbinary vectors carrying the virulence region of pTiBo542 (Hood *et al.*, 1986; Komari *et al.*, 1986). Strain A281 (Hood *et al.*, 1987; Komari, 1989) with a wide host range and higher transformation efficiency was used by both Raineri *et al.* (1990) and Chan *et al.* (1993). The strain EHA101 (Hood *et al.*, 1986) was developed with two vector versions, one harboring a “disarmed” version of pTiBo542 and another with the “superbinary” vector, where a DNA fragment with the *virB*, *C*, and *G* genes was introduced into a small T-DNA-carrying plasmid that is used in a binary vector system (Komari, 1990). Hiei *et al.* (1994) tested the efficacy of both the supervirulent EHA101 and another ordinary strain LBA4404 in combination with pIG121Hm, a derivative of a “normal” binary vector pBIN19 (Bevan, 1984), and pTOK233, a derivative of “superbinary” vector pTOK162 (Komari, 1990). The LBA4404 (pTOK233) combination was slightly more effective than LBA4404 (pIG121Hm) and EHA101 (pIG121Hm), while EHA101 (pTOK233) alone was not very effective. Dong *et al.* (1996) demonstrated that LBA4404 (pTOK233) was most effective in *Javanica* rice. In general, an “ordinary” vector/strain combination proved effective in transforming cultivars that are easy to grow in tissue culture, and in case of cultivars difficult or recalcitrant to tissue culture, the choice of vectors and strains is more crucial. More recently, Jeon *et al.* (2000) showed that a combination of LBA4404 with common binary vectors resulted in callus transformation efficiencies of up to 40% from which transgenic plants were regenerated at a frequency of 85%.

2.3.2 Induction of *vir* genes

Agrobacterium was found to be attracted to wounded plant cells in response to several phenolic signal compounds, including 4-acetyl-2, 6-dimethoxy phenol (commonly known as acetosyringone) and 4-(2-hydroxy acetyl)-2,6-dimethoxy phenol (α -hydroxy acetosyringone) that activate the *vir* genes on Ti plasmids (Stachel *et al.*, 1985). Seven phenolic compounds were found to induce the expression of *vir* genes (Bolton *et al.*, 1986) but monocots appear not to produce, or produce at insufficient levels, these compounds to act as signals (Smith and Hood, 1995). Rice cells

may be capable of producing sufficient levels of inducing agent, as the first report of transgenic calli (Raineri *et al.*, 1990), did not require any phenolic compound addition in the co-cultivation media. The transfer of T-DNA is enhanced at an early stage of co-cultivation, and the efficiency of gene transfer is also increased when both the bacteria and the tissues are pretreated with acetosyringone (Aldemita and Hodges, 1996). Certain other factors, such as an acidic pH (Turk *et al.*, 1991), culture temperatures below 28 °C (Alt-Moerbe *et al.*, 1988), high osmotic pressure (Usami *et al.*, 1988), the presence of opines (Veluthambi *et al.*, 1989), and aldoses like D-glucose (Cangelosi *et al.*, 1990) also increase *vir* gene induction.

2.3.3 Tissues amenable to transformation

Binns and Thomashaw (1988) pointed out a distinct correlation between cell wounding and competency of such cells for transformation, and proposed that processes related to DNA synthesis and cell division are critical for the integration of delivered DNA into the host genome. The wound responses of monocots differ significantly from those of dicots, with the cells at the wound sites of monocots becoming lignified or sclerified with no apparent cell division (Kahl, 1982). This renders the choice of dicot explants such as leaf discs and hypocotyls useless *vis-à-vis* their monocot counterparts; hence, actively growing tissues need to be supplemented with *vir*-inducing compounds to ensure successful transformation. For generating transgenic rice, the favored choice has been mature or immature embryo (scutellum)-derived callus cultures (Hiei *et al.*, 1994; Dong *et al.*, 1996; Rashid *et al.*, 1996; Toki, 1997). Shoot apices (Park *et al.*, 1996) and immature embryos (Aldemita and Hodges, 1996) have also been employed with limited success.

The choice of tissue for transformation is also dependent on the rice genotype. *Japonica* and *Javanica* varieties are usually highly responsive to tissue culture and actively dividing callus cultures are the tissues of choice for these varieties (Hiei *et al.*, 1994; Dong *et al.*, 1996). A majority of *Indica* varieties falling under a different group, designated group I based on isozyme analysis (Glaszmann, 1987) are recalcitrant to tissue culture and transformation procedures. Aldemita

and Hodges (1996) used immature embryos to achieve transformation of two group I *Indica* cultivars, namely TSC10 and IR72, a method extended by Hiei *et al.* (1997) to many other group I varieties.

2.3.4 Co-cultivation and culture media conditions

The composition and incubation conditions of the co-cultivation and subsequent callusing media during the transformation process play a critical role in the initial transient transformation efficiencies and subsequent stable transgene integration. A modified N6 medium with B5 vitamins, 2,4-dichlorophenoxy acetic acid (2,4-D) supplemented with acetosyringone during co-cultivation was the medium of choice for both *Japonica* and *Indica* varieties (Hiei *et al.*, 1994; Rashid *et al.*, 1996; Toki, 1997), while MS-based media was used for *Javanica* varieties (Dong *et al.*, 1996). Media solidified with a gelling agent was preferred over liquid media, where β -glucuronidase (GUS) expression was low (Hiei *et al.*, 1997). The optimization of media (e.g., choice of N6 over MS) can be monitored based on transient expression of reporter genes like *gus*, as conditions allowing for a high level of transient expression are generally associated with a high frequency of stably transformed calli and subsequent transgenic plants (Hiei *et al.*, 1997). The *gusA* gene containing an intron in the coding region has been a very useful reporter as the GUS expression is limited to plant cells (Ohta *et al.*, 1990; Vancanneyt *et al.*, 1990).

2.3.5 Reporter genes and selectable marker genes

The most commonly used reporter gene in rice transformation was initially the β -glucuronidase gene (*gusA*) (Jefferson *et al.*, 1987), which served as a valuable tool to monitor transient expression early after transformation, providing an indication of the expected frequency of stable transgenic lines. Histochemical GUS activity was primarily localized at or around the vascular tissue in leaf, root, and flower organs and was also detected in the embryo and endosperm of dormant and germinating seeds (Terada and Shimamoto, 1990). Battra and Hall (1990) also observed GUS

activity in transgenic rice in the leaf epidermis, mesophyll, vascular bundles, in the cortex and vascular cylinder of the root, and marginal activity in the root epidermis. Fluorometric assay of various organs showed that GUS activity in transgenic rice plants was comparable to that seen in transgenic tobacco plants. A more recent innovation in the GUS reporter system involves the development of GUS *plus*TM (CAMBIA), which is a new reporter gene isolated from *Staphylococcus* sp. with superior properties to *E. coli gusA*. A version with the rice glycine-rich protein signal peptide for extracellular secretion providing rapid, *in vivo* GUS assays has been tested extensively in transgenic rice, and the gene has been codon optimized for high expression in plants. Vickers *et al.* (2003) developed a synthetic, codon-optimized xylanase gene (*sXynA*) as a reporter gene in conjunction with GUS *plus* for quantitative transient analyses in plants, which allows for sensitive assays at the low levels of transgene protein found in transiently transformed tissue extracts. The xylanase protein is stable, activity kinetics is linear over long time periods, and assays are cost effective. Kirby and Kavanagh (2002) developed a synthetic reporter gene called *NAN*, a codon-optimized derivative of *nanH*, a *Clostridium perfringens* gene, which encodes the so-called “small” cytoplasmic sialidase. This reporter system is aimed at providing a compatible and functionally equivalent reporter gene to *gusA*, which would facilitate dual promoter studies and internal standardization of expression analyses in the same transgenic plant.

In recent transgenic studies, the favorite reporter gene encodes the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994). The lack of requirement for an exogenous substrate, cofactors or histochemical fixation makes GFP a particularly valuable reporter for plant cells. Several modifications of the native GFP have improved its thermotolerance and fluorescence, especially in plants (Siemering *et al.*, 1996; Haseloff *et al.*, 1997). For these reasons, GFP and its variants are widely used as attractive alternatives to *gusA* for assessment of promoter function and in optimizing plant transformation (Stewart, 2001). However, in leaves of transgenic rice, a dramatic decrease of GFP fluorescence was observed as the leaves aged. This was found to be associated with increases in chlorophyll

concentration (Zhou *et al.*, 2005) that, if not appreciated could lead to underestimating GFP expression levels. The removal of chlorophyll by etiolation or extraction with ethanol substantially restored GFP fluorescence in transgenic rice seedlings.

Selection marker genes are employed to select transformed tissues in culture media owing to their ability to confer resistance to chemical components added to the tissue culture media. Early rice transformation studies used the neomycin phosphotransferase (*nptII* or *aph(3')II* or *neo*) as a selection marker, that conferred resistance to aminoglycoside antibiotics. The selection agent used was kanamycin in the protoplast transformation studies, but it was not effective as many calli recovered after kanamycin selection yielded nontransformed plants (Toriyama *et al.*, 1988; Ayres and Park, 1994). A related antibiotic G418 proved more effective as evidenced by high-frequency regeneration of transgenic plants from biolistic transformation (Ayres and Park, 1994), but was still ineffective in *Agrobacterium*-mediated transformation where no transgenic regenerants were recovered (Aldemita and Hodges, 1996), possibly suggesting that prolonged exposure to G418 inhibits regeneration.

For *Agrobacterium*-mediated transformation, the selection marker of choice was hygromycin phosphotransferase (*hpt* or *aphIV* or *hph*), that confers resistance to the aminoglycoside antibiotic hygromycin (Hiei *et al.*, 1994; Dong *et al.*, 1996; Rashid *et al.*, 1996; Aldemita and Hodges, 1996). Hygromycin resistant transformed tissues typically give rise to fertile transgenic plants with few visible phenotypic abnormalities, such as albinos.

Herbicide-resistant genes are also used as selection markers, and for transgenic rice, the most widely used is the *bar* (basta-resistant) gene, that confers resistance to L-phosphinothricin (PPT), glyphosate (an ammonium salt of PPT), and bialaphos (a derivative of PPT) (Christou *et al.*, 1991; Cao *et al.*, 1992; Datta *et al.*, 1992; Rathore *et al.*, 1993).

Recent research has focused on developing selectable markers based on positive selection systems that exploit the plant's own metabolic pathways. The first in line amongst such positive selection systems was that for phosphomannose isomerase (PMI)/mannose (Privalle, 2001). PMI, an enzyme not present in many plants, catalyzes

the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. Plant cells lacking this enzyme are incapable of surviving on synthetic medium containing mannose. Introduction of this gene thus enables selection of transgenic plants on media supplemented with mannose. This selection method was successfully employed for rice (Lucca *et al.*, 2001b; Datta *et al.*, 2003; He *et al.*, 2004). Another novel selection system for rice is based on galactose as the selective agent with an UDP-glucose: galactose-1-phosphate uridylyltransferase gene providing resistance (Joersbo *et al.*, 2003). Yamada *et al.* (2004) developed a selection system based on a mutant rice gene for a feedback-insensitive α -subunit of anthranilate synthase (OASA1D) for the transformation of rice. Expression of OASA1D conferred the ability to detoxify the tryptophan analog 5-methyltryptophan (5MT) in transformed cells of rice. Dai *et al.* (2001a) employed the bacterial gene *codA* encoding cytosine deaminase (CD), which converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a compound that is toxic to cell growth. Transgenic rice plants containing the *codA* coding sequence fused with the phloem-specific promoter of rice tungro bacilliform virus were selected by the addition of 5-FC to the medium. Upadhyaya *et al.* (2000b) generated transgenic rice with the *tms2* gene, a conditional negative selection marker that encodes indoleacetic acid hydrolase (IAAH), which converts naphthaleneacetamide (NAM) to the potent auxin naphthaleneacetic acid, a phytotoxic derivative. This gene, under the control of the manopine synthase 2 promoter from *A. tumefaciens* and exogenously applied NAM was used in selecting transgenic rice. Oreifig *et al.* (2004) demonstrated the use of aminoglycoside-3'-adenyltransferase gene (*aadA*)-mediated streptomycin resistance for nonlethal selection of transgenic rice, which resulted in plant regeneration frequencies under selection pressure as high as those in nontransformed controls without selection.

Recent efforts to improve the rice transformation system have focused on marker-free selection, as this should alleviate environmental safety concerns regarding the release of unwanted resistance genes into the food crop chain. Selection systems often have other limitations. These include negative effects in culture media that decrease the

ability of transgenic cells to proliferate and to differentiate into transgenic plants. Additionally, their inclusion often precludes the use of the same marker gene for gene stacking through retransformation. Tu *et al.* (2003) demonstrated that in the recipient transgenic genome of the elite Chinese CMS restorer line Minghui 63, two independent loci are involved in the integration of the insecticidal protein gene *cryIAb/cryIAC* and selectable marker gene *hph*. The independent locus integration of the transgenes facilitated the removal of *hph* from the gene of interest simply by segregation. Cotsaftis *et al.* (2002) generated transgenic rice with the gene of interest driven by the maize ubiquitin promoter between minimal terminal inverted repeats of the maize *Ac-Ds* transposon system, which was cloned in the 5' untranslated sequence of a *gfp* gene used as an excision marker. This strategy is aimed at transposon-mediated repositioning of transgenes to generate plants that are free of selectable markers and T-DNA inserts. By using a minimal number of transformation events a large number of transgene insertions in the genome can be obtained so as to benefit from position effects in the genome that can contribute to higher levels of expression. These techniques have one major pitfall: the time-consuming exercise of progeny crossing to generate the desired marker-free transgenic plants.

Another advance in the generation of marker-free rice plants involves the MAT vector system that exploits the oncogenes of *Agrobacterium* as a positive selection marker to select transformed tissues in combination with the site-specific *R/RS* system (Endo *et al.*, 2002). The original MAT vector contains the chimeric *ipt* gene that enables regeneration of marker-free shooty phenotypes from which transgenic plants are regenerated through cytokinin-dependent organogenesis (Ebinuma *et al.*, 1997). However, this method was unsuitable for plants dependent on auxin-induced embryogenesis. The system developed by Endo *et al.* (2002) consists of a vector in which the chimeric *ipt* and *gfp* genes fused with the CaMV 35S promoter are combined with the site-specific recombination *R/RS* system, to remove them from the transgenic cells after transformation. Thus, a single-step transformation with scutellar tissues including the excision of the *ipt* gene caused the regeneration of marker-free transgenic rice plants through

embryogenic tissues. Lu *et al.* (2001) adapted an effective “twin T-DNA” binary vector (Komari *et al.*, 1996) to develop a double right border (RB) binary vector, through which transgenic plants would contain two types of T-DNA inserts, i.e., one with both the selectable gene and the gene of interest, and the other with the just the gene of interest, which were then segregated in subsequent generations to recover marker-free transgenic rice. Breitler *et al.* (2004) developed a two T-DNA vector system where the first T-DNA of the vector, delimited by *A. tumefaciens* borders, contains the *hpt* selectable gene and the *gfp* reporter gene while the second T-DNA, delimited by *Agrobacterium rhizogenes* borders, bears the *bar* gene, featuring the gene of interest. Statistical analysis identified in most of the transgenic rice lines a most likely linkage configuration theoretically allowing genetic separation of the two T-DNA types and out segregation of the T-DNA bearing the *bar* gene. The technology was successfully employed in three elite rice cultivars (ZhongZuo321, Ariete and Khao Dawk Mali 105) known to exhibit contrasting amenabilities to transformation.

In another novel dual binary vector system, pGreen/pSoup (Vain *et al.*, 2003), where, pGreen is a small Ti binary vector unable to replicate in *Agrobacterium* without the presence of another binary plasmid, pSoup, in the same strain, marker-free transgenic rice was generated. Co-transformation experiments were conducted using a pGreen vector containing the *bar* and *gusA* expression units (no transgene in pSoup) or with a pSoup vector containing an *aphIV* and *gfp* expression units (no transgene in pGreen) to generate transgenic plants with multilocus unlinked transgene integration, which would allow recovery of selectable marker-free plants in subsequent progeny.

2.4 Efficient Transgenic Plant Regeneration

A prerequisite for a high-efficiency transformation technique is a highly efficient and robust tissue culture system. Transformation processes often involve a long course of tissue culture, subcultures on medium with and without antibiotics, during which the quality of the calli may be lost. The selection of a most suitable subculture medium to improve the quality of calli and later an efficient

regeneration medium might be a key step for the success of transformation.

Transgenic rice regeneration media were mostly MS based with benzyl-aminopurine (BAP) and naphthalene acetic acid (NAA) in *Japonica*, *Indica* (Hiei *et al.*, 1994; Aldemita and Hodges, 1996; Dong *et al.*, 1996), and *Javanica* varieties (Dong *et al.*, 1996). Hormone-free MS media were used for rooting of the regenerated shoots. Inclusion of sorbitol, kinetin and casamino acids also increase efficiency of regeneration (Toki, 1997), and in some cases, an increased amount of sucrose in a pregeneration medium increases efficiency (Uze *et al.*, 1997). For increasing transgenic plant regeneration efficiency in *Indica* varieties, using MS-based media as a starting point, the relative proportions of the nutrients and the ratios of macronutrients and micronutrients were changed taking into account the bioavailability of the nutrients under a given pH range (Lin and Zhang, 2004). Maltose proved to be better than sucrose as the carbohydrate source in both the subculture and differentiation media for *Indica* rice (Lin and Zhang, 2004). Toki *et al.* (2006) designed an efficient protocol, where 1-day precultured seeds were used for transformation, from which transformants were regenerated within 30 days of the beginning of the culture. Regenerated plantlets were transferred to soil at 45 days from the beginning of culture and further grown to maturity. The addition of proline in the culture media in both co-cultivation and regeneration steps, and maintaining cultures at 32 °C was further thought to assist in the recovery of rice cell growth after co-culture with *Agrobacterium*. To recover multiple independent transgenic events from a single immature embryo, Hiei and Komari (2006) devised a procedure, where a rice embryo was sectioned into as many as 30 pieces after nonselective cultures following co-cultivation. Following selection, more than seven independent transgenic plants per original embryo (transformation frequency of 738%) were produced.

Nishimura *et al.* (2005) conducted conventional crosses of low-regeneration rice strain Koshihikari with high-regeneration rice strain Kasalath and identified some QTLs, which control the regeneration ability in rice. Using a map-based cloning strategy, a QTL encoding ferredoxin nitrite reductase (NiR) that determines regeneration

ability in rice was isolated. Molecular analyses revealed that the poor regeneration ability of Koshihikari is caused by lower expression than in Kasalath and the specific activity of NiR. Using the *NiR* gene as a selection marker, transgenic rice was regenerated without exogenous marker genes. This study demonstrates that nitrate assimilation is an important process in rice regeneration and also provides an additional selectable marker for rice transformation.

2.5 Transgene Expression: The Phenomenon of Transgene Silencing

Successful plant genetic engineering relies on stable integration, desired level of expression, and predictable inheritance of the introduced transgenes. A comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment (Dai *et al.*, 2001b) showed that *Agrobacterium* method was a good system to obtain transgenic plants with lower copy number (an average of 1.8 as compared to 2.7 in case of particle bombardment), intact foreign gene, and stable gene expression. T-DNA generally integrates into gene-rich transcriptionally active regions of the rice genome (Barakat *et al.*, 2000; Sha *et al.*, 2004), and on occasions cause integration of multiple elements in various patterns of inverted or tandem repeats (De Buck *et al.*, 2000; Eamens *et al.*, 2004).

Transgenic lines from particle bombardment generally have multiple copy integrations at a single locus frequently displaying non-Mendelian segregation (Pawlowski and Somers, 1996; Kumpatla *et al.*, 1997; Kohli *et al.*, 1998; Kumpatla and Hall, 1999) and are prone to the phenomenon of gene silencing. Transgene silencing could be a consequence of transcription inactivation by promoter methylation, known as transcriptional gene silencing (TGS) or through post-transcriptional gene silencing (PTGS) characterized by specific reduction of cytoplasmic RNA through sequence-specific degradation, with normal rates of nuclear transcription being maintained (Vaucheret *et al.*, 1998). Gene silencing is frequent in transgenic rice generated by particle bombardment (Iyer *et al.*, 2000; Tyagi and Mohanty, 2000).

Kohli *et al.* (1998) proposed that transgene organization in rice engineered through direct

DNA transfer favors a two-phase integration mechanism mediated by the establishment of integration hotspots. This model, together with the exonuclease/ligase model (McElroy *et al.*, 1990) suggests a possible explanation for the occurrence of multicopies of genes located at a single locus in transgenic plants generated by particle bombardment. Kloti *et al.* (2002) demonstrated tissue-specific silencing in rice spread to various organs over generations.

An extensive investigation of several hundred transgenic plants generated by electroporation or particle bombardment revealed that transformants bearing multicopy inserts frequently displayed non-Mendelian segregation (Kumpatla *et al.*, 1997; Kumpatla and Hall, 1999), and characterization of several R₁ progeny revealed that *mUbl1/bar* and *35S/Bt* genes were extensively methylated and transcriptionally inactivated in the silenced lines. The epigenetic modification of the transgene sequences was further confirmed by the reactivation of the *bar* gene expression in R₂ seedlings (from the silenced lines) germinated on medium containing the demethylating agent 5-azacytidine (AzaC) or trichostatin (Kumpatla *et al.*, 1997). It appears that, once a locus is sensitized to methylation, it is rendered very prone to methylation. This was exemplified in an investigation of 34 R₂ plants in which bialaphos resistance was reactivated by seedling exposure to AzaC; however, all except one were resilenced by 50 days after germination. The single line that retained resistance to maturity did not set seed, and after maintenance in the greenhouse for over a year, set seed which when germinated yielded the silent phenotype. Re-establishment of silencing in all the lines was found to correlate in each case with methylation of the ubiquitin promoter (Kumpatla and Hall, 1998a, b), and in a homozygous, nonsilenced R₁ line, promoter methylation and silencing may arise in R₃ generation (Kumpatla and Hall, 1998a, b). Several sites in the 35S promoter that is widely used in transformation are susceptible to fragmentation (Kohli *et al.*, 1999a; Kumpatla and Hall, 1999). The possibility that certain sequences are especially prone to silencing was proved with the root-specific rice *Rcg2* promoter (Xu *et al.*, 1995), which showed transgene silencing even though *Agrobacterium*-mediated transformation was used (Dong *et al.*, 1996). The reactivation of silencing by AzaC

and a meiotic resetting of *Rcg2-uidA* construct was not uniform amongst transgenic lines or even throughout individual plants, indicating that various silencing mechanisms may operate and that the chromosomal position of the transgene may affect the silencing processes (Hall *et al.*, 2001). Fu *et al.* (2000b) discovered that three transgenes at a co-integrate locus along three generations exhibited differential silencing and differential methylation patterns, which did not spread to the flanking transgene, thus confirming that various silencing mechanisms may be operative within the locus.

Other DNA features such as sequential and structural differences between the prokaryotic and eukaryotic DNA, have been suggested as signals that could lead to transgene sequences being recognized by host surveillance systems as intrusive (Kumapatla *et al.*, 1998a, b). Fu *et al.* (2000a) demonstrated that the use of linear transgene constructs lacking vector backbones was very effective in reducing the occurrence of transgene silencing in rice transformation.

Multicopy insertions are susceptible to silencing, and epistatic interactions between multicopy and single copy inserts were shown to silence transgenes in rice, when a single copy gene at one locus was silenced in the presence of locus bearing multiple transgene copies (Hall *et al.*, 2001). The segregation from the multiple transgene locus resulted in reactivation of the gene at the single copy locus reminiscent of paramutation where a paramutated locus regains expression when segregate away from a paramutagenic locus (Matzke *et al.*, 1996).

Alleviation of transgene silencing can be achieved by various strategies such as introduction of sequence heterogeneity in promoters (Kumapatla *et al.*, 1998a, b), suppression of methylation systems and reduction for heterochromatization by insertion of enhancer elements (Francastel *et al.*, 1999), and inclusion of MARs resulting in improved transgene expression in copy numbers up to 20 copies (Vain *et al.*, 1999).

2.6 Transgenic Rice Biotechnology: Important Milestones

Introduction of agronomically important genes into rice by breeders worldwide has contributed to record yields and resistant crops tiding over

both biotic and abiotic stresses to combat global food shortage to meet the predicted demands of increasing population, a fact corroborated by the achievements of the green revolution movement. It is now projected that by the year 2012, rice production needs to increase by 755 million tons (Brookes and Barfoot, 2003). The housing and habitation demands of the increasing population in the developing world has led to sacrificing the farming and crop cultivation areas in favor of residential development (Fischer *et al.*, 2000), and hence the viable option of increasing the areas of rice cultivation is becoming more and more difficult. This is the reason for shaping a vision for a second green revolution (Sakamoto and Matsuoka, 2004) where, rice breeding efforts need to be supplemented with advances in biotechnology especially the introduction of agronomically important genes through genetic transformation. The technology of genetic transformation when the first reports of introducing agronomically important genes in rice dates back to 14 years and the advances in the field show a significant progress (Bajaj and Mohanty, 2005; Kathuria *et al.*, 2007). The preceding account highlights the important milestones in this area.

2.6.1 The first reports

Hayakawa *et al.* (1992) generated the first transgenic rice plants with an agronomically important gene by introducing the CP gene of RSV into two *Japonica* varieties by electroporation of protoplasts. The resultant transgenic plants expressed the CP at high levels (upto 0.5% of total soluble protein) and exhibited a significant level of resistance to virus infection transmitted by an insect vector (planthopper). Fujimoto *et al.* (1993) developed insect-resistant transgenic rice by introducing a truncated δ -endotoxin gene, *cryIAb* of *B. thuringiensis* (*Bt*), which has specific biological activity against lepidopteran insects into *Japonica* rice. To highly express the *cryIAb* gene in rice, the coding sequence was extensively modified based on the codon usage of rice genes. Transgenic plants efficiently expressed the modified *cryIAb* gene at both messenger-RNA (mRNA) and protein levels. Bioassays using R₂ generation plants with two major rice insect pests, striped stem borer (*C. suppressalis*) and leaf folder (*C. medinalis*). Uchimiya *et al.* (1993) developed

bialaphos-resistant rice plants expressing a *bar* gene under the control of the maize ubiquitin promoter, which when inoculated with mycelia of the sheath blight disease pathogen, *R. solani*, and subsequently treated with the herbicide were completely protected from symptomatic infection. This report demonstrated the possibility to design new agronomic strategies for the simultaneous control of weeds and fungal pathogens in fields of transgenic plants expressing a *bar* gene.

2.6.2 Rockefeller Foundation's rice biotechnology program: the saga of Xa21-engineered transgenic rice

The story of the bacterial blight resistance gene *Xa21*-engineered transgenic rice epitomizes the International Program on Rice Biotechnology (IPRB) initiated by the Rockefeller Foundation to exploit the scientific tools of biotechnology in improving rice, and extends the knowledge generation from the program goals to production of improved rice varieties (O'Toole *et al.*, 2001). The scientific saga also serves as an illustrative example of how the knowledge of traditional breeding and biotechnology complement each other in generating disease-resistant rice.

In 1977, *Oryza longistaminata* lines originating from Mali, Africa were identified to carry broad-spectrum resistance to bacterial blight. Khush *et al.* (1991) transferred *Xa21* into *O. sativa* background through interspecific hybridization. Ronald *et al.* (1992) mapped the *Xa21* locus by the restriction fragment length polymorphism technique. Map-based cloning of the gene via BAC library construction (Wang *et al.*, 1995) was followed by the *Xa21* isolation by positional cloning, sequencing, and demonstration of resistance to multiple *X. oryzae* pv. *oryzae* isolates in transgenic rice plants (Song *et al.*, 1995; Wang *et al.*, 1996). The *Xa21* gene was then pyramided with other *Xa* R genes by marker-assisted selection using RFLP and PCR (Huang *et al.*, 1997; Reddy *et al.*, 1997). Tu *et al.* (1998) transferred *Xa21* into the IR72 variety, and later Zhang *et al.* (1998) developed other elite transgenic *Indica* varieties resistant to *X. oryzae* pv. *oryzae*. The first field trials of *Xa* genes, including *Xa21* were reported in China, India, Indonesia, and the Philippines (Rockefeller Foundation, 1999). The final achievement of this scientific saga climaxed with the development

of a commercial hybrid restorer line genetically improved by marker-assisted selection of *Xa21* and the resulting hybrid rice "Minghui 63" demonstrating field level efficiency in combating bacterial blight (Chen *et al.*, 2000).

2.6.3 Insect-resistant transgenic rice

After the first report of insect-resistant *Bt*-transgenic rice (Fujimoto *et al.*, 1993), Wunn *et al.* (1996) developed transgenic *Indica* rice breeding line IR58 expressing a synthetic *cryIAb* gene providing tolerance to several lepidopteran insect pests. Duan *et al.* (1996) engineered the potato proteinase inhibitor II (*pinII*) gene in Nipponbare, Tainung, and Pi4 varieties for resistance to a major insect pest, pink stem borer. The first report of *Agrobacterium*-mediated rice transformation for an agronomically important gene was with insect-resistant *cryIAb* and *cryIIAc* genes to generate transgenic Nipponbare varieties resistant to striped stem borer and yellow stem borer (Cheng *et al.*, 1998). The first field trials of *Bt*-transgenic rice were with transgenic Minghui 63 and Shanyou 63, which showed high insect resistance with no reduction in yield toward leaf folder and yellow stem borer (Tu *et al.*, 2000). The same year was witness to successful field trials of the transgenic KMD1 line of a Chinese *Japonica* cultivar, Xuishi 11, which was resistant to eight lepidopteran pests (Shu *et al.*, 2000). KMD1 and related transgenic lines were also used as insect-resistant rice germplasm for hybrid rice production (Wang *et al.*, 2002).

High *et al.* (2004) suggested that *Bt* rice could contribute in circumventing up to 10% of the Asian rice yield losses caused by the lepidopteran pests. For providing wider and durable resistance to insect pests, combinations of *Bt* with other insecticidal genes sourced from plants themselves such as lectins, protease inhibitors, or ribosome inactivating proteins proved effective (Sharma *et al.*, 2004; Bajaj and Mohanty, 2005).

2.6.4 Nutritionally enhanced "Golden Rice"

Research focused on nutritional enhancement of rice will have a significant impact on the needs of global population, as close to 3.8 billion

people consume it as a staple component of their daily diet. The nutritional component, which attracted major research attention, was β -carotene (provitamin A) due to its absence in milled rice. The increased consumption of this component in daily diet could potentially prevent an estimated 3 million childhood eye defects and even deaths annually (Bouis *et al.*, 2003). Engineering rice with enhanced micronutrient content is hoped to circumvent the global micronutrient underconsumption, as the high costs of supplementary micronutrient-rich nonstaple foods like fruits, vegetables, animal and fish products, and pulses are beyond the reach of millions in the developing countries (Zimmermann and Hurrell, 2002; Bouis *et al.*, 2003).

The research groups of Ingo Potrykus at Swiss Federal Institute of Technology and Peter Beyer, University of Freiberg pioneered the research on provitamin A-enriched rice popularly known as “Golden Rice.” The first step in this research was the generation of transgenic rice with daffodil phytoene synthase gene (Burkhardt *et al.*, 1997). Later, the entire biosynthetic pathway for provitamin A was engineered in the rice endosperm in a single transformation step using phytoene synthase gene (*PSY*) and lycopene β -cyclase gene (*LCY*) from daffodil and phytoene desaturase gene (*crt1*) from bacterium, *Erwinia uredovora* (Ye *et al.*, 2000). Later, it was found that the entire pathway could be reconstituted using only the *PSY* and *crt1* genes in *Indica* and *Japonica* varieties (Hoa *et al.*, 2003), using nonantibiotic mannose-based selection systems (Datta *et al.*, 2003). Later research focused on tiding over limitations and the debate on carotenoid requirements formed in Golden Rice (Bouis *et al.*, 2003; Zimmermann and Qaim, 2004). The use of a codon-optimized *crt1* gene under the control of an endosperm-specific promoter (Al-Babili *et al.*, 2006) and use of maize *PSY* gene (Paine *et al.*, 2005) enhanced the transgenic rice carotenoid content up to 37 $\mu\text{g/g}$ in “Golden Rice-2”, a move now proposed to further improve provitamin A production in rice (Grusak, 2005). An international effort is currently underway to exploit this improved pathway technology in popular rice cultivars of the developing countries (Potrykus, 2001). As a significant step in this direction, Datta *et al.* (2006) developed transgenic high-yielding elite *Indica* rice varieties IR64 and

BR29 with carotenoid content of as high as 9.3 $\mu\text{g/g}$.

2.7 Transgenic Plant Regulation: Biosafety Regulations and Environmental Risks of Transgenic Rice

Release of genetically modified food-crop plants has been a sensitive issue with various stakeholders and the public opinion on it is divided. Most developing countries, where rice is the staple crop for a large number of poor people, welcome transgenic rice both at the public and the government level. Main evidence for this is that most Asian and African countries already have working biosafety regulations for field-testing transgenic crops. Transgenic rice resistant to insect, bacteria, and herbicides has undergone field trials to test for economic profitability and environmental impacts (Oard *et al.*, 1996; Tu *et al.*, 2000; Ye *et al.*, 2001, 2003; Bashir *et al.*, 2004; Huang *et al.*, 2005). Although most of these trials were conducted with transgenic rice containing the vector sequences including the marker and reporter genes, there is indeed a shift to generating transgenic rice without the unnecessary vector and marker sequences. The tools and techniques used toward this aim have been outlined earlier in the chapter. Additional strategies to use endogenous benign selection markers have been explored, for example, genes specific for allowing regeneration in the absence of phytohormones (Zuo *et al.*, 2002), the phosphomannose isomerase (Lucca *et al.*, 2001b), the betaine aldehyde dehydrogenase (Daniell and Dhingra, 2002) or the NiR (Nishimura *et al.*, 2005). A number of reports suggest that there may be no unintended effects of the transgenic rice either within the plant or in the environment (Momma *et al.*, 2000; Wang *et al.*, 2000; Xu *et al.*, 2002; Li *et al.*, 2004; Wang *et al.*, 2006). However, the complexity of the matter in combination with intellectual property rights (IPR) issues is evident from the fact that “Golden Rice” is still not close to being commercialized.

3. FUTURE ROAD MAP

The importance of rice as one of the three important cereal crops in the world can be seen

from the fact that the worldwide produce amounts to more than 600 million tons, and more than 90% of the produce is for human consumption (Kathuria *et al.*, 2007). Since, more than half of the global population derives a significant portion of their caloric intake from rice, it is of utmost importance to focus biotechnological innovations in this crop toward continuous efforts to isolate novel genes and promoters with a view to generating novel transgenic lines with enhanced plant vigor, maximized crop yields, and better tolerance to various abiotic/biotic stresses. The World health Organization estimates that global population would reach 9 billion by 2050, the vast majority forecasted in developing countries of South-East Asia and sub-Saharan Africa. This necessitates biotechnological research in rice toward development of rice cultivars of higher yield potential to achieve higher food production on essentially the limited available land prone to drought, salt, and temperature stresses. Efforts in adapting transgenic rice technology into the mainstream population can already be seen in China, which was the first country to commercialize transgenic rice. The main focus of their efforts was to develop insect-pest-tolerant transgenic rice varieties, and already large-scale field trials since 1998 (Jia *et al.*, 2004) were carried out with several transgenic varieties tolerant to lepidopteran pests, bacterial blight, rice blast fungus, drought, and salt stress. The wide acceptance of transgenic rice in China is hoped to trigger a worldwide acceptance of the transgenic rice especially in the developing countries where rice is a staple crop. The success stories of transgenic rice varieties GM Xianyou 63 and GM-II-Youming 86 (Huang *et al.*, 2005) that require only 20% pesticides compared to untransformed controls, combined with a 6% increase in yield, is a clear testimony of creating environment-friendly rice plants that are of economic benefit to the farmers of the developing countries. A projection of a net gain of 4 billion dollars by China from *Bt* rice by 2010, has triggered cascade of effects in acceptance of transgenic rice in Iran, where 4000 ha are now growing transgenic rice (www.isaaa.org) and in India, where transfer of the “Golden Rice” trait is being taken up in popular rice cultivars (Datta *et al.*, 2003; Hoa *et al.*, 2003). It is now expected that by 2012, up to seven transgenic rice varieties including traits

like herbicide tolerance, disease resistance, abiotic stress tolerance, and nutritional enhancement will be marketed worldwide.

3.1 Expected Products

Transgenic rice technology has crossed the point of a laboratory proof of concept to actually supplement traditional breeder innovations to meet global nutritional demands. This is reflected in the form of the wide spread field trials of insect- and herbicide-tolerant rice in United States, China, South Asia, and the Mediterranean (Bajaj and Mohanty, 2005).

The committed research focus of developing countries like India toward adopting “Golden Rice” technology (Potrykus, 2001; Sharma *et al.*, 2003), has resulted in a projection that provitamin A-enhanced rice would be available for consumers in the developing world within the next 7–8 years (Brookes and Barfoot, 2003).

Efforts made in improving yield by targeting genes like ADP-glucose pyrophosphorylase, which increases seed weight per plant (Smidansky *et al.*, 2003), and transfer of C4 plant genes into rice, which is a C3 plant with an aim to increase photosynthetic efficiency and yield (Matsuoka *et al.*, 1993; Ku *et al.*, 1999), expression of *Arabidopsis* phytochrome A gene to increase yield (Garg *et al.*, 2006) have opened doors for future research in improving grain yields.

3.1.1 Rice as a biofactory

Due to the genomics, transformation and tissue culture resources available for rice and its simple life cycle under varied climatic conditions and standardized agronomic practices, rice has recently acquired the status of biofactory for the production of high-value pharmaceutical or nutraceutical compounds. Additional advantages of producing such compounds in plants rather than the microbes are post-translational modification of the proteins, increased safety for human consumption, extraction and purification cost benefits and storage benefits in seed endosperm (Stoger *et al.*, 2000; Twyman *et al.*, 2003). A number of high-value products have been produced in transgenic rice like a single chain Fv antibody against

a tumor-associated carcinoembryonic antigen (Stoger *et al.*, 2000), human lactoferrin (Nandi *et al.*, 2002; Suzuki *et al.*, 2003), transglutaminase (Claparols *et al.*, 2004), N-hydroxycinnamoyl transferase (Jang *et al.*, 2004), lupin acid phosphatase (Hamada *et al.*, 2004), human lysozyme (Huang *et al.*, 2002a, b; Hennegan *et al.*, 2005), glycogenlike peptide for type II diabetes treatment (Yasuda *et al.*, 2005), recombinant pollen allergen (Okada *et al.*, 2003); linolenic isomers, which reduce fat and hypertension (Kohno-Murase *et al.*, 2006); allergen-specific T-cell epitope (Takagi *et al.*, 2005), and the human granulocyte colony stimulating factor (Hong *et al.*, 2006), antihypertensive peptide fused with rice glutelin (Yang *et al.*, 2006). These examples establish transgenic rice as an ideal model for future pharmaceutical and nutraceutical applications.

3.2 Expected Technologies

Technological innovations in transgenic rice technology have always been geared toward achieving a maximized and targeted expression of transgenes. Novel promoters to achieve high levels of transgene expression like the bacteriophage T7 polymerase (Nguyen *et al.*, 2004), TM2, a novel strong MAR isolated from tobacco (Xue *et al.*, 2005) were shown to increase transgene expression to higher levels compared to traditional promoters like CaMV 35S promoter.

3.2.1 Rice as a model for cereal genome function studies

The complete rice genome sequence has opened up enormous possibilities of map-based cloning of several agronomically important genes. The most significant example of exploiting the information from the completely sequenced rice genome is the analysis throwing up several orthologs of *Arabidopsis* flowering time genes (Goff *et al.*, 2002), which would be of significance when developing high-yielding rice varieties. A high-throughput fluorescent tagging of full-length *Arabidopsis* gene products *in planta* (Tian *et al.*, 2004) could be adapted to analyze proteins of unknown function in the now available complete rice genome sequence. Hirochika *et al.* (2004)

developed a rich resource for mutants for gene discovery in rice, which would enable functional analysis of most of the rice genes. The Rice Full Length cDNA Consortium developed in 2003 has enabled access to nearly 28 000 full-length cDNAs, which when transgenically analyzed aided by the RNA interference (RNAi) approach would aid in deciphering important gene functions.

Sequencing of the rice genome along with generating populations of genome saturation mutagenesis, provides the opportunity to take our understanding of cereal cellular processes and networks to the next level and integrate rice improvement as a crop, as a model plant, and as a plant biofactory. Nearly 38 000 proteins have been annotated in the rice genome, and for more than half of them, functions have not been elucidated. It is also clear that a number of proteins are a part of one or more cellular pathway(s) and hence their function linked to, and to a certain extent dictated by, the function of other proteins. Elucidating these functions and interconnections is most easily possible through specific mutagenesis and/or silencing studies. For mutagenesis studies as well as for generating appropriate transgenic lines, homologous recombination would be the ideal tool. However, very limited success has been achieved with this approach in rice using the recombinase systems available (Cotsaftis and Guiderdoni, 2005). The largely random integration methods of *Agrobacterium*- and biolistics-mediated transformation systems have been used to generate saturation-mutagenized populations of *Indica* and *Japonica* rice. Different reporter genes such as the *gus*, *gfp*, *luc*, and the anthocyanin synthesis *R* gene have been used in coordination with different retrotransposon systems, e.g., *Ac/Ds* (Chin *et al.*, 1999; Enoki *et al.*, 1999; Kohli *et al.*, 2001; Greco *et al.*, 2001; Kolesnik *et al.*, 2004; Upadhyaya *et al.*, 2006), *Tos17* (Hirochika, 2004) or *T-DNA* (Jeon *et al.*, 2000; An *et al.*, 2005a, b) to generate these populations. A number of novel and important genes have been identified using these populations (reviewed in Hirochika *et al.*, 2005; Kathuria *et al.*, 2007), including genes controlling plant height, cellulose synthesis, cell elongation (Zhou *et al.*, 2006), gibberellic acid synthesis gene (Margis-Pinheiro *et al.*, 2005), blast resistance gene (Kim *et al.*, 2003), and pollen development gene (Han *et al.*, 2006).

The now reliable method of gene silencing as a supplementary technology to gene mutation for confirming their role and effects on the plant was discovered as an undesirable trait. Transgenes integrated in plants would stop expressing the protein product or even the mRNA. The phenomenon was first reported in plants such as petunia and tobacco in the early 1990s (Decarvalho *et al.*, 1992; Renckens *et al.*, 1992). The first reports of transgene silencing and its reactivation by 5-azacytidine in rice (Kohli *et al.*, 1996; Kumpatla *et al.*, 1997) paved the way for further gene silencing studies in rice (Kumpatla and Hall, 1998a, b; Fu *et al.*, 2000b). Recent reviews (Kohli *et al.*, 2003, 2006) describe comprehensively various DNA, mRNA, and protein-related factors that contribute to transgene silencing. As a result of extensive studies on transgene and endogenous gene silencing in transgenic plants, methods of RNAi were established to interrogate the role of endogenous genes. RNAi vectors for transient and stable suppression of gene function in rice have been reported (Miki and Shimamoto, 2004; Wang *et al.*, 2004) and the technology has been used to investigate the role of agronomically important genes in rice, for example the silencing of *OSGEN-L*, which causes male sterility (Moritoh *et al.*, 2005). Recent reviews by Mansoor *et al.* (2006) and Kusaba (2004) cite additional examples where the RNAi technology of gene silencing has led to information on the role of different genes in different plants including rice.

3.3 Public, Industrial, Political, and Economic Consequences

There is no doubt about the powerful role of molecular biology and biotechnology in rice improvement for future generations since it directly feeds into the fast-tracked and marker-assisted breeding efforts to generate varieties that yield enough to feed the rising populations. The transgenic technologies can indeed directly generate varieties with desired characteristics but more importantly the transgenic technology is useful for discovering, isolating, and assessing new genes and regulatory elements to understand the genomics and proteomics networks. Such an understanding can in turn lead to fast-tracked

molecular breeding efforts. For what it can deliver in terms of alleviating hunger and poverty, the technology cannot be allowed to stagnate when its use is already showing its potentials (Brookes and Barfoot, 2003; James, 2005). However, at the same time, a rigorous safety, regulatory, surveillance, and risk management strategy must be formulated to ally any public fears.

Major National agencies such as the Animal and Plant Health Inspection Service (APHIS), Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA) in the United States and institute level bodies such as the Institutional Biosafety Committee (IBC) play a role in evaluating the transgenic crop plants for release. For issues involved in biosafety of the transgenic crops, a good resource is the <http://usbiotechreg.nbi.gov> Web site. Research on and release of a transgenic crop spans an entire spectrum of personnel that are in any way connected to such research as for example, starting from the green house manager at the research center, up to the national certification bodies. One of the recent concerns in any applications seeking research or field test permission is the requirement to submit maximum information scientific and otherwise, whether relevant to risk assessment or not. This is not in the interest of the technology or the public. Guidelines are now being increasingly available on what aspects of the research and research product information must be included to make reasonable risk assessment without having to provide reams of unnecessary documentation (www.pubresreg.org). A number of European Union (EU) framework program grants have been funded over the last 8 years under the Quality of Life and Management of Living Resources to address the science and social science aspects of biosafety of GM crops including rice. A collection of reports and papers of the five working groups addressing safety testing, detection of unintended effects, gene transfer, traceability, and societal aspects has been published in two special volumes of the Food and Chemical Toxicology (Kuiper *et al.*, 2004). Surveys conducted at the EU level at regular intervals indicate that the overall public attitude to GM crops is neutral although people in some specific countries may be more for or against the technology (Frewer *et al.*, 2004).

Field tests for “Golden Rice” have been conducted in India, the Philippines, and Vietnam.

However an overzealous precautionary approach has limited the opportunity of commercializing it despite major hurdles on the IPR front being deftly negotiated through the involvement of a consortium of private and public stakeholders including major rice research and breeding centers in Asia.

Acceptance of transgenic rice in developing countries will enable governments of other neighboring countries also to take a stand considering the potential economic and human resource benefits. Additionally, rice is being increasingly used in molecular farming enterprises and its genomics resources are once again erecting it as a model to study cell wall synthesis and breakdown for the biofuels industry. Most plant-based bioethanol projects are centered on cereals/grasses such as *Miscanthus*, sweet sorghum, maize, etc. In view of these developments the transgenic technology of rice crop points toward a bright future.

4. CONCLUSION: RICE—A MODEL MONOCOT

A small genome, an efficient transformation technology, a comprehensive physical map and more recently, the complete genome sequence made rice achieve the status of a “model monocot”. Information gleaned from the genome is currently being utilized to understand fundamental aspects of growth, development, and physiology of the plant kingdom. The functional aspects range from elucidation of individual gene function to deciphering and engineering metabolic pathways. The extensive ancestral synteny shared by rice with other cereals enables its exploitation in designing research to genetically improve other related cereals with larger genomes like maize, wheat, and barley.

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Maize

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1. INTRODUCTION

The commercial application of agricultural biotechnology in corn has primarily focused on the development of “input traits”, which provide attributes beneficial to the grower. Examples of licensed products comprise herbicide and pest resistance traits with others such as disease, drought, salinity, and cold resistance, and enhanced photosynthesis and nitrogen fixation under development. Because input traits improve crop production, agricultural companies have leveraged the technology to maintain and expand market share.

Biotechnology is also enabling the creation of new products, called “output traits”, aimed at the farmer’s downstream customers. Such traits may provide improved nutritional qualities for animal feed, healthier and more flavorful foods, specialty chemical and biologic compounds, or improved processing capabilities (Shoemaker *et al.*, 2001). Two specific examples are Syngenta’s corn amylase and phytase varieties with embedded enzymes for improved ethanol production and increased utilization of phytate in animal feed (Kemble, 2006). Such opportunities remain largely unmet in the market place, thereby providing new growth areas for agricultural biotechnology companies and promising to provide benefits for agricultural processors and consumers. While output traits

will still be used to expand market share, they will provide potentially greater downstream value and will require new business models based upon thorough analyses of each specific target industry to determine the best approaches for value capture.

Corn ethanol production in the United States is one area where biotechnology may be applied to dramatically decrease costs, improve energy input requirements, and expand capacity. Numerous opportunities exist for improving important characteristics of the corn plant to decrease the cost of producing ethanol. However, the cost of developing a biotechnology crop with approval from the appropriate regulatory authorities is high. Therefore, these opportunities must be analyzed in an industrial context, understanding the market size, growth and potential return to ensure that the most value is created in the shortest period of time.

Regardless of the plant chosen to be manipulated through biotechnology for ethanol production, four technological platforms must be in place. Firstly, robust tissue culture protocols must be in place that couple the production of large numbers of regenerated plants with substantive reductions in the timing of the life cycle. Secondly, this tissue culture technology in turn must be linked to high-frequency DNA transfer whereby the cell competent to regenerate is likewise competent with respect to nucleic acid uptake. Thirdly, the genes and/or their promoters

that increase some aspect of ethanol production should be amenable to expression optimization. Finally, a processing technology must be in place that maximizes the recovery of ethanol. In this chapter, the advances in maize tissue culture and transformation, in gene discovery and expression optimization, and maize ethanol processing are reviewed. The position affirmed here is that technical advances in maize biotechnology make it today's model plant for ethanol recovery.

1.1 History, Origin, and Distribution

Maize (*Zea mays* ssp. *mays*) originated from "teosinte" following mutation and recognition by man that this change should be capitalized upon through a conscious breeding effort. In fact, it has been argued that the derivation of corn from teosinte is man's first and most significant achievement of genetic engineering (Grun and Federoff, 2004). Maize is thought to have originated in Mexico and can be traced minimally back to at least 5000 years BC. (Jugenheimer, 1976; Brenner, 1991). Cultivation of maize and the expansion of its food products are the base structure of the pre-Colombian Mesoamerican civilizations. Commercial maize is cultivated from latitude 50°N to 40°S. Given this, modern maize can adapt to different climates and soil types. Currently, maize is cultivated throughout the world, in tropical and temperate climates, and it is considered to be the third most planted crop after wheat and rice (Wallace and Brown, 1988; Brenner, 1991).

1.2 Botanical Description

Maize is a cross-pollinating species and thus morphological variability is common among the different geographies where it is grown. The height of the plant may range from 0.6 to 6 m but usually it reaches 2.4 meters. Most of the varieties mature in 100 to 140 days from planting and produce one to three ears per plant, averaging 800 kernels per ear. The kernel number, in part, depends on ear length and yield ranges from 0.5 to 23.5 tons of grain per hectare.

Soil moisture and temperature are important factors in maize germination and early plant growth. At temperatures of 10°C and lower, maize growth is significantly impaired. Optimal maize

growth occurs at 27°C, under conditions of limited soil moisture, and 35°C, when soil water content is optimal. Cool, humid, and cloudy conditions will inhibit pollen production. In general, pollen shedding begins approximately 2 to 3 days before the silks emerge. The tassel continues to shed for another 5 to 8 days. The silks, in contrast, begin the elongation 7 to 10 days before emergence. Maize requires approximately 50 cm of water during the growing season and drought conditions, as expected, seriously impair seed set (Aldrich *et al.*, 1975). In this connection, America's Corn Belt drives its productivity through the fortuitous combination of moderate temperatures coupled with substantial rainfall and soil with ideal moisture-holding capacity.

The genus *Zea* is a grass that belongs to the family Gramineae. *Zea mays* is the most common cultivated species among other species including *Z. diploperennis*, *Z. luxurians*, and *Z. perennis*. *Zea* species are diploids with $n = 10$ chromosomes. A wide assortment of chromosome knobs, inversions, translocations, duplications, polyploids, and trisomic lines have been long available to maize workers and have, in no small way, contributed to the rapid progress of maize genome mapping. Indeed, it was the availability of this material that allowed Creighton and McClintock (1931) to show that crossing over accompanied the physical exchange of chromosome segments to show that genomes were not stable but segments thereof could move from one location to another. The utility of these chromosomal aberrations is limited in that it gives a global understanding as to how the genome is organized but little information of the organization of gene distribution.

The sophisticated information gathered from the publicly funded maize sequencing and database efforts has contributed greatly to our detailed understanding (Chandler and Brendel, 2002; Lawrence *et al.*, 2004). The corn genome is large, in fact six times larger than that of rice, and comprises 2.5 Mb. Notably, the genes comprise only 20% of the genome. The genes are not distributed randomly as a function of chromosome length but rather in a clusterlike fashion. Between these clusters are conserved high copy number retrotransposons, in addition to other repetitive sequences. The gene blocks themselves vary in size and may be likened to isolated islands surrounded by oceans of recurring DNA sequences. Indeed,

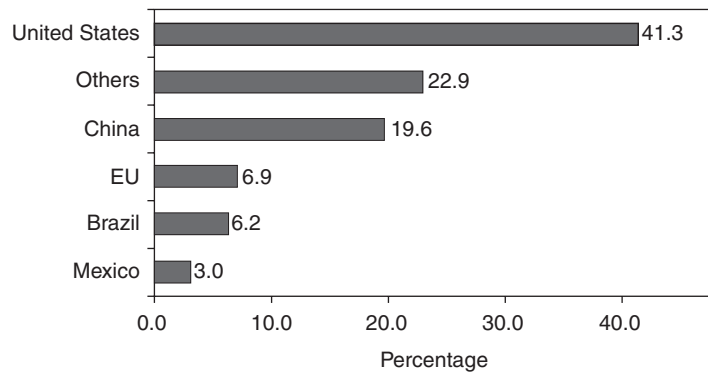


Figure 1 Corn world production for 2005–2006 [Reproduced from NCGA (2006)]

Messing *et al.* (2004), using 475 000 bacterial artificial chromosome end sequences, concluded that a significant portion of the maize gene set consists of randomly arrayed gene families.

1.3 Economic Importance

Maize is one of the most important commercial cereal crops in the world. The major maize production concentrated in the United States, China, Europe, Brazil, and Mexico collectively. These countries account for approximately 77% of the world's annual maize production. The United States alone is responsible for 41.3% of this production (Figure 1).

The most useful part of maize is the kernel. The kernel consists of the endosperm and the embryo and is produced following double fertilization. One sperm fertilizes the egg and gives rise to the diploid embryo, whereas the second sperm fertilizes the

polar nuclei, resulting in the production of a triploid endosperm. The pericarp is the tissue that surrounds the seed and is of maternal origin.

The endosperm is the primary storage tissue for starch and proteins that are essential for seed germination and is equal to approximately 82% of kernel's dry weight. The starch is a source of food, sweeteners, bioplastics, and fuel in addition to other industrial uses. Maize is the most significant crop in the United States with a value of 21.1 billion dollars. Nearly 81.8 million acres were planted throughout the United States in 2005. About 55.5% of the grain corn produced in the United States is fed to livestock, whereas approximately 17.1% of grain corn is exported either as grain or corn product. The remaining 14.6% is used to produce ethanol and 12.8% is processed. Processed products include starch, corn sugar, corn oil, gluten feed and meal, whiskey, alcohol, and for corn meals, corn flakes, etc. (Figure 2).

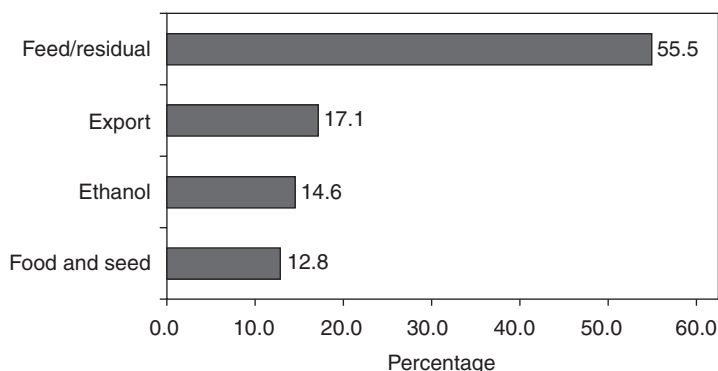


Figure 2 Usages of corn in the United States for 2005 [Reproduced from NCGA (2006)]

The maize market is experiencing change due to a high demand for ethanol. In consequence, the per-bushel price of corn is likely to rise as the grain is shifted from its traditional uses over to biofuel production. Indeed, recent estimates hold that corn directed toward ethanol production is expected to bring \$4.00/bushel.

1.4 Traditional Breeding

While classical breeding programs have produced high-income seed lines, tolerant and/or resistant to abiotic and biotic stresses, their synthesis is time consuming and beset many difficulties including linkage drag. Marker-assisted breeding (MAB) has vastly simplified plant breeding. From an operational point of view, molecular markers define regions of the chromosome to which a gene is linked. The lower the frequency of crossing over, the fewer the recombinants and the tighter the linkage is between the marker and the gene of interest. Thus, in the absence recombination, the transmission of a particular DNA marker co-segregates with a gene 100% of the time. Hence, the presence of marker, as determined by DNA hybridization, establishes the inheritance of the gene.

The application of MAB in maize is well established. Since the earlier 1990s, maize workers had available to them a core set of restriction fragment length polymorphism markers that marked the maize genome, available from the University of Missouri. Hosts of other markers have since been developed and their availability and description are posted on the Maize GDB Web site (www.maizegdb.org). Many examples are available for discussion.

Recently, Ribault and Ragot (2007) have followed the introgression of favorable alleles at five target regions in maize. Characteristics selected included increased yield, reduced water dependence, and increased synchrony between male and female flowering. Between three and four generations were needed to produce plants that recovered 85% of the parental genotype. BC₂F₃ were backcrossed to tester and then evaluated for drought resistance. When multiple hybrids were tested, the best performers increased the response to drought by 50%.

1.5 Limitations of Conventional Breeding

The product of multiple generations between the initial cross and a hybrid expressing the desired phenotype, whose parental alleles are largely preserved, is obviated by cloning. First, parent inbreds of high-performing hybrids can be altered by a single gene, thus conserving 100% of the hybrid genotypes selected through breeding. Second, it is possible to deliver single genes of commercial significance from a species totally unrelated to maize in a single transformation cycle. From a practical and economic point of view, this means that the labor-intensive, multigeneration breeding protocols needed to select for a disease-resistant phenotype are reduced, under ideal conditions, to a series of established tissue culture/transformation protocols (Sairam *et al.*, 2003). This is no small consideration given the increasing demands that have been put on alternative uses for maize, which will likely reduce its availability to its traditional markets.

1.6 Development of Transgenics

A number of cereal transgenics are available that have both social and commercial value. For example, the development of golden rice that is rich in vitamin A can combat the development of infant blindness in countries too poor to have the vitamin widely available for purchase. The development of this rice required no less than the transfer of nine different genes; a process that would have been cumbersome using traditional breeding. The production of genetically modified maize has found a number of unique economic niches. Strains of maize resistant to the European corn borer (ECB) are available for purchase. In consequence, billions of harvest dollars are saved annually in the United States (Hyde *et al.*, 1999; Wu, 2006). However, not all transgenic production is without controversy. Some issues related to yield were raised with respect to lines segregating the *Bt* gene (*Bacillus thuringiensis*). However, when incremental losses in *Bt* plants were compared to losses resulting from diseased hybrids lacking this gene, the presence of the *Bt* gene was proven to significantly increase yield (Lauer and Wedberg, 1999; Dillehay *et al.*, 2004). The efficacy of the *Bt* gene lies in the fact that the

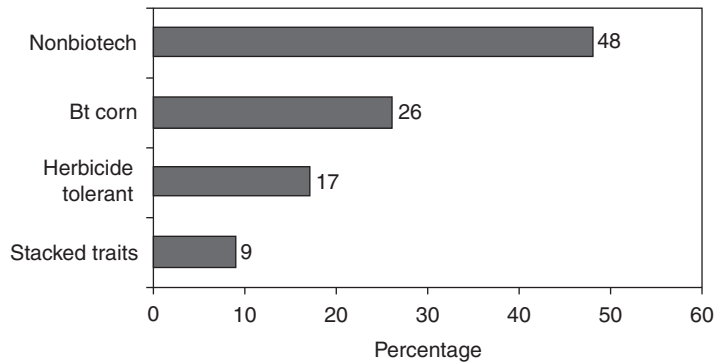


Figure 3 Percentages of biotech and nonbiotech corn planted in the United States in 2005 [Reproduced from NCGA (2006)]

protein encoded is nontoxic to humans, animals, and honeybees (Ostlie *et al.*, 1997). The use of *Bt* corn carries with it additional social advantages, not the least of which is a reduction on the dependence of environment-challenging insecticides (Jauhar, 2001). Despite the obvious benefits of *Bt* corn, only 26% of the corn planted in the United States is genetically modified against ECB (Figure 3). Additional transgenics that have been developed for their potential economic advantages include enhanced antioxidant and essential amino acid production (Xuehui *et al.*, 2001; Dörmann, 2003) in addition to edible vaccine production (Guerrero-Andrade *et al.*, 2006).

2. GENE DISCOVERY

2.1 Introduction

The recovery of ethanol from maize starch is a first-generation technology. It has been estimated that there are 998 million dry tons of agricultural resource materials available, exclusive of an additional 368 million tons of forest resource. Regardless of the plant source, a successful ethanol biotechnology requires the ease of access to cellulose *per se* in order to solubilize hemicellulose sugars. Subsequently, cellulose is hydrolyzed to sugar, which in turn can be fermented to alcohol.

Gene discovery will make possible the designing of transgenic plants that frees cellulose from the complex polymer matrix that associates it with lignin. In principle, genes can be selected that affect some aspect of lignin biosynthesis such that either the amount synthesized and/or deposited

is reduced or the binding altered such that lignin is more easily released during biomass treatment with heat, enzymes, or acids. Once released, the cellulose itself can be hydrolyzed to its component sugars following the identification, cloning, and modification of high-performance cellulase genes and ethanol production optimized by any number of adapted genes specific to the fermentation process. A host of different protocols have been developed for maize and other plants that facilitate the isolation of genes with the expressed goal of altering some aspect of metabolism leading to the commercialization of an agri-value-added product.

The first step for transgenic maize product development is gene discovery. Gene discovery is the process of isolating a gene sequence and defining its biological function. The biological function or trait targeted for improvement in the product will determine the most optimal method used for gene discovery. In general, there are two approaches available for gene discovery: forward genetics and reverse genetics (Settles, 2005). Forward genetics starts with a phenotypic variation, most often a loss-of-function phenotype caused by a monofactorial recessive mutation, which defines the biological function of the underlying gene. For example, if the transgenic product target is earlier flowering maize varieties, then the desired gene would be one that induces or promotes flowering and whose loss-of-function phenotype is late flowering. Using forward genetic techniques, the gene sequence underlying the late flowering mutation is identified and the functional gene is used in the transgenic product. Alternatively, reverse genetics begins with a known gene sequence with a presumed relevant gene

function. Various reverse genetic techniques are employed, often relying on gene disruption, to determine the biological function of the known gene. Several forward and reverse genetic methods are available to the maize researcher for gene discovery and these are described briefly here.

2.2 Forward Genetics

2.2.1 Transposon tagging

Maize transposable elements or transposons have been used for decades for gene isolation and functional studies. Several two-component transposon families exist in maize, including the *mutator* (*Mu*), *associator/dissociator* (*Ac/Ds*) and *suppressor-mutator* (*Spm*) families, to name a few of the most often utilized (Masson *et al.*, 1991; Tacke *et al.*, 1995; Long and Coupland, 1998; Brutnell, 2002; Lisch, 2002; Kumar *et al.*, 2005; Weil, 2005). In general, insertion of the transposon into a gene disrupts its function leading to a loss-of-function mutant phenotype. Such a phenotype provides the phenotypic variation used for forward genetic gene discovery. Since the application of transposons for gene disruption and for gene isolation, also known as transposon tagging, are very similar regardless of the family of transposable element used, two different schemes for transposon tagging are described herein using the *Mu* family of elements, given that *Mu* elements have been the most successfully used family for tagging (Bensen *et al.*, 1995; Winkler and Helentjaris, 1995; Gao *et al.*, 1998; Papa *et al.*, 2001; Fu *et al.*, 2002; Dinges *et al.*, 2003; Shen *et al.*, 2003; Shi *et al.*, 2003; Carey *et al.*, 2004; da Costa e Silva *et al.*, 2004; Xiaoli *et al.*, 2004; Ada *et al.*, 2006).

Transposon tagging can be either targeted or random depending on whether the desired type of mutant phenotype already exists. If the desired type of mutation exists, then a targeted transposon tagging strategy would be used. If the desired type of mutation has not previously been identified, then a random transposon tagging strategy would be employed. For targeted transposon tagging, a reference mutation with the desired phenotype is crossed with active *Mu* lines that are wild types for the reference mutation. Again, using flowering time as our target trait, plants homozygous for a described late flowering mutation, such as *delayed*

flowering1 (*dlf1*), for example, would be crossed onto diverse active *Mu* lines that have a normal flowering phenotype (Muszynski *et al.*, 2006). *Mu* elements will transpose inserting into new genomic locations, disrupting gene function. Therefore, at low frequencies, an element will insert into the functional copy of the *dlf1* locus in the *Mu* active parent leading to a new nonfunctional *dlf1-mu* insertion allele. To uncover such an event, a large F₁ population of between 20 000 and 30 000 individuals is required, as the rate of forward mutation in active *Mu* lines varies on average from 10⁻⁴ to 10⁻⁵. The F₁ population is screened for rare individuals, which display the reference mutant phenotype; in this case, late flowering. Once identified, the rare mutant individuals are outcrossed to produce segregating families. The map position of the reference mutation must be known, as linked DNA markers are used to differentiate the reference mutant allele from the new *Mu*-insertion allele after outcrossing. Co-segregation of the new *Mu*-insertion mutant allele with *Mu* element sequences is then used to isolate the cognate gene. Targeted *Mu* tagging has been used successfully to isolate a number of maize genes affecting a variety of kernel, seedling, and adult plant traits.

Random *Mu* tagging is used if a reference mutation with the desired phenotype does not already exist. In this strategy, active *Mu* lines are outcrossed to non-*Mu* inbred lines creating F₁ seeds. F₁ plants are self-pollinated to create F₂ families, which are then screened for segregation of the desired novel mutant phenotype. Once identified, co-segregation analysis of the new *Mu*-insertion mutant allele with *Mu* element sequences can be used to isolate the cognate gene. Given the forward mutation rate of *Mu*, tens of thousands of F₂ families might need to be screened in order to identify the desired mutant phenotype. Depending on if the mutant phenotype affects kernel, seedling, or adult plant traits, this strategy may not be efficient. An alternative mutagenesis strategy utilizing chemical or radiation mutagenesis in connection with transposon tagging may be more efficient. Chemical or radiation mutagenesis methods often have a higher forward mutation rate than *Mu*. For example, ethyl methane sulfonate (EMS) mutagenesis of maize pollen can produce mutations at rates from 10⁻³ to 10⁻⁵. In addition, screening a chemically mutagenized

inbred population offers the advantage of being able to identify mutations with more subtle mutant phenotypes, as the background variation is expected to be uniform. Therefore, as an alternative strategy to random transposon tagging, random EMS mutagenesis and screening for the desired mutant phenotype can be used as a first step. After the desired mutant phenotype is identified, a targeted transposon tagging strategy can be used as previously described.

2.2.2 Positional cloning

With recent improvements in the density of markers on the maize genetic map and integration of the genetic map with the high-coverage physical map, positional or map-based gene cloning in maize is now feasible (Coe and Schaeffer, 2005; Rudenko *et al.*, 2005). The development of a complete or near complete genome sequence in *Arabidopsis* and rice has enabled positional cloning to be the standard method for gene isolation in these species. Although a complete maize genomic sequence is not yet available, efforts to sequence the maize genome are currently underway (<http://www.maizegenome.org/>). Despite this, the requisite tools for positional cloning in maize are available. In general, positional cloning relies on reiterative mapping of DNA markers, either simple sequence repeats or single-nucleotide polymorphisms (SNPs), against a segregating phenotype, typically a mutant phenotype. Co-segregation of markers linked to the mutant phenotype defines a recombination interval wherein the underlying gene is located. Reiterative mapping with evermore tightly linked markers and an ever-increasing population size defines a smaller and smaller recombination interval. Mapping is repeated until a single gene or open reading frame is identified. A review detailing the steps for positional cloning in maize has recently been published (Bortiri *et al.*, 2006). Therefore, we refer the reader to this article for information on positional cloning considerations and examples of recently map-based cloned maize genes.

2.2.3 Reverse genetics

As mentioned previously, a reverse genetics approach begins with a known gene sequence having

a presumed relevant gene function. The goal of reverse genetics is to determine or confirm the suspected gene function. This is typically accomplished by disrupting the function of the gene to produce a loss-of-function phenotype. From the nature of the mutant phenotype, one can infer a biological activity for the candidate gene. For example, disruption of the sequence homologs of the meristem identity genes *FLORICAULA* (*FLO*) from *Antirrhinum* and *LEAFY* (*LFY*) from *Arabidopsis* identified the duplicate *Zea FLO/LFY1* (*zfl1*) and *Zea FLO/LFY2* (*zfl2*) genes as the functional orthologs in maize (Bomblies *et al.*, 2003). This example highlights an important consideration when using reverse genetics. Because of gene duplication and functional redundancy, both mutated *zfl1* and *zfl2* paralogs had to be combined as a double mutant in order to display an obvious loss-of-function phenotype. Moreover, each single *zfl* mutation did display an altered phenotype but each was subtle, requiring replicated measurements and statistical analyses to detect them. Thus, reverse genetics moves from a gene to a phenotype from which gene function can be inferred. Several reverse genetic techniques have been successfully utilized and are briefly described here.

2.2.4 Trait utility system for corn (TUSC)/maize targeted mutagenesis (MTM)

Two reverse genetics methods are available, which rely on the high mutagenic activity of *Mu* transposons: the Pioneer Hi-Bred TUSC and MTM (Till *et al.*, 2003a; Henikoff *et al.*, 2004). TUSC is a proprietary collection of indexed active *Mu* x inbred F₁ DNA linked to segregating F₂ progeny seed. MTM is a similar publicly available collection of indexed F₁ DNA and F₂ progeny seed (<http://mtm.cshl.edu/>). Both collections are sufficiently large that they are expected to contain an instance of a *Mu* insertion in every gene in the maize genome. Both collections are used in the same manner. For each candidate gene of interest (GOI), both forward and reverse polymerase chain reaction (PCR) primers are designed to specifically amplify defined regions of the candidate GOI. Each forward and reverse primer is paired with a degenerate primer designed from the consensus *Mu* terminal inverted repeat (TIR) sequence in

PCR amplification reactions. Thus, gene-specific primer and *Mu*-TIR primer amplifications are performed on the complete F₁ indexed DNA collection, with appropriate controls. Amplification of a gene-primer + *Mu*-primer product identifies an F₁ plant that carried a *Mu* insertion in the candidate GOI locus. Sequencing the amplification product will identify the exact insertion site of the *Mu* element in the genomic sequence and will aid downstream analysis. The F₂ progeny seed from the identified F₁ DNA sample is obtained, grown, and observed for segregation of any mutant phenotype(s). Characterization of additional independent *Mu*-insertion alleles and co-segregation analysis will confirm the suspected biological function of the candidate GOI. Several maize genes, homologous to genes from other plant species, have been confirmed to mediate the same biological function or have identified novel functions. As mentioned earlier, due to extensive duplication of the maize genome, gene redundancy is often an issue with any reverse genetics approach.

2.2.5 Targeting induced local lesions in genomes (TILLING)

Another reverse genetics tool available to the maize community is TILLING (Till *et al.*, 2003b, 2004; Weil *et al.*, 2005). TILLING relies on the high forward mutation rate of EMS mutagenesis and the ability to detect the resultant transversions or transitions as SNPs in PCR amplification products. First established for use in *Arabidopsis*, it has now been adapted as a maize functional genomic tool (<http://genome.purdue.edu/maizetilling/>) (Singh *et al.*, 2003; Kolkman *et al.*, 2005). TILLING is done in a manner similar to TUSC/MTM. Candidate GOI-specific primers are used to query a collection of DNA from a mutagenized population for loss-of- or altered-function alleles. Different algorithms aid in the positioning and design of PCR primers, aimed to optimize identification of nucleotide changes that would impact gene activity. Once different point-mutation alleles are identified, the corresponding F₂ or F₃ families are grown. Analysis of F₂ or F₃ families for a mutant phenotype that co-segregates with the new EMS allele will determine gene function.

2.2.6 Mapped activator

Recently, a reverse genetics method utilizing the tendency of *Activator* (*Ac*) transposable elements to insert into linked sites has been developed (Brutnell, 2002; Cigan *et al.*, 2005; McGinnis *et al.*, 2005). Regional mutagenesis utilizing *Activator* in maize (http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_tagging.html) aims to place an active *Ac* element every 10 cM (centi-Morgans) throughout the maize genome. The system relies on the tendency of *Ac* to transpose to linked sites combined with a reporter allele, which indicates *Ac* dosage. The map position of the candidate GOI must be known in order to choose the closest linked *Ac* line with which to initiate the mutagenesis screen. In brief, the mapped *Ac* line linked closest to the GOI is outcrossed to the reporter allele line. Kernels with a transposed *Ac* are selected, based on their increase in *Ac* dosage, and grown. PCR screening with GOI-specific and *Ac* termini-specific primers will indicate if the newly transposed *Ac* has inserted in or near the GOI. Plants carrying an *Ac* insertion in or near the GOI are self-pollinated producing segregating families. Families are observed for an altered phenotype co-segregating with the *Ac*-insertion allele, thereby confirming gene function. Although primarily used as a reverse genetics tool, the mapped *Ac* lines can also be used for targeted transposon tagging using a reference mutation that maps within 10 cM of a mapped *Ac*. While still being developed, this technique provides the maize community with another functional genomics tool for gene discovery and functional analysis.

2.3 Verification

Once a gene has been identified and assigned a biological function, the next step in product development is to verify that modulation of the gene's expression in transgenic maize plants does indeed affect expression of the target trait in a desirable and predictable manner. Modulation of gene expression is typically done using one of the following three methods: gene overexpression, gene silencing, or gene shuffling. The method of expression modulation depends on the nature of the gene's endogenous expression and the desired phenotype of the product. For example, to

produce early flowering in maize, overexpression of a floral induction gene, like *dlf1*, would be tested in different transgenic constructs by driving expression of *dlf1* with constitutive promoters of varying strengths. Once a method of expression modulation is selected, the next step is to generate a sufficiently large number of events of each construct to adequately evaluate their efficacy. This step is dependent on having an efficient and high-capacity transformation system. Moreover, transformation in an inbred background facilitates phenotype screening in either inbred or hybrid backgrounds. The final step in verification is to develop and apply suitable molecular and phenotypic assays to ensure that the construct meets acceptable efficacy criteria to warrant its advancement through the product development process. Each of these steps is discussed in more detail below.

2.3.1 Modulate gene expression

Modulation of gene activity for product development involves gene overexpression, gene silencing, or gene shuffling. Each produces a new dominant trait to be incorporated into a commercialized product. Overexpression produces more of a gene product, usually in additional tissues and for a longer period of time than the endogenous gene. Typical constitutive promoters, such as *ubiquitin* or *actin*, are used, although there are many other choices available. Additionally, constitutive promoters of differential strengths—mediating high, medium, or low expression levels—are tested to determine an optimal degree of regulation. Gene silencing is another method used to modulate gene expression and serves to down-regulate or terminate gene activity. This can be achieved by several methods. Typical silencing constructs rely on inverted or direct repeats to create double-stranded hairpin transcripts of coding or promoter sequences. Other silencing strategies use RNA interference (RNAi), antisense RNA (asRNA), or small interfering RNA (siRNA) approaches, to name a few (Waterhouse *et al.*, 1998; Boubakri *et al.*, 2006; Hecky *et al.*, 2007). The third method of expression modulation is gene shuffling. Gene shuffling is a process of *in vivo* mutagenesis and selection for altered or novel protein activity (i.e., increased thermotolerance, altered enzyme

specificity, or improved kinetics). The process for gene shuffling is well described (Castle *et al.*, 2004; Pattanaik *et al.*, 2006). Shuffled genes are usually selected in microbes, such as bacteria or yeast, as this facilitates the automation and screening of sufficiently large and complex shuffled libraries. Gene shuffling has only recently been applied to agricultural improvement, as there are inherent limitations with selection *in planta* at each round of shuffling. Even so, some success has been reported. Once an appropriate method for gene expression modulation is determined, the resultant promoter–gene combination is constructed and maize transformants are generated.

3. PLANT TISSUE CULTURE AND TRANSFORMATION

3.1 Introduction

The development of robust plant regeneration protocols from single cells capable of being transformed at high frequency that combines speed, output, genotype independence, and host genome stability has been long recognized as the holy grail of plant biotechnology. In order to meet this goal, many different explants have been tested for their regeneration capacity. These include shoot apices, leaf and stem segments, hypocotyls, epicotyls, immature embryos, and mature intact seed. Regeneration of cells derived from these explants has been attempted and achieved through manipulation of a number of developmental pathways. Plants can be regenerated by multiplying preexisting shoot meristems, by direct shoot morphogenesis, and indirectly and/or directly from undifferentiated cells, such as those associated callus and through somatic embryogenesis (Thorpe, 1994).

Historically, the development of plant tissue culture, especially in the cereals, developed independently of any associated transformation endeavors. Indeed, the first transformation attempts by Coe and Sarkar (1966) attempted to produce genetically modified pollen following treatment of the developing tassel with DNA in solution. In consequence, as new transformation technologies were developed for tobacco, these were tested in turn for their applicability with respect to the cereals. Hence, in the early days of

cereal biotechnology, tissue culture development and transformation did not develop in parallel.

Different transformation systems have been tested on maize cells. These include the direct DNA uptake technologies embracing polyethylene glycol (PEG) Ca^{++} enhancing, silicone carbide whiskers, microinjection, and electroporation-mediated nucleic acid transfer. The conspicuous advantage of these technologies in aggregate is that DNA delivery is genotype independent. However, in order to be used at all, totipotent protoplasts must be available for transformation across a wide variety of genotypes within a given species. This has yet to be achieved. Biolistics is also a direct transfer technology whose use is protoplast independent but whose maximal utility requires genotype-independent regeneration. The use of *Agrobacterium tumefaciens*-mediated transformation requires suitability of host range and that the genetically modified cells are regenerable following infection.

This section summarizes the major advances that have been made toward the production of maize transgenics. In particular, it will be argued that the community is positioned to produce, in large numbers, transgenic cereals independent of genotype (Sairam *et al.*, 2002, 2003; Al-Abed *et al.*, 2006) and whose commercial limitations will be a function of the cloned genes available for exploitation and commercialization. Finally, the most recent progress in this lab using the newly reported split-seed technology is reviewed: a tissue culture procedure from which 50 plants can be regenerated from a single seed, each originating from a single shoot bud.

3.2 Explants Used for Maize Regeneration and Transformation

Minimally, there are four requirements needed to produce a transformation protocol that have equal utility in academia and industry. First, those cells targeted for DNA uptake must be capable of regenerating fertile plants. Second, regeneration should be genotype independent, particularly with respect to inbred lines. Third, regeneration should be rapid, producing large numbers of plants arising from either single cells or shoot buds. Finally, the system should be user friendly, minimizing growth chamber space, greenhouse footprint area,

and labor. In the following sections, the different explants are reviewed that have been tested for regeneration and transformation in maize and have been evaluated according to these criteria.

3.2.1 Immature embryos

Immature embryos have been extensively used for maize regeneration and transformation since the first report on the establishment of totipotent callus from which maize plants regenerated (Green and Phillips, 1975). Plant regeneration from immature embryos can be obtained by somatic embryogenesis and organogenesis. In the case of somatic embryogenesis, callus is induced from immature embryos and with further culturing the callus becomes embryogenic and produces somatic embryos, which in turn germinate to produce a complete plant with a shoot and root. In organogenesis, the shoots are either directly induced from embryos or directly regenerated from callus (Springer *et al.*, 1979; Lu *et al.*, 1982; Lowe *et al.*, 1985; Vasil *et al.*, 1985). Thus, the utility of this explant depends upon a readily available supply of embryos that commits greenhouse space to this effort. This dedication can be significant; especially if a large number of inbreds are being tested for their suitability in the production of transgenic hybrids.

The callus from maize cultures is mainly classified into three types. Nonembryogenic callus is watery, usually turns brown and loses its ability to regenerate and therefore is unsuitable either for propagation or transformation. Type I embryogenic callus is usually compact and white in color and plants can be regenerated directly through organogenesis. Unfortunately, the utility of this type of callus is diminished as it cannot be maintained for long periods of time. Type II embryogenic callus is friable, soft, and yellowish (Green *et al.*, 1983).

Both types of embryogenic callus are mostly induced from the middle and the coleorhizal end of the scutellum (Lu *et al.*, 1982; Vasil *et al.*, 1985). Immature embryos of 1–2 mm size were found to be the optimal for induction of embryogenic callus (Green and Phillips, 1975). The regeneration process is primarily divided into three stages. The embryos are cultured on either modified MS (Murashige and Skoog, 1962) or N6 (Chu

et al., 1975) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and incubated in the dark at 26–28 °C for callus induction. The embryogenic callus is then transferred to media containing 2,4-D and transferred to the dark, thus allowing the maturation and germination of somatic embryos. Alternatively, the cultures may be returned to the light and cultured on a medium lacking 2,4-D. Under these conditions, regeneration from direct organogenesis occurs.

The use of immature embryos as a starting point for the development of transgenic maize, indeed for any plant, comes at a price. There are technical problems to consider. These include strict limitations with respect to the size and stage of embryo used (Armstrong, 1994), genotype restrictions (Tomes and Smith, 1985; Horn *et al.*, 2006), and somaclonal variation and albinism (Armstrong and Phillips, 1988). In addition to these limitations and those related to greenhouse space, the establishment of accountable embryogenic suspension cultures requires a minimum of 1 year, to say nothing of the continuous, labor-intensive maintenance (Bhaskaran and Smith, 1990). Then, too, the recovery of fertile plants, either from protoplasts or from the embryos themselves, can likewise take an additional 9 to 12 months. Hence, either universities or companies seeking to employ this labor-intensive technology must be prepared to invest a minimum of 2 years before the first transgenic seed is harvested.

Immature embryo-based regeneration systems have proven to be effective for the genetic transformation of maize. Nearly 15 years passed between Green and Phillips' (1975) initial report of regeneration using immature embryos and the first report of fertile transgenic plants obtained from embryogenic suspension cultures derived from immature embryos (Gordon-Kamm *et al.*, 1990). Different methods have been used to transform immature embryos. These include particle bombardment, electroporation, *Agrobacterium*-mediated transformation, and whiskers-mediated transformation (D'Halluin *et al.*, 1992; Ishida *et al.*, 1996; Songstad *et al.*, 1996; Petolino *et al.*, 2000). These different transformation methods report a wide frequency of transformation efficiencies ranging from 0.8% to 45% (Wright *et al.*, 2001; Zhao *et al.*, 2001).

Given the wide range of transformation frequencies and the number of methods by which

DNA can be transferred either to embryogenic tissue or protoplasts, the choice of delivery remains an issue. There have been a stunning number of improvements made in the last 15 years that especially adapt *Agrobacterium* for use in maize. Among these has been the development of the supervirulent strains (Hood *et al.*, 1993) wherein multiple copies of specific genes of the *vir* regulon have been introduced into the bacterium resulting in heightened rates of T-DNA transfer. Accompanying the increase in transfer is low copy integration, thus insuring simple Mendelian transmission while eliminating gene silencing associated with multiple integrative events (Shou *et al.*, 2004). The numerous improvements associated with *Agrobacterium* have proven most effective using the inbred A188 and its derived hybrid progenies such as Hi-II (Frame *et al.*, 2006; Hiei *et al.*, 2006; Horn *et al.*, 2006).

Depending on the maize line and how it is manipulated prior to DNA transformation, certain cultivars may prove refractory to *Agrobacterium* infection, and hence alternative delivery strategies must be employed. Following biolistic treatment or whisker-mediated transformation of either embryogenic callus or cell suspension cultures, transgenic plants may be regenerated. Lowe *et al.* (1995) also reported transformation of shoot apices but progeny were not obtained. It was concluded that further manipulations were needed to overcome this problem. A number of reviews are available for particle bombardment and *Agrobacterium*-mediated transformation discussing the different parameters to consider in using each method (Taylor and Fauquet, 2002; Cheng *et al.*, 2004).

3.2.2 Embryogenic cell suspension cultures

Embryogenic cell suspension cultures for maize cells are initiated from type II friable callus derived from immature embryos (Vasil and Vasil, 1986). To initiate a suspension, fresh soft embryogenic callus is dispersed in liquid MS or N6 media supplemented with 2,4-D, and cultures are kept in shaker in dark for a period of 4 to 6 months with routine subculturing and adding fresh media every 3–4 days till they can be used for transformation. The establishment and the regeneration process of cell suspension culture are well described by

Vasil and Vasil (1986). Cell suspension cultures were successfully used for obtaining transgenic plants by particle bombardment and whisker-mediated transformation (Gordon-Kamm *et al.*, 1990; Frame *et al.*, 1994).

The advantage of using the cell suspension cultures lies in the ability to maintain the cultures for a long time (cryopreservation) when time is a critical factor especially for industrial production of medicinal compounds. In addition, cell suspension cultures are the only source for the isolation of maize protoplast that can be regenerated to plant (Vasil and Vasil, 1986). Most importantly, cell suspension cultures are the only source for the isolation of maize protoplasts capable of regeneration (Vasil and Vasil, 1986).

3.2.3 Protoplasts

In principle, protoplasts are attractive targets not only for gene delivery but also for regeneration. Maize regeneration from isolated protoplasts using type II callus was first reported by Rhodes *et al.* (1988). The resulting plants lacked utility as these were sterile. Shortly after this initial report, fertile maize, derived from protoplasts, was regenerated (Prioli and Söndahl, 1989; Shillito *et al.*, 1989). Competent protoplasts are maximized following 9 months of subculture of the original mother culture (Shillito *et al.*, 1989). In order to generate protoplasts, suspension culture cells are added to a mixture of cellulose degrading enzymes. After cell wall degradation, the protoplasts are harvested, stabilized on an MS medium containing high molar sucrose, centrifuged, washed with salt solution, and resuspended in protoplast culture media (Shillito *et al.*, 1989). The protoplasts are then plated on a layer of nurse cells derived from the original suspension culture seeded beneath filter paper to allow division and proliferation. The protoplast-derived embryogenic callus is then cultured on MS media supplemented with cytokinins, and regeneration occurs. The utility of this technology is genotype specific and hence limited.

Since protoplasts lack a wall, this cell population is amenable to transformation by direct DNA uptake. Methods include electroporation, PEG-mediated transformation, and Agrolistic

transformation (Fromm *et al.*, 1986; Armstrong *et al.*, 1990; Hansen *et al.*, 1997). Despite all of the intensive activity using protoplasts, their utility as a generator of fertile plants remains limited (Golovkin *et al.*, 1993). Indeed, when protoplast-mediated transgenic plant technology is evaluated as a whole, the number of fertile engineered plants produced remains unalterably low. When this limited output is considered against the labor-intensive activities associated with their fabrication, the cost-benefit ratio is minimally suspect. In reality, the recent advances in transformation methodology that produce transgenics in greater numbers and at faster speeds using a wider range of genotypes argue that the routine use of protoplasts serves no particular advantage.

3.2.4 Explants from mature seeds

Given the restrictions associated with protoplast usage and despite reports testing immature embryos derived from numerous maize inbreds and hybrids, routine production of transgenic plants from commercial cultivars remains limited using these starting materials (Horn *et al.*, 2006). In recent years, researchers have shifted focus and are testing tissues derived from mature seed for regeneration and transformation (Zhong *et al.*, 1992; Li *et al.*, 2002; Sairam *et al.*, 2002, 2003; Al-Abed *et al.*, 2006). In one approach, the apical meristems are dissected following germination and cultured on MS media supplemented with cytokinins to induce multiple shoots (Gould *et al.*, 1991; Zhong *et al.*, 1992). The process of plant regeneration from shoot meristems mainly consists of three steps: induction, multiplication, and elongation of shoot buds, resulting in mature plant development in the greenhouse.

Excised shoot apical meristems have also proven to be efficient explants for maize transformation using either particle bombardment or *Agrobacterium*-mediated transformation (Gould *et al.*, 1991; Zhong *et al.*, 1996; Zhang *et al.*, 2002). Congruent with apex excision are reports of chimeric transformants (Zhong *et al.*, 1996). However, Sticklen and Oraby (2005) have pointed out that chimerism of transgenic plants can be avoided when transformed meristems are multiplied for 2–3 months.

Alternatively, Sairam *et al.* (2003) have shown that a multiplicity of shoots can be proliferated from complete maize meristems following incubation on a germination medium containing 2,4-D. If these meristems are in turn subcultured in the light on a modified MS medium containing 6-benzylaminopurine, six to eight shoots are produced per meristem, independent of all genotypes tested. Seed was recovered in 6 to 9 months from the original time of explant implantation. If the meristems are placed in the dark and cultured on a modified MS medium containing 2,4-D, somatic embryogenesis is formed and fertile plants are subsequently recovered yielding harvestable seed in 6 to 9 months. Albinos and plant chimeras are notably absent using this procedure.

This technology also holds great promise for recovering transgenics in large numbers following *Agrobacterium*-mediated transformation. If the maize seeds are germinated on a medium containing auxin, 60–86% of shoot meristems show uniform transient expression of either β -glucuronidase (GUS) or green fluorescent protein (GFP) across the entire surface of each explant. This observation was confirmed following PCR analysis and the GFP marker segregated in a 1:1 ratio in the pollen. The high transfer rates were independent of the use of supervirulent *Agrobacterium* strains provided the seed was first germinated on auxin-supplemented, modified MS media.

Huang and Wei (2004) have reported regeneration of plants through callus initiation from mature embryos. The embryos were sliced and cultured on N6 media supplemented with 2,4-D and induced callus was further cultured on media containing cytokinin and silver nitrate. The number of regenerated shoots per callus, however, was small, i.e., two shoots. A more recent report from Sidorov *et al.* (2006) showed that nodal sections of 7- to 10-day-old maize seedlings can be transformed with *Agrobacterium* and subsequent transgenic plants were regenerated from type I callus. Whether this system continues to be explored depends upon the extension of the totipotency of type I callus, to say nothing of increasing the shoot output.

Most recently, the utility of “split seeds” as a novel explant has been explored by Al-Abed *et al.* (2006). By splitting the seed longitudinally,

three different tissues containing meristematic cells are exposed simultaneously. These tissues include the scutellum, coleoptilar ring, and shoot apical meristem (Figure 4). Split-seed explants from the inbred lines R23 and B73 and the hybrids Hi-II and LH198 x LH227 were tested for regeneration output. An average regeneration frequency of 76% is observed, and in each explant expressing regeneration the mean number of shoots regenerated is 28 (Figure 5). Hence, potentially, more than 50 plants can be rapidly produced per seed. Fertile seed can be recovered in 6 months from split-seed implantation. Finally, based on electron microscopy analysis, each plant has been shown to be originated from a single shoot bud (Figure 6). Since plant production is high, conditions are currently being optimized that will link high-frequency DNA transfer to this robust regeneration protocol.

Indeed split-seed explants have been successfully used to introduce the *Arabidopsis CBF3* gene under the control of the inducible promoter rd29A by particle bombardment (Al-Abed *et al.*, under review). The stable integration of the *CBF3* gene has been confirmed in T₀ and subsequent generations (Figure 7). The performance of these maize transgenics is currently being evaluated under conditions of abiotic stress. Finally, with the continuing perfection of the split-seed technology, it should be possible to automate this process, thus allowing its rapid production of value-added transgenics while reducing the dependence on the need for labor and space.

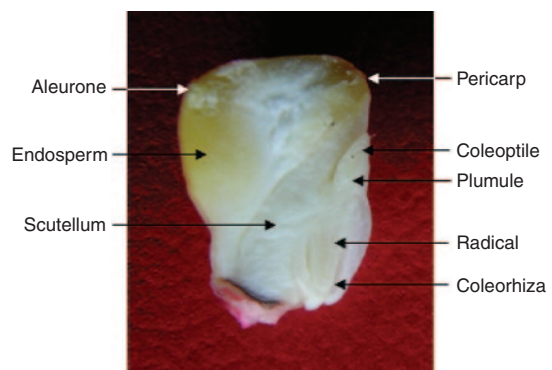


Figure 4 A vertical section of split-seed explant, showing arrangement of tissues and organs [Reproduced from Al-Abed *et al.* (2006). © Springer]

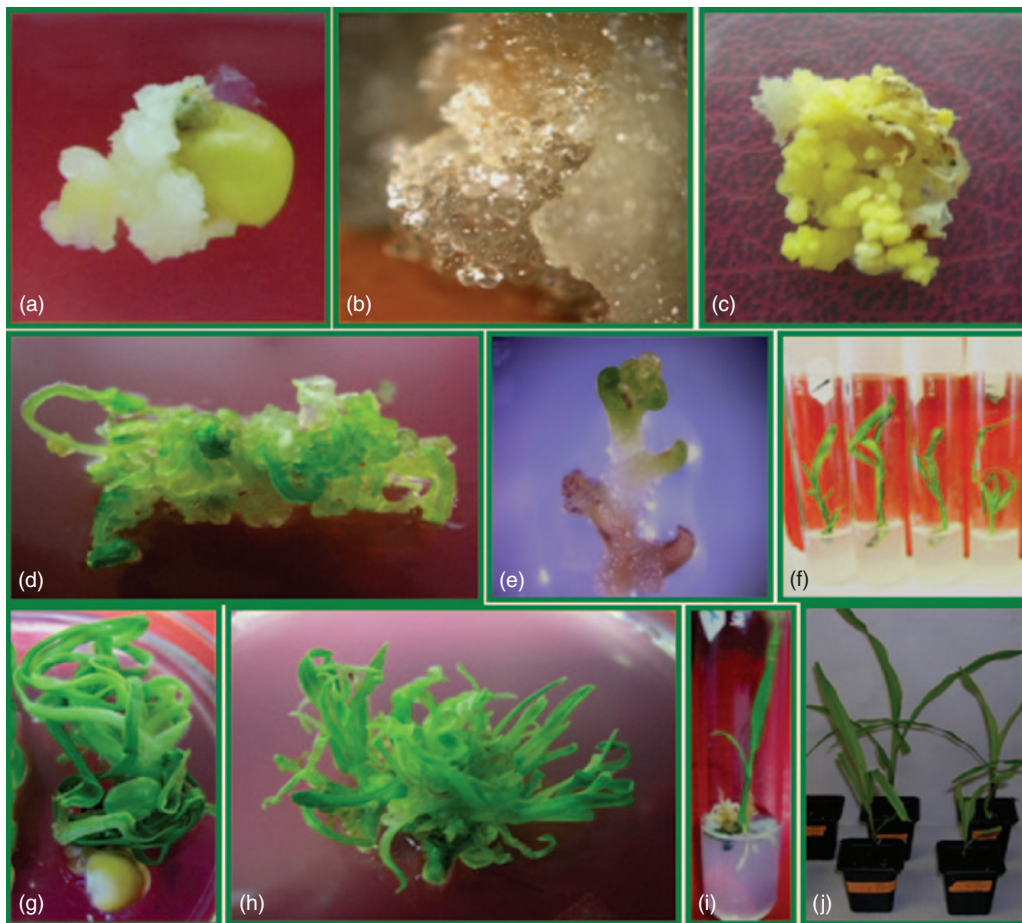


Figure 5 Regeneration of maize plants from split seed: (a) callus induced from split seed; (b) callus proliferation; (c) embryogenic callus; (d) shoot regeneration from callus; (e) somatic embryo; (f) shoot elongation; (g, h) direct multiple shoots regenerating from split seed; (i) regenerated plantlet in a rooting medium; (j) maize plants regenerated from split-seed explant [Reproduced from Al-Abed *et al.* (2006). © Springer]

3.3 Transformation

A high-efficiency, high-capacity transformation system is necessary to generate a sufficient number of primary transformants (T_0) for efficacy testing. Typically, upward of 20 to 30 primary transformants are made to adequately test a construct. Additionally, 3 to 8 different constructs may be tested for a given gene. Therefore, 60 to 240 T_0 must be generated to adequately assess a reasonable number of constructs. Although transformation with the “gene gun” or biolistic methods is common, transformation mediated by *Agrobacterium tumefaciens* is the method of choice today. As described below, recent evidence suggests

that shoot meristems and “split seeds” may be ideal explants for inbred transformation using *Agrobacterium* when competence is primed using a modified MS medium supplemented with auxin (Figure 8). Not only is the rate of transfer DNA (T-DNA) transfer high but none of the genotypes tested have proven refractory to regeneration by either somatic embryogenesis or direct organogenesis (Sairam *et al.*, 2002, 2003; Al-Abed *et al.*, 2006).

3.4 Optimization

The next step in product development is to optimize the transgenic construct. This step

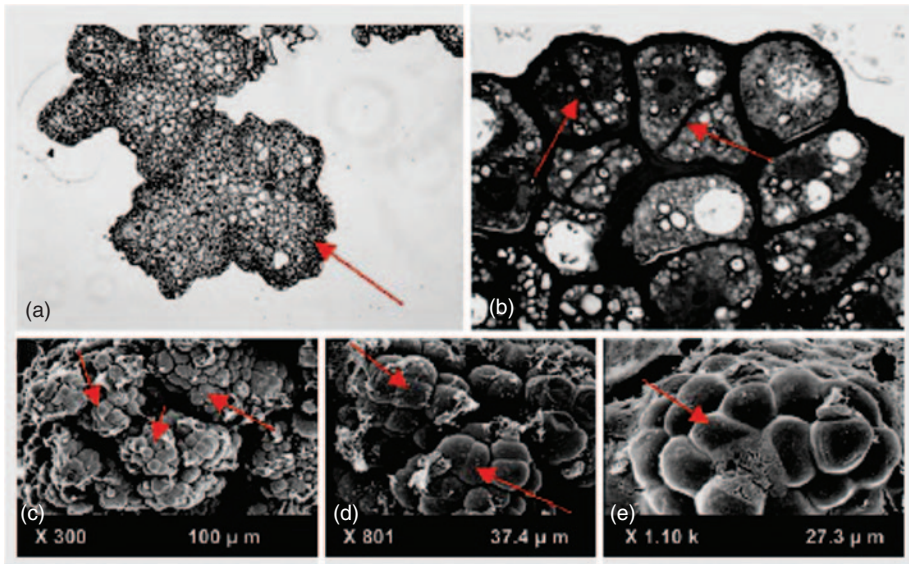


Figure 6 Microscopy images of embryogenic callus and initiating shoot buds: (a) embryogenic callus showing meristematic cells on the peripheral sides; (b) actively dividing cells; (c–e) scanning electron microscope of meristematic cells grouping to form shoot buds [Reproduced from Al-Abed *et al.* (2006). © Springer]

usually requires (1) testing alternative promoters to produce an optimal expression level and degree of phenotypic change, (2) choosing suitable selectable markers, (3) evaluation of phenotypic expression, and (4) event sorting, where a large number of events are tested to obtain the most efficacious and

stable insertion. Optimization requires expanding field evaluations to test additional constructs effectively and to determine commercially acceptable levels of phenotype improvement and stability without a deficit in any other agronomic trait.

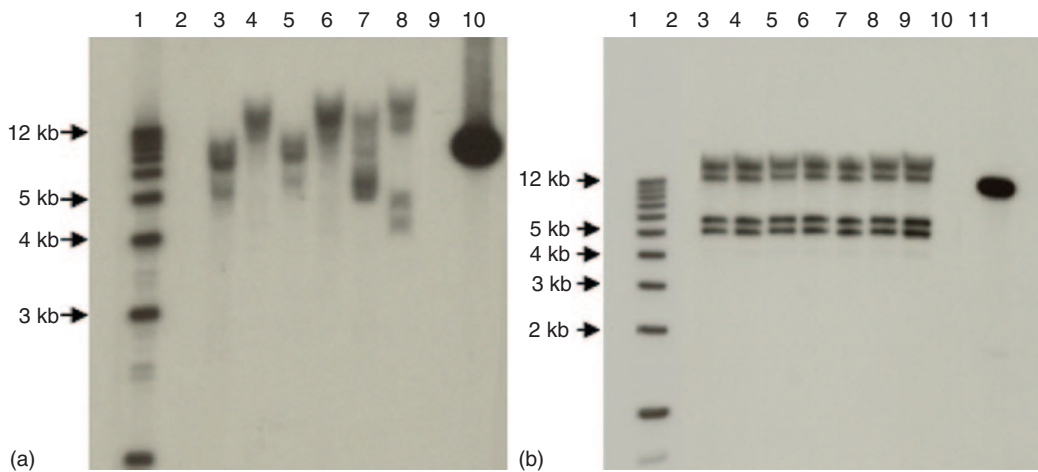


Figure 7 Southern blot analysis of T_0 and T_1 *CBF3* plants, recovered from transformed split-seed explants. (a) Southern blot analysis of T_0 plants: lanes 1: kb ladder; 2: nontransformed plant digested with *KpnI*; 3, 5, and 7: plants 7, 8, and 28 digested with *HindIII*; 4, 6, and 8: plants 7, 8, and 28 digested with *KpnI*; 10: plasmid positive control digested with *KpnI*. (b) T_1 plants that originated from plant 28: lanes 1: kb ladder; 2: nontransformed plant; 3–9: plants 1, 3, 4, 7, 11, 13, and 14 digested with *KpnI*; 11: plasmid positive control digested with *KpnI*

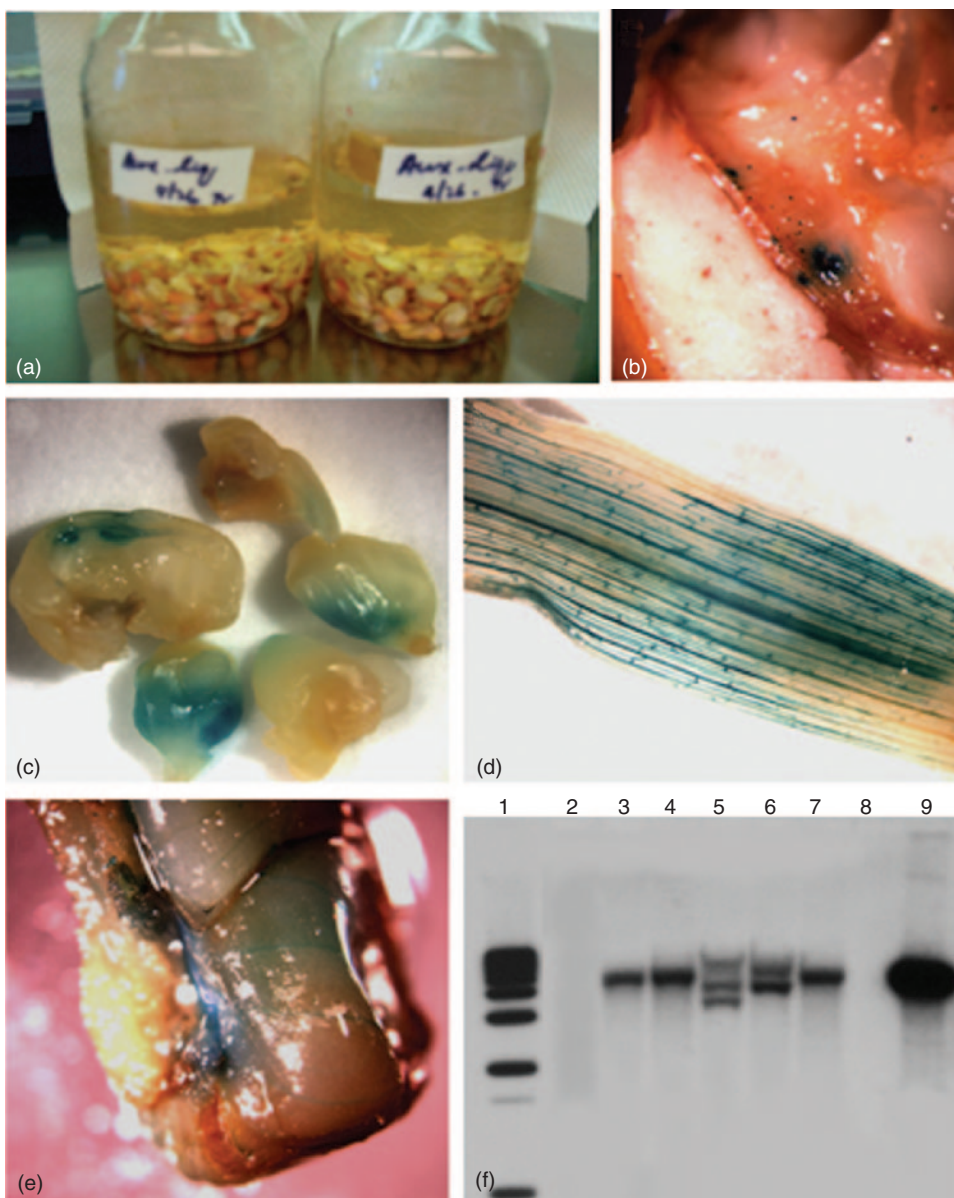


Figure 8 *Agrobacterium*-mediated transformation of split-seed explant. (a) Split-seed explants pretreatment prior to Agro infection. (b, c) Transient GUS expression from Agro infection of split-seed explants from mature and immature seeds. (d) GUS expression in leaf of regenerated transgenic plant. (e) GUS expression in immature seeds from T_0 plant. (f) Southern blot analysis of transgenic GUS plants: lanes 1—kb ladder; 2—a nontransformed plant; 3–7—transformed plants; 8—empty; 9—plasmid positive control

3.4.1 Promoter testing

Once an efficacious promoter–gene combination has been identified from the verification stage, alternate promoters are tested to achieve a commercially acceptable change in the target trait.

One of the originally tested promoters may be sufficient but typically testing several different promoters is required. Alternative promoters may mediate different levels of constitutive expression, may restrict expression temporally and/or spatially, or may be inducible. The choice

of promoters to test depends on the target trait and strategy for modification in the product. Early and rigorous field testing is also required to determine the most optimal promoter–gene combination for product advancement.

3.4.2 Selectable markers

Selectable and screenable marker genes are key factors in discriminating between putative transformants and unaltered plants. Most of the selectable markers that have been used in maize transformation are genes that give resistance to antibiotics or to herbicides (Spencer *et al.*, 1990; Walters *et al.*, 1992). Some of the concerns related to public acceptance and the chance of environmental contaminations from using those types of marker genes have led the industry to focus on eliminating these genes while replacing them with more environmentally friendly markers that can potentially contaminate any rung in the food chain. An example is the use of the phosphomannose isomerase gene (*PMI*) (Wright *et al.*, 2001). A list of selectable marker genes used in maize transformation along with their respective promoters is summarized in Table 1. The use of a specific promoter is determined by whether expression is to be constitutive, as when selecting for initial transient expression in the target cell, or selective with respect to tissue and/or timing.

Table 1 Some of the common promoters and selectable markers used for corn transformation^(a)

Promoter/selectable marker gene	Reference
<i>Ubi1:pmi</i>	Negrotto <i>et al.</i> , 2000
<i>CaMV 35S:bar</i>	Zhao <i>et al.</i> , 2001
<i>CaMV 35S:bar</i>	Frame <i>et al.</i> , 2002
<i>LIR:RepAll</i>	Gordon-Kamm <i>et al.</i> , 2002
<i>CaMV 35S:barI</i>	
<i>Ubi1:moGFP:PinII</i>	
<i>CaMV 35S:bar+Ubi1:FLP:PinII</i>	
<i>Ubi1:PPO (Y426M + S305L)</i>	Li <i>et al.</i> , 2003
<i>Ubi1:pmi/ubi:PPO</i>	
<i>e35S/HSP70:nptII</i>	Zhang <i>et al.</i> , 2003
<i>Act1:cre//35S:nptII</i>	
<i>e35S/HSP70:11//</i>	
<i>HSP17.5E:cre</i>	
<i>Act1:epsps-cp4</i>	Huang <i>et al.</i> , 2004

^(a)Reproduced from Shrawat and Lörz (2006)

3.4.3 Evaluation of phenotypic expression in transformants

After generating a sufficient number of transformants for each construct, construct efficacy, or the ability of the construct to mediate the desired change in the target phenotype, is evaluated. In order to efficiently evaluate a construct, several molecular and phenotypic assays are developed that determine if a construct is mediating the desired phenotype to an acceptable degree. Usually, a number of criteria must be met or exceeded to allow a construct to move to the next stage or phase of product development. Although the types of assays are particular to the trait targeted, there are a few standard metrics that must be met. Analysis of the primary transformant or T₀ plant is desirable, as this will allow only events meeting the criteria to advance to field testing. Molecular assays to assess the number and fidelity of integration of the transgene construct in each T₀ event are conducted. Also, quantitative expression analyses are needed to confirm that the transgene transcript is present and accumulates to the expected level in the correct tissue. Additional expression analysis of the protein may also be performed. Minimally, one should identify intact, single-copy transgene integration events that produce detectable full-length transcript. T₀ events meeting these criteria are pollinated to produce a segregating family for field testing. Back crossing the inbred T₀ to the same inbred will produce a T₁ family segregating 1:1 for the presence or absence of the transgene for each event. Alternatively, crossing to an unrelated or heterotic inbred will produce segregating T₁ hybrids for field evaluations.

There are many options for the degree and rigor of the initial field evaluation. Determination of the essential objectives at this stage will help to decide the types of measurements, number of replications, level of detection of effect, and statistical design of the field evaluation. Most often, the primary limiting factor for T₁ field evaluations is the amount of T₁ seed produced. Typical T₀ plants yield only a few hundred kernels, half of which are azygous for the transgene. Therefore, highly replicated destructive measurements are precluded in first-generation field evaluations. The main objective of T₁ field evaluations is usually to determine if 50% or more of the events within a

construct are mediating a measurable or detectable change in the target trait or component of the trait in the transgene-containing plants compared to their nontransgenic sibs or other controls. The degree of change may not be what is needed for a commercial product but this issue is addressed in the optimization stage. A primary verification goal is to identify constructs that are having no effect or a detrimental effect on plant performance. Such constructs are filtered out, which allows more resources to be directed to fewer efficacious constructs. Once efficacious constructs and events are identified, seed increases at the T₂ stage allow for more rigorous field testing. Results from replicated, multiyear, multilocation field tests produce enough empirical outcomes to warrant advancing a construct. At the conclusion of the verification stage, a promoter–gene combination that mediates a detectable, predicted change in the target trait is identified. Once this is achieved, the construct advances to the optimization stage of product development.

3.4.4 Event sorting

After the most optimal promoter–gene construct has been identified, a very large number of transgenic events must be generated. Typically 100 or more events of the optimized construct are generated and evaluated in replicated field tests. At this stage, position effects of the transgene are being evaluated. Local genomic position influences the amount and stability of expression. Event sorting for stable and optimal expressivity and penetrance of the transgenic phenotype through several generations occurs at this step. A high-capacity, efficient transformation system and ample field testing resources are needed at this stage. The effect of different growing environments can also be assessed on the optimized event(s) by testing in different locations. This helps determine the stability of the phenotype in different geographic locations.

3.5 Regulatory Rules for Transgenic Maize

The United States Department of Agriculture Animal and Plant Health Inspection Service (APHIS) have strict rules regulating the release

and planting of transgenic plants. These have been summarized by Ellstrand (2003). Plants producing any industrial/chemical products now come under regulation. Indeed, APHIS has taken great pains to protect the public from the possibility of nontransgenic seed being contaminated with transgenic seed. In consequence, perimeter fallow zones are required. Moreover, the planting of food and field crops at the same location in subsequent seasons is prohibited in order to avoid contamination by volunteers. Farm implements must be dedicated either for use with transgenics or nontransgenics. This includes both planting and harvesting equipment in addition to prescribed storage depots. Both machine and personnel training programs are mandated for all workers coming in contact with transgenic seeds.

With respect to producing pharmaceutical active compounds in corn, the regulations are particularly strict. APHIS requires that no other corn be grown within 1.6 km if the transgenics being field tested are open pollinated. If controlled pollinations are made by placing pollen bags on top of the tassels, no other corn can be grown within 800 m. Moreover, if the transgenic corn in question expresses a pharmaceutical product, then the planting must be time staggered. This corn must be planted either 28 days before or after any other and in a zone enforced between 800 and 1600 m of the test site.

4. COMMERCIALIZATION

4.1 Introduction

The final stage in transgene product development is commercialization. At this stage, the best optimized events are either backcrossed or retransformed into elite germplasm in order to produce competitive hybrids with the new or improved trait. In addition, the new transgene trait is stacked with other genetically modified (GM) or native traits into a total product “package” carrying all of the trait improvements offered to the customer. Finally, the total package of improved traits is tested across diverse geographies under standard grower’s input and tested for trait improvement and high yield. Typically, only the top one or two events are advanced to this stage. Integrating or backcrossing the trait into

current elite inbreds is expensive and must be done quickly, as elite inbreds are usually replaced by superior inbreds every 5 to 10 years. Alternatively, once the top event is introgressed into a few key inbreds, it can be incorporated into the breeding germplasm for development and selection of new inbreds. Doing so raises regulatory issues, as all inbred development and testing would need to be regulated as GM material. However, this is the current practice for soybeans and will likely be the future for maize breeding as well. As the number of improved GM and native traits increases, the number of combinations of these traits into potential products increases exponentially. Most will be combined together as a “stacked” product package with several GM and native improvements to a single hybrid. Different GM traits may be combined at one or a few transgene integration sites or possibly carried on artificial chromosomes. Selection for improved native traits with markers (marker-assisted selection, MAS) can be done during the integration of the transgene into elite inbreds. Stacked products are tested in comparison with standard and competitor hybrids for improved trait performance and hybrid yield across geographies. Hybrids with superior yield and improved traits are advanced for commercial production and sale to farmers to meet the ever-growing need for food, feed, and biorenewable materials.

4.2 Stress Tolerance

The *Arabidopsis* transcriptional factor *DREB1A/CBF3* under the control of the inducible R29a promoter has been successfully transformed in maize (2007Al-Abed *et al.*, under review). Corn expressing *CBF3* gene showed significant tolerance to cold, drought, and salinity compared to wild-type plants. In the case of maize, segregating *CBF3* can be grown for weeks at 10 °C with no ill effects and can survive below freezing. Indeed, more than 60% of the corn survives under drought conditions of 21 days and under conditions of 400 mM salt. The availability of these genes and others that confer resistance to biotic stressors will have the effect of increasing the acreage under corn cultivation, thus providing more seed. This is no small consideration given that seed is likely to be diverted from its traditional commercial uses

to a market place allowing higher profit margins for farmers. In this connection, maize bioreactor pharmaceutical production and ethanol synthesis are but two of the markets to which maize seed is likely to be redirected. The availability of these genes and others that confer resistant biotic stressors will have the effect of increasing the growing range of corn.

4.3 Vaccines and Antibodies

Collectively, the pharmaceutical industry must be prepared to accept the inevitable pressure of designing new production designs that will reduce drug costs to consumers, especially in developing countries. Today, state-of-the-art production of genetically engineered proteins is done in mammalian cell culture. A minimum investment of 100 million dollars is needed to build a factory that will produce them, depending on the product-limited quantity.

Moreover, the use of mammalian cell culture as bioreactors for production of human interest proteins (HIPs) carries with it certain intrinsic health risks. Specifically, there is an inherent danger of viral contamination associated with mammalian-derived materials that necessitates exhaustive safety testing and validation of production processes. Animals infected with certain zoonotic viruses have transmitted fatal illnesses to humans. Numerous mouse-derived cell lines are known to contain endogenous retroviruses and some demonstrate species-specific tumorigenic potential. Oncogenic xenotropic murine retroviruses are of particular concern because of the many theoretical risks they present to humans. New viruses with altered pathogenicity or host range could be generated through genetic recombination. Tumors also may form through integration of the viral genome in close proximity to a host oncogene, thus activating the oncogene.

Within the plant biotechnology sector, there is a great interest in expressing mammalian proteins in plants in a way that would allow their commercial exploitation. The advantages of producing therapeutic recombinant proteins in plants are many. These include the ability to fabricate HIP on an agricultural scale, thus lowering manufacturing costs significantly, and the ability, for example, to export highly sought and needed therapeutic

proteins that remain stable in dry seed for extended periods of time. HIPs cover a broad range of commercially important, value-added products, which include vaccines, antibodies, hormones, peptides, cytokines, enzymes, and bioactive lipids. Most importantly, no human or animal pathogens have ever been reported that infect plants. Thus viral contamination that is observed in animal cell culture is absent in plants.

Transgenic plants have been produced that express a number of different HIP molecules using a variety of plant species (Mason and Amtzen, 1995; Arakawa *et al.*, 1998; Mor *et al.*, 1998, 2001; Zeitlin *et al.*, 1998). In maize, the B-subunit of heat-labile enterotoxin has been produced (Lamphear *et al.*, 2002). Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of diarrhea in children under the age of 5 in third-world nations. ETEC pathology is profound and results in 650 million cases of diarrhea that kills 800 000 children annually (Black, 1986). The ability to translate the expression of the B-subunit and proteins like it into edible vaccine will likely figure prominently into the commercialization of maize transgenic seed.

Monoclonal antibody production should likewise do the same. Recently, Meristem Therapeutics has announced the expression of IgA1 in maize. The protein made has been shown to regress tumors in lungs, breasts, and pancreases of model animals. Large amounts of antibodies are needed equal to several grams/day/patient. Since 200 monoclonal antibodies are in trial, an additional 600 are in the preclinical phase. The utility of a storage vessel like maize seed coupled with abundance is likely to create a bioreactor of choice in the future.

4.4 Ethanol

The fuel ethanol industry is the second largest user of corn grain in the United States following livestock feed. Twenty percent of domestic corn was fermented into fuel ethanol in 2006 (USDA, 2007). While the process technology has existed in the past century (Bothast and Schlicher, 2005), the industry has experienced unprecedented growth over the past 5 years, with the annual production capacity in the United States more than doubling to over 5 billion gallons (Figure 9). An additional

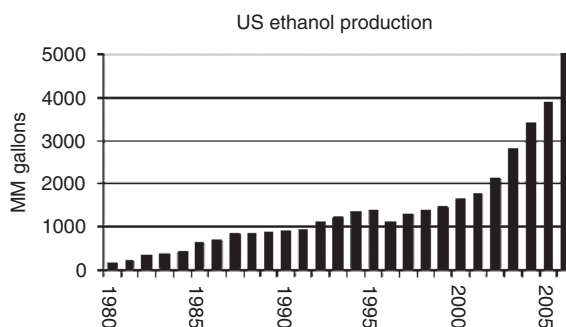


Figure 9 Growth of ethanol production in the United States (RFA, 2007)

3.4 billion gallons of capacity are currently under construction (RFA, 2007).

This growth has been propelled by three primary drivers. (1) Refiners uniformly switched from methyl tert-butyl ether (MTBE) to ethanol as a fuel additive to meet the octane and emission requirements for reformulated gasoline set by the 1990 Clean Air Act Amendments. While MTBE had been banned in over 20 states because of concerns over drinking water contamination, the complete changeover occurred following the Energy Policy Act of 2005 (EPACT, 2005), which did not include a liability waiver protecting refiners against future MTBE contamination lawsuits. (2) Federal incentives for ethanol production include an excise tax credit of \$0.51/gallon ethanol awarded to blenders for using ethanol in gasoline and a \$0.10/gallon income tax credit for small-scale ethanol producers (defined as producers making less than 60 Mgal/year and limited to \$1.5 million annually). Many states offer additional incentives for ethanol production while some provide rebates for purchasing vehicles equipped with fuel flexible engines that enable operation on high ethanol content blends (RFA, 2007). (3) Technology maturation has decreased the cost of producing ethanol while high petroleum costs have driven up its selling price resulting in large profits for ethanol producers.

Legislation has played a key role in influencing demand and will continue to do so as researchers have highlighted its positive impact on energy security, environmental performance, and rural development. Corn-derived ethanol is a renewable, domestically available fuel that can reduce the

country's dependence on imports of foreign petroleum (Farrell *et al.*, 2006). Moreover, since ethanol produced in the United States is primarily based on corn, the carbon dioxide emitted during combustion was originally taken up by the corn plant during growth. Therefore, ethanol utilization plays a role in lowering domestic greenhouse gas emissions. Increased ethanol production also contributes to the rural economy by boosting corn prices and creating jobs for construction and manufacturing (Swenson, 2005). For these reasons, EPACT 2005 initiated the development of a Renewable Fuel Standard that requires a minimum of 7.5 billion gallons per year in renewable fuel production by 2012 (EPACT, 2005). With the call by President Bush to replace 75% of petroleum imports from the Middle East by 2025 (State of the Union Address January 31, 2006), many policy makers and groups have called for further legislation to greatly expand the minimum fuel production.

4.4.1 Process descriptions

Over 95% of ethanol produced in the United States is made from corn (RFA, 2007). However, the production processes vary considerably and desirable output traits will be dependent upon the mode of processing. Thus, to properly size the market for a given trait, the specific process and economic benefit that the trait targets must first be quantified.

Corn grain is fermented into ethanol through two primary pathways: the wet mill and the dry grind processes. Historically, large corn processors have built wet mills as they have the flexibility to produce a portfolio of different products including high fructose corn syrup, corn gluten feed, corn germ, corn oil, and others in addition to ethanol. Wet mills operate similarly to a petroleum refinery as they can optimize output depending on market conditions, but they are also much more capital and energy intensive. By contrast, dry mills are optimized for ethanol production and therefore they are considerably less expensive and more efficient, albeit less flexible. Most of the increase in capacity over the past decade has been supplied by dry mills. Other feedstocks, such as milo and wheat starch, can be used in processes similar to the dry grind. It is expected that lignocellulosic

biomass will be used for ethanol production in the future. As corn stover is a potential feedstock for the cellulosic process, this process is also described below.

4.4.2 Wet mill process (EPA, 1995)

In the wet mill process (Figure 10), delivered and screened corn grain is initially steeped in dilute sulfur dioxide to degrade the protein in the grain and remove the soluble fractions. The steep water from this first step passes through multieffect evaporators to recover the protein, which can be sold as feed supplements. The steeped corn continues through mills that strip out the germ and some of the starch and fiber. The germ is removed from the pulp using a liquid cyclone and the oil is extracted to be sold as a product. The remaining corn flows through more washes, screens, and mills to separate out the bran, which is combined with the spent germ from the oil extraction and sold as corn gluten feed. At this point, the primary corn grain stream now consists mostly of the gluten and starch. The next step is a centrifugation stage where corn gluten meal, a high-protein (60–70%) animal feed, is produced. The starch and residual gluten and solubles are then processed downstream into starches, sugars, high fructose corn syrup, ethanol, or other fermentation products. For ethanol production, the process of converting the starch is very similar to the dry mill process described below.

4.4.3 Dry mill process (Kwiatkowski *et al.*, 2006)

In the dry grind process (Figure 11), the delivered corn grain is screened and ground in a hammer mill. The ground corn is mixed with thermostable α -amylase enzyme, ammonia, lime, and water in a slurry tank for the liquefaction step. A jet cooker is used to inject steam into the process to begin gelatinization where the starch is hydrolyzed into oligosaccharides. The liquefaction conditions are typically 88 °C, pH 6.5, with a residence time of 1 h. After liquefaction, the mash is cooked in a downstream tank for 15 min at 110 °C before being cooled down to 60 °C for the saccharification step. During saccharification, glucoamylase enzyme is

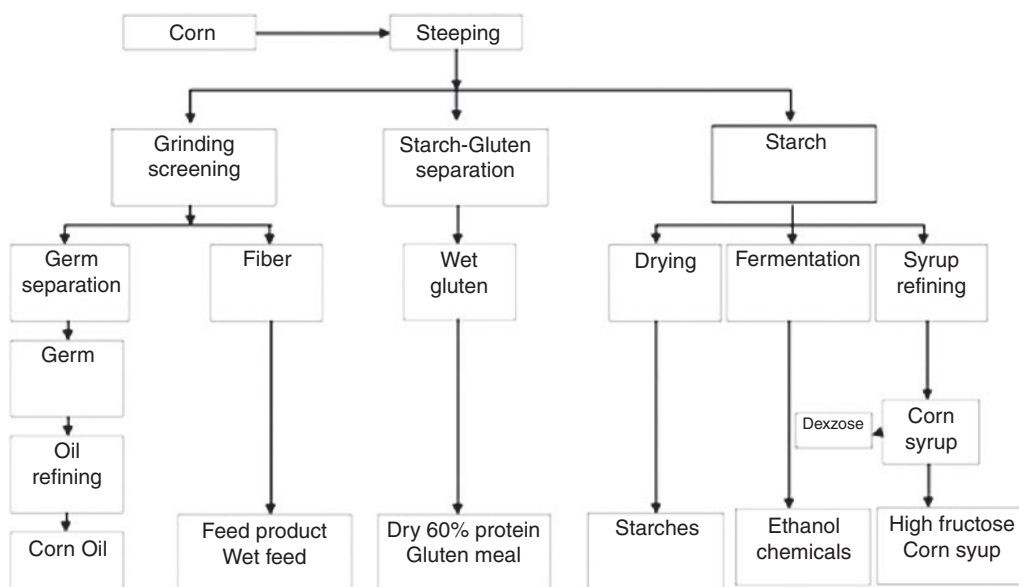


Figure 10 Process flow diagram for the wet mill process [Reproduced from RFA (2007)]

added to the slurry to further hydrolyze the oligosaccharides into their component glucose sugars. Sulfuric acid is added to lower the pH to 4.5 during the 6 h required for saccharification. After saccharification, the slurry is cooled again to 32 °C for the fermentation. Yeast is added during fermentation to convert the sugars into ethanol

and carbon dioxide in large fermentation tanks. The tanks are continuously cooled and mixed using the compressed off gas carbon dioxide, which contributes to pH control. Typical residence times for the fermentation range from 36 to 72 h. The outlet beer has an ethanol content of about 10% (w/w).

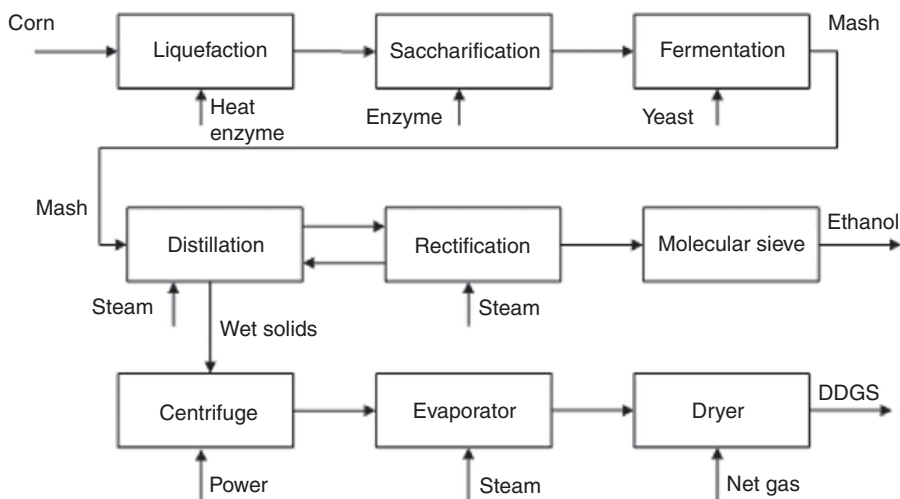


Figure 11 Process flow diagram for the dry grind process

The beer stream is fed to a steam reboiled distillation column where the ethanol purification begins. In the beer column, nearly all of the ethanol and most of the water is separated from the unfermented proteins, oil, fibers, and byproduct chemicals. The top of the beer column contains the ethanol/water mixture and this stream continues on through another distillation, a two-step rectification/stripping process before final dehydration in a molecular sieve. The stillage or “bottom” fraction from the beer column, containing the unfermented proteins, oil, fibers, and byproducts, is dewatered using a centrifuge and then dried further in a series of evaporators before final drying in a drum dryer. The final product from the drum dryer is called distiller’s dried grain and solubles (DDGS), which can be sold as animal feed.

A variation of the dry mill process is the raw starch process (Wang *et al.*, 2005). In a raw starch dry mill, the high-temperature liquefaction is replaced with a low-temperature operation that requires significantly more glucoamylase enzyme. The increase in enzyme offsets the elimination of the gelatinization step, which is normally used to improve the liquefaction efficiency. The primary advantage of raw starch processing is a reduction in capital and energy costs.

4.5 Cellulosic Ethanol (Aden *et al.*, 2002)

Ethanol can also be produced from lignocellulosic biomass. While this process has not been demonstrated on a commercial scale, many believe that it is the most promising alternative ethanol process because of the wide availability of inexpensive cellulosic feedstock. This process may become valuable for corn producers because corn stover, the stalks, leaves, and cobs, are a cellulosic feedstock that could be collected and converted into ethanol. The actual process has many of the same steps as the dry grind process but there are slight differences.

In cellulosic ethanol processing (Figure 12), the corn stover is delivered to a processing facility where the material is degraded into sugars and then fermented into ethanol. The corn stover is initially milled and treated with dilute sulfuric acid and high temperature steam in the pretreatment and hydrolysis stage. These initial reactions break apart the cellulose–hemicellulose–lignin matrix into a hemicellulose hydrolyzate along with the remaining cellulose and lignin. Because this step is performed at extreme conditions, the material requirements for the reactor can lead to exorbitant costs. The hydrolyzate stream is filtered of the solids and treated in a detoxification step where

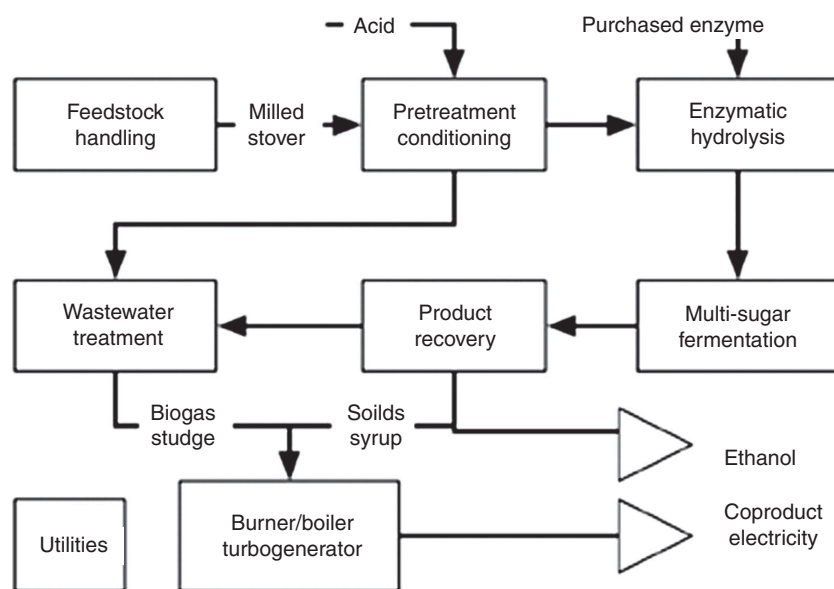


Figure 12 Process flow diagram for the cellulosic ethanol process [Reproduced from Sheehan (2003)]

lime is added to neutralize the remaining acid with gypsum as the waste product. The liquid stream is then recombined with the solids from pretreatment and pumped to the saccharification reactor where enzymes are added. These enzymes hydrolyze the cellulose into its component glucose sugars. The saccharification is performed in cascading tanks and is followed by fermentation tanks, where both the glucose and the pentose sugars from the hemicellulose are converted to ethanol. The ethanol is purified using a process similar to the dry and wet mill processes. Solids from the initial distillation are separated and dried using a centrifuge and evaporators before being combusted for steam production and power generation for the processing facility. Excess electricity can be sold to the grid as a co-product.

The cellulosic ethanol process can have several variations. The most typical difference is in the pretreatment and hydrolysis processes. While the description above highlights the dilute acid pretreatment followed by enzymatic hydrolysis, a number of other technologies are being investigated. Examples of other processes are concentrated acid hydrolysis, hot water pretreatment, and ammonia explosion pretreatment (Mosier *et al.*, 2005). The process of breaking open the biomass to liberate the sugars is both technically and economically challenging, so developing a process that more closely follows the dry mill liquefaction process is important.

4.6 Ethanol Process Economics

The wet mill and dry grind processes are relatively mature and while opportunities to optimize both exist, economic viability relies primarily on the commodity prices of the corn grain, ethanol, and various co-products. Conversely, the cellulosic process remains uncommercialized and significant process development is required for large-scale deployment. Short descriptions of the economic performance are given here for the dry mill and the cellulosic process as these are likely to provide the majority of current and future industry growth.

The ethanol production costs for the dry mill process can be roughly divided into five categories: corn, energy, other raw materials and utilities, fixed costs, and capital costs, with revenue from co-product DDGS being a negative cost.

Table 2 Example production costs for the dry mill process^(a)

Inputs	\$/gallon EtOH
Shelled corn	1.02
Natural gas	0.13
Electricity	0.03
Other raw materials, utilities	0.12
Labor, supplies and overheads	0.12
Capital charges	0.13
DDGS credit	−0.24
Total costs	1.32

^(a)Source: Tiffany and Eidman, 2003; Kwiatkowski *et al.*, 2006

Based on studies investigating the economics of dry mill processing (Tiffany and Eidman, 2003; Kwiatkowski *et al.*, 2006), the production cost ranges from \$1.00–\$1.50/gallon of ethanol and is highly dependent upon corn grain prices. Table 2 gives an example of these relative costs and shows that the two largest cost contributors are the corn grain and the DDGS. Moreover, the prices for these two commodities are impacted directly by increased ethanol production, with higher demand for corn leading to higher prices and a larger supply of DDGS leading to lower prices. Both of these changes have negative impacts on the ethanol production costs.

The growth of the industry in the first decade of the 2000s significantly impacted corn utilization. Corn grain used for ethanol production increased to a market share of 20% of the total US corn production (USDA, 2007). With continued expansion, the ability to supply enough corn grain may become constrained. Late in 2006, competition for corn from the livestock and ethanol industries had driven the price of the grain to unusually high levels (USDA, 2007).

Increased dry grind ethanol production also impacts the supply of DDGS. The co-product can partially displace corn demand as a feed alternative; however, this replacement is limited by DDGS nutritional composition and transportation challenges. Ethanol industry growth of the 2000s led to a potential oversupply of DDGS and drop in DDGS prices, which decreases corn ethanol profit margins. This was demonstrated during the late 2006 surge in corn prices as DDGS, typically sold at a premium to corn in ruminant markets, began to be priced at a discount to corn (Lohrmann, 2006). These two economic parameters, the price of corn and the price of

DDGS, will have a considerable impact on the future ability for ethanol producers to operate a profitable enterprise.

Other factors influence the economics of dry mill processing to a lesser degree. The overall conversion of corn grain to ethanol is important; however, with mature processing technology, most new facilities achieve a conversion of 2.75 gallons of ethanol per bushel of corn, which is over 95% of the theoretical limit and leaves little room for conversion improvement. Opportunities for reduced energy utilization include lower temperature liquefaction, improved ethanol/water separation technology, and decreased drying requirements for the co-product DDGS. Moreover, while ethanol producers primarily use natural gas for energy, alternative fuels such as coal, waste biomass, and methane gas from anaerobic digestion have been used to lower energy costs. Maximizing the energy utilization efficiency of the process and finding inexpensive energy sources will remain a critical aspect of the economics. Capital and operating costs are important as well, but because of the rush to build new facilities and relative maturity of the process technology, new technologies are being implemented slowly. In fact, the capital costs for dry grind manufacturing facilities increased during the ethanol boom in the early 2000s as demand for engineering, construction, and materials led to premium costs.

4.7 Opportunities for Improved Ethanol Production Using Transgenic Technology

Many opportunities exist for biotechnology to improve the economics of ethanol production. Bothast and Schlicher (2005) list the following priorities: new feed stocks and co-products; fermentation, saccharification, and hydrolysis improvements; fiber and germ recovery; DDGS optimization; and distillation and other separations optimization. New trait development can have a positive impact in all of these improvement areas; however, biotechnology improvements not based on plant traits will not be discussed here. New traits are a valuable tool for expanding corn production and ensuring crop harvest, both of which help maintain an adequate supply of grain at affordable prices for ethanol production.

Input traits have the potential to decrease ethanol processing costs indirectly by enabling the harvest of an adequate supply of corn grain that meets both ethanol and feed requirements at competitive prices. Traits focused on yield, pest resistance, herbicide resistance, and stress tolerance all affect the available corn supply and thus the price of corn grain. For example, pest-resistant corn helps protect the crop from infestation, which on average should increase the yield per acre versus pest susceptible corn. Transgenic yield improvements also would indirectly benefit the economics of ethanol production and complement breeding improvements. Additionally, new traits, such as cold, salinity, drought, and other stress tolerance, enable expanded production by allowing farmers to grow corn in less ideal soils and climates.

The processing of the corn grain to ethanol can be improved with a number of different transgenic output traits. An example is the development of breeds of corn with higher starch and fermentable sugar content, which would be beneficial for both wet and dry mill processes, assuming that the cost of fermentable sugar on a mass basis decreases. An increase in the available sugars for fermentation could increase the theoretical maximum of 2.85 gallons of ethanol per bushel of corn to a higher value, thereby decreasing the per gallon raw material cost. Initial work by Monsanto and Pioneer has resulted in the development of “Processor Preferred Fermentable Corn” and “High Total Fermentables” ethanol optimized varieties (Bothast and Schlicher, 2005). While these products have been developed using traditional breeding, compositional changes, such as increased starch content, can be accomplished by modifying genes that impact the synthesis, degradation, and accumulation of starch in plants. Further modifications to the starch composition of the corn grain could change the ratio of amylase and amylopectin, which affects the degradation and viscosity properties of the starch.

Other genetic modifications can underlie output traits that impact the efficiency of various unit operations in the ethanol process. A primary example is the amylase corn developed by Syngenta, which produces α -amylase enzyme in the endosperm of the corn kernel (Singh *et al.*, 2006). The thermostable enzyme increases its activity in the presence of water and elevated temperature, enabling the hydrolysis of starch

into oligosaccharides. This corn variety has been developed for the dry mill industry and decreases the requirement for addition of exogenous α -amylase enzyme; thus lowering the raw material costs for the ethanol production. The same corn could be used in the raw starch process variation to decrease α -amylase requirements and expanded to include thermostable glucoamylase enzymes embedded within the corn grain. Providing both enzymes within the corn may eliminate the need for exogenous enzyme while enabling low-temperature processing that can reduce capital and operating expenses. The cost savings enabled by using corn that produces its own enzyme are estimated to be between \$0.03–\$0.07/gallon for the dry mill process. This modest estimate (from 2% to 7% of dry mill processing costs) may be expanded in the raw starch process, where eliminating the jet cooker and high-temperature liquefaction and consolidating the saccharification could provide a saving of approximately \$0.20/gallon or more if fewer inhibitory compounds or the level of maillard products were produced.

The nonfermentable segment of the corn grain consists of fiber and protein, a byproduct that is sold as animal feed. While a number of technologies have been developed to separate out the other subcomponents of the corn kernel, such as germ and oil, or to convert the fiber into additional ethanol, the remaining protein could be augmented with additional modifications to the corn to increase its value. Possibilities are the modification of the protein or amino acid composition and the addition of other nutrients or embedding enzymes such as phytase, which are added into the animal feed downstream. Depending upon the specific modification, increasing the value of the DDGS stream (which currently provides an approximately \$0.15–\$0.25/gallon credit) can have a large impact on the ethanol cost structure.

4.8 Enabling Cellulosic Ethanol

The most significant opportunity for increasing ethanol production from corn is by enabling the utilization of the stover and fiber as feedstock. Similar compositional modifications and processing capabilities can be applied to corn stover for

improved cellulosic ethanol production as were described for corn grain ethanol production.

There is significant potential for producing ethanol from lignocellulosic materials such as corn stover, and research in this area has become a major focal point for the US Department of Energy (DOE) and United States Department of Agriculture (USDA). Thus far, commercial production has been limited to a couple of small-scale pilot plants and demonstration facilities because of the expensive processing costs (DOE, 2006b). The economics of cellulosic ethanol production are considerably different than that of the dry mill process, with the cost per gallon estimates ranging from \$1.50 to \$2.20 using current technologies (Aden *et al.*, 2002; DOE, 2006b). The primary advantage of cellulosic ethanol production is the lower feedstock costs provided by corn stover and other biomass materials; however, this advantage is overcome by increased processing costs. The recalcitrance of the lignocellulose requires a severe pretreatment and an adequate supply of enzymes is much greater than the dry mill process leading to exorbitant capital and operating costs.

Many of the same transgenic opportunities described above for starch processing can also be utilized for enabling the production of ethanol from corn stover. The production of plant structural components, including lignin, hemicellulose, and cellulose, can be altered to increase the polysaccharide content and lower the lignin content. Such changes in cell wall biochemistry would provide greater levels of sugar in a form that could be processed more easily. Unfortunately, cell wall biosynthesis and the mechanisms of pretreatment reactions and cell wall degradation are still not well-understood phenomena. Preliminary annotation of the *Arabidopsis* genome has identified at least 1000 known or putative genes encoding cell wall related proteins (Yong *et al.*, 2005). Among the targets currently studied are genes in the cellulose synthase family (*CESA*), cellulose synthaselike (*CSL*), xyloglucan endotransglycosylases/hydrolases (*XTH*), expansins (*EXPA*, *EXPB*), expansinlike (*EXLA*, *EXLB*), p-Coumarate co-enzyme A ligase (*CCL*) and endoglucanases or plant-produced cellulases (Cosgrove, 2005).

The cost advantages of optimized compositional modifications are hard to predict. For example, while decreasing lignin composition may improve

ethanol production costs by decreasing the pretreatment conditions (resulting in up to a \$0.20/gallon cost reduction and importantly decreasing the upfront capital requirements for processing facilities) and decreasing enzyme requirements (estimated at \$0.15–\$0.50/gallon), it may also reduce the lignin available for energy generation, which is a valuable byproduct (providing \$0.10–\$0.25/gallon for energy purchase offsets and electricity credits). Compositional modification may also enable process improvements in purification or allow higher sugar streams to be used, which would decrease capital costs. However, until plants are actually made with these modifications and their processing capabilities are tested, it is unclear what the economic impact will be. Furthermore, if compositional modifications to corn affect agronomic performance, the plant is unlikely to be commercially viable and may have difficulties progressing through the regulatory process. While compositional modifications can be identified in traditional breeding programs and some have been studied in depth (e.g., the maize brown mid-rib mutants), so far none have found widespread commercial use and have not yet enabled cellulosic ethanol production.

Similar to the improvements made by amylase production for grain ethanol facilities, improvements in cellulosic ethanol processing can be expected from plant production of cellulase, hemicellulase, and ligase enzymes. From a cost perspective, these enzymes are responsible for \$0.15–\$0.35/gallon but in a mature cellulosic ethanol market are likely to be in high demand, which could drive enzyme costs higher. Consider that 0.020 g cellulase/g cellulose is required for the process and if cellulase production is approximately 100 g/l, then the fermentation capacity required for enzyme production for a 30 billion gallons cellulosic ethanol market would be 17.6 billion gallons, which creates a severe problem, considering the amount of glucose that will be required to support enzyme production at this scale.

Producing the cellulase enzymes in plants may enable low-cost cellulosic processing, saving enzyme costs, and potentially reducing the pretreatment requirements. One of the concerns of this option is that the enzymes may become active and hinder normal plant growth. To date, most studies expressing cellulase enzymes in plants have investigated the plant as a

source of enzymes, as opposed to creating a low-cost processing trait. Despite the difference in experimental objective, the production of unmodified lignocellulosic degrading enzymes has previously been demonstrated (Dai *et al.*, 1999; Ziegelhoffer *et al.*, 1999) and some studies showing high-protein expression have also been associated with hindered crop growth (Hood *et al.*, 2003; Clough *et al.*, 2006). Other studies have been performed with reportedly normal plant growth but these have shown low enzyme expression, have used enzyme catalytic domains lacking a binding domain, or have been performed in a less relevant model system than corn (Dai *et al.*, 2000a, b, 2005). None of these referenced reports have demonstrated the ability to produce multiple cell wall degrading enzymes in the same plant, which is known to have a synergistic effect on the activity of cell wall degrading enzymes (Wood and McCrea, 1986; Wood *et al.*, 1986; Woodward, 1991; Mansfield *et al.*, 1999). Additionally, studies conducted thus far have yet to challenge the plants at temperatures where the enzymes are active. The lack of a relevant temperature challenge is important because some enzymes may become active in the field, thus threatening harvest yields and agronomic performance, which is a critical consideration for maize traits. Finally, the targeting of the enzymes within cells or organelles has been practiced as an approach to ensure proper maturation of the plants (Jin *et al.*, 2003). This approach limits the enzyme accumulation potential, does not work for all enzymes (Hood *et al.*, 2003; Clough *et al.*, 2006), and distances the enzyme from its substrate providing a lower impact on the pretreatment costs.

While producing processing enzymes in the plant can have a dramatic impact on cellulosic ethanol production costs (estimated at between \$0.15/gallon and over \$0.65/gallon), obtaining high enough enzyme levels that still allow normal plant maturation and do not interfere with agronomic performance is critical. One solution often practiced is the use of thermophilic enzymes, whose activity is decreased at moderate temperatures and increased at high temperatures. While the activity modulation may be adequate to avoid adverse effects on plant physiology, this requires high-temperature processing, which prevents further process optimization that may occur at lower temperatures. Mechanisms that

control plant enzyme activity *in vivo* would be valuable to producing lignocellulosic degrading enzymes, and other enzymes in plants.

Given the rapid expansion in ethanol production over the last 5 years and the projected continued expansion over the next 5 to 20 years, agricultural biotechnology promises to have a strong impact on ethanol processing costs. Unlike improvements in single unit operations, transgenic traits have the potential to affect every unit of operation from the field to the final product. Input traits can impact yield and ensure availability, which ultimately affects the cost of sugar used in the ethanol fermentation. Output traits can change the cost structure of any targeted unit operation. Traits such as compositional modification and enzyme expression, depending upon the affected processing steps, could ultimately decrease the cost of producing cellulosic ethanol by over 35% using today's technology as a baseline.

In this review, the status of maize biotechnology has been reviewed and its likely impact on energy production evaluated. In 2004, 11% of the corn harvest produced 3.4 billion gallons of ethanol (DOE, 2006a). This volume supplies but 1.7% of the nation's fuel demand. Nevertheless, this output was sufficient to generate 147 000 that translated into \$2 billion of additional tax revenue.

If corn remains the principal engine of ethanol production in this country, then an unhealthy competition ensues between its utility as a food, feed and manufacturing additive, and as an energy source. In recent years, corn has dipped below \$2.00/bushel when purchased for its traditional use and is expected to bring more than \$4.00/bushel when purchased for ethanol production. In order to solve this problem, new technology needs to be developed that taps into a significant portion of the more than 1.3 billion dry metric tons of agricultural resources available whose ethanol is produced from cellulose. The cost to fund the laboratories that will provide impetus for gene discovery, for isolation of genetic regulatory elements that specify the time and tissue in which gene expression occurs, the delivery of newly synthesized heterologous genes to produce the transgenics, and their subsequent evaluation, to say nothing of the bulking of seed material, is high. However, if one considers that in 2005, more than \$250 billion was spent on oil imports, perhaps the investment in the development of cellulosic biotechnology is indeed a bargain.

5. CONCLUSION

In this review, an attempt has been made to provide an overview of the advances in maize biotechnology that involve from a concept to product development. In the last 10 years maize tissue culture has advanced to the point where it is robust with respect to speed and to the number of regenerants per explant. Most important are the observations that suggest that cell regeneration competence can be induced, making the procedure genotype independent. Of equal importance is the fact that the cells capable of regeneration are likewise competent with respect to gene transfer. Complementing the development of linked regeneration/DNA transfer protocols are the vigorous gene discovery programs. In principle, now any gene should be transferable making it possible to produce a large number of commercially available corn hybrids. These have been produced after many generations of selection and breeding, and now the technology exists to make them transgenic by the addition of single genes to the parental inbreds.

Hence with an investment that is small relative to potential economic recovery transgenic maize can be readily produced that range from its capacity to grow in alternative environments to serving as a bioreactor. It is likely in fact that maize because of the seed's large storage capacity will be used to produce a wide range of HIPs to service drug delivery in those parts of the world that are too poor to establish an alternative infrastructure. Then too the capacity to make transgenic maize to grow in a wide variety of environmental challenges will stabilize food resources as corn is shunted into the production of biofuel. Because of its importance, globally transgenic maize is a tool for good and will remain so in the hands of all who are of good will.

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Wheat

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1. INTRODUCTION

Global population growth in the next 50 years would occur essentially in the developing countries where malnutrition is already prevalent and over 800 million people face daily hunger. Forty percent of the world's land use for agriculture is already seriously degraded and to meet the nutritional needs of this growing population, cereal production in general and wheat production in particular will need to be increased by 40% in the next 20 years. Wheat is one of the world's most important food grains that provides more than 20% of the calories and protein in human diet, and the staple food for over 40 countries, accounting for 35% of the world's population (Bushuk, 1998). It is thus alarming to note that since 2002, world wheat stocks have declined gradually from 237 to 174 million tons in 2006 (FAO, 2006). Therefore, increases in wheat productivity in a sustainable way using conventional breeding methods anchor its limitations. A biotechnological tool for sustainable food supply is needed to meet these requirements in the near future.

1.1 Wheat Improvement

From its first introduction in 1996 till date, genetically modified (GM) crops have been adopted by more than 8.5 million farmers in 21 countries covering around 400 million hectares of cultivated land. Remarkably, the global biotech crop area increased by more than 50-fold in the

first decade of commercialization of GM crops. However, wheat is still not part of GM crops grown in the field (International Service for the Acquisition of Agri-biotech Applications, 2005). Wheat grain yield is a complex trait, reflecting the culmination of vegetative and reproductive growth and development, and their interactions with the edaphic and aerial environments. Stably expressed genes leading to higher grain yield are important targets of wheat breeding. Quantitative genetics now offers the prospect of dissecting and characterizing the genetic complexity of yield. Moreover, the coincidence of quantitative trait loci (QTL) for yield and those for other traits provides information on traits likely to be responsible for yield. Recently, Uauy *et al.* (2006) reported the positional cloning of *Gpc-B1*, a wheat QTL associated with increased grain protein, zinc, and iron content. The ancestral wild wheat allele encodes a NAC transcription factor (*NAM-B1*) that accelerates senescence and increases nutrient remobilization from leaves to developing grains, whereas modern varieties carry a nonfunctional *NAM-B1* allele.

Increasing grain yield can be achieved by increasing either the total biomass produced by the crop or the proportion of the total biomass that is invested in grains (greater harvest index). Grain yield also represents the product of grain number and mean weight per grain. Grain number *per se* can be broken down into its components: ear number per plant and grain number per ear, which are determined by the number of spikelets per ear and grains per spikelet. Therefore, by studying how

these yield components vary within a particular genetic background, it is possible to gain an insight into the possible function of a gene that influences yield and when, as well as how, they are likely to exert the effect(s).

1.2 Need for Genetic Engineering of Wheat

One of the major limitations in conventional breeding involves the barriers for gene transfer through incompatibility and species differences. Progress in biotechnology has resulted in insertion of desired foreign gene(s) to overcome problems of sexual incompatibility and species barriers between organisms. This technology helps breeders and molecular biologists to introduce the gene of interest with more selective modification and represents a significant advance. In addition, transgenics have many other advantages, namely, the potential to resist biotic and abiotic stresses, add nutritional quality to the product, herbicide and pesticides resistance, etc., resulting in increased productivity. Recent genetic engineering technology allows not only transfer of a single gene, but also a couple of genes in a much more precise, controllable, and predictable way than is achievable with conventional breeding. Genetic engineering offers a useful supplement to plant breeding like the killing protein (KP4) from maize has been transferred to wheat (Clausen *et al.*, 2000) for imparting resistance against stinky smut (*Tilletia tritici*) and similarly a potent antimicrobial protein Ace-AMP1 from onion (*Allium cepa*) was transferred for enhancing fungal disease resistance (Roy-Barman *et al.*, 2006). Improvements can thus be achieved in much shorter time frames and of selected traits.

2. DEVELOPMENT OF TRANSGENIC WHEAT

2.1 Technical Advances in Wheat Transformation

2.1.1 Particle bombardment mediated transformation

The majority of wheat transformation investigations reported (Vasil *et al.*, 1992, 1993; Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*,

1994; Zhou *et al.*, 1995; Altpeter *et al.*, 1996; Barro *et al.*, 1997; Zhang *et al.*, 2000; Melchiorre *et al.*, 2002; Chugh and Khurana, 2003; Patnaik and Khurana, 2003; Yao *et al.*, 2006) have utilized microparticle bombardment technology for wheat transformation.

Vasil *et al.* (1991, 1992) for the first time stably transformed wheat suspension cells and callus lines through particle bombardment. However, from suspension cells regeneration could not be achieved and regenerated plants from the callus lines were sterile. Perl *et al.* (1992) reported that for bombardment the regeneration of scutellar calli can be rendered efficient and the time required considerably accelerated by a liquid culture phase. The expression of reporter gene following DNA delivery can be improved by maintaining the scutellar calli in 0.25 M mannitol before and after bombardment, by bombardment in the presence of silver thiosulfate and $\text{Ca}(\text{NO}_3)_2$ (rather than CaCl_2) and by the elimination of spermidine from the DNA/microparticle mixture. A protocol that includes all these features leads to several-fold higher transient expression of the reporter gene.

Weeks *et al.* (1993) for the first time reported production of fertile transgenic wheat through particle gun by using 5-day-precultured immature embryo of 0.5–1.0 mm size. The frequency was, however, very low as 1–2 transgenic plants could be recovered out of the 1000 explants bombarded. Moreover, it takes around 5–6 months to seed setting of T_0 plants according to the protocol followed by them. In a similar study by Becker *et al.* (1994), particle bombardment was done with pDB1 plasmid in scutellar tissue. They also optimized several parameters such as distance between different gun components, gas pressure, partial amount of vacuum, and size and amount of particles per bombardment. Around 100 transient transformation events per embryo could be observed. However, after selection on Basta, 12 plants were recovered out of 1050 bombarded explants. Nevertheless, the transgenics obtained showed Mendelian inheritance for the genes introduced.

Takumi and Shimada (1997) studied the genotypic influence on regeneration and transformation. They used six genotypes in the study and found that although transient gene expression was similar among the genotypes, recovery of stably transformed plants varied significantly. The better

responding genotypes, namely, Akadurama and Norin 12, showed 1.4% and 1.7% transformation frequency, respectively, but no transgenic plants were recovered. This was attributed to their *in vitro* culture response variability rather than the transformation procedure. Similarly, Ingram *et al.* (1999) optimized parameters for transient gene expression in microspore-derived embryos of wheat. A preculture of 4 h in 0.4 M maltose was found to enhance the transient expression by a magnitude of 3.

One of the major drawbacks of particle bombardment-mediated genetic transformation is the integration of multiple copies of the transgene. Srivastava *et al.* (1999) demonstrated a possible way to obtain single copy transgenics based on site-specific recombination of the *cre-lox* systems (Sauer, 1994). They designed a plasmid pVS11 harboring *bar* gene flanked by *lox* recombination sites and thus four multiple copy loci were resolved successfully into single copy transgenes.

In a subsequent work by Pastori *et al.* (2001), two elite wheat varieties were transformed with a relatively high embryogenic capacity by particle bombardment. A strong correlation between transformation frequency and the age of wheat donor plants was observed in both varieties. The mean transformation frequency increased from 0.7% to 5% when using immature embryos from old and young donor plants, respectively. The efficiency of biolistic transformation strongly depends on the condition of the donor plant and the plant genotype chosen for the transformation process. Pellegrineschi *et al.* (2002) analyzed the transformation efficiency of 129 wheat sister lines. Of the 129 genotypes evaluated, 8 demonstrated transformation efficiencies above 60% (60 independent transgenic events per 100 immature embryos bombarded). Bobwhite SH 98 26 was identified as a supertransformable wheat line. Recently, Yao *et al.* (2006) reported low copy gene transfer but stable expression of transgene in a commercial wheat via particle-gun method. They made linear gene constructs lacking vector backbone and containing a promoter, ORF, and terminator. Despite the transformation frequency was low, being 0.5–0.6% only, the expression of transgene *1Ax1* (coding for a unit of HMW glutenin) was conformed in the T₁ progeny by sodium dodecyl sulfate polyacrylamide gel electrophoresis and reverse transcription polymerase chain reaction (RT-PCR) analysis.

2.1.2 *Agrobacterium*-mediated transformation of wheat

Agrobacterium-mediated transformation has several desirable features over naked DNA delivery, such as introduction of few copies of genes and more chances of recovery of single copy transgene, high co-expression of introduced genes, easy manipulation of transgene and the ability to transfer large segments of DNA with minimal rearrangements, and lower cost of experimentation (Hiei *et al.*, 1994; Gheysen *et al.*, 1998; Hansen and Wright, 1999; Shibata and Liu, 2000; Jones *et al.*, 2005). Though several attempts were made to transform cereal crops with *Agrobacterium* as early as in 1988 by Grimsley *et al.* (1988), the first rice transformation was reported by Raineri *et al.* (1990). Stable wheat transformation through *Agrobacterium* could only be achieved in 1997 (see Table 1), when Cheng *et al.* (1997) successfully transformed a variety of wheat explants with a transformation frequency ranging from 0.14% to 4.3%. They studied several factors affecting transformation frequency, such as explant type (leaf tissue from young seedling, immature inflorescence, freshly isolated and precultured immature embryos, embryogenic callus derived from immature embryos, and cells in suspension culture), *Agrobacterium* cell density for inoculation, inoculation and co-cultivation time period, co-culture medium and presence of surfactant and inducer in the inoculation medium. However, they did not use any gene of interest but inheritance and expression of *nptII* and *uidA* genes were analyzed till T₂ generation, and in most of the transgenic events the transgenes behaved as dominant loci exhibiting normal Mendelian segregation with 98% co-expression as determined by paromomycin leaf paint and β -glucuronidase (GUS) histochemical assay. Nevertheless, presence of inducer acetosyringone and glucose in the inoculation and in co-cultivation medium was necessary for freshly isolated immature embryo transformation, while these could be omitted for transformation of suspension culture cells suggesting differing competence of various explants for *Agrobacterium*.

As an alternative explant, Amoah *et al.* (2001) used immature inflorescence from a commercial wheat cultivar of spring wheat, Baldus, known for its hard endosperm and bread making quality. They took 0.5–1.0 cm immature inflorescence and precultured them on media containing

Table 1 Progress in wheat transformation

Transformation method	Explant	Vector	Selection marker	Gene of interest	References
Particle gun	Immature embryo	pAHC25	<i>Bar</i>		Weeks <i>et al.</i> (1993)
Particle gun	Immature embryo	pBARGUS	<i>Bar</i>		Nehra <i>et al.</i> (1994)
Particle gun	Immature embryo	pDBI	<i>Bar</i>		Becker <i>et al.</i> (1994)
Particle gun	Immature embryo	pAHC25	<i>Bar</i>		Barro <i>et al.</i> (1997)
Particle gun	Immature embryo	pACT1F	<i>Bar</i>		Takumi and Shimada (1997)
Particle gun	Microspore callus	pAHC25	<i>Bar</i>	<i>1Ax1, 1Dx5</i>	Ingram <i>et al.</i> (1999)
Particle gun	Immature embryo	Many	<i>Bar</i>		Srivastava <i>et al.</i> (1999)
Particle gun	Immature embryo	pUBARN	<i>Bar</i>	<i>PbyA</i>	Brinch-Pedersen <i>et al.</i> (2000)
Particle gun	Immature embryo	pRQ101/pESWSMV-N1b	<i>Bar</i>	<i>N1b</i>	Sivamani <i>et al.</i> (2000a)
Particle gun	Immature embryo	pAct:bar/pUbi:KP4	<i>Bar</i>	<i>KP4</i>	Clausen <i>et al.</i> (2000)
Particle gun	Immature embryo	pAHC25	<i>Bar</i>		Rook <i>et al.</i> (2000)
Particle gun	Immature embryo	pAHC20/pSF1	<i>Bar</i>	<i>Ferritin</i>	Drakakaki <i>et al.</i> (2000)
Cellular permeabilization	Mature and immature embryo	pBI121/pActGUS	<i>np1II</i>		Mahalakshmi <i>et al.</i> (2000)
Particle gun	Microspore callus	pAHC25/pDM803	<i>Bar</i>		Brisbe <i>et al.</i> (2000)
Particle gun	Immature embryo	pAHC25	<i>Bar</i>	<i>1Dx5</i>	Alvarez <i>et al.</i> (2000)
Particle gun	Immature embryo	pLZ12	<i>Bar</i>	<i>mc70</i>	Zhang <i>et al.</i> (2001)
Particle gun	Immature inflorescence	pHMW-GUS/pAHC20	<i>Bar</i>	Promoter <i>Glu1D-1</i>	Lamacchia <i>et al.</i> (2001)
Particle gun	Immature embryo	pAHC25	<i>Bar</i>		Pastori <i>et al.</i> (2001)
Particle gun	Immature embryo	pRQ101A	<i>Bar</i>	<i>sh2</i>	Smidansky <i>et al.</i> (2002)
Particle gun	Immature embryo	pAHC17/pRQ103	<i>Bar</i>	<i>WSMN CP</i>	Sivamani <i>et al.</i> (2002)
Particle gun	Scuteller calli	PGU/Snos	<i>np1II</i>	Promoter <i>GBSS1</i>	Kluth <i>et al.</i> (2002)
Particle gun	Immature embryo	pAHC25	<i>Bar</i>	<i>HKT1</i>	Layrie <i>et al.</i> (2002)
Particle gun	Immature embryo	PAHCUBi	<i>Bar</i>	<i>Chitinase/glucanase</i>	Anand <i>et al.</i> (2003a)
Particle gun	Immature embryo	pAHC20	<i>Bar</i>	<i>Lr21</i>	Huang <i>et al.</i> (2003)
Particle gun	Immature embryo	pAHC20	<i>Bar</i>	Promoter <i>H2B</i>	Rasco-Gaunt <i>et al.</i> (2003)

Particle gun	Immature embryo	pGL2	<i>hpt</i>	Permingeat <i>et al.</i> (2003)
Particle gun	Mature embryo calli	pDM302/pAct1F	<i>Bar</i>	Patnaik and Khurana (2003)
Particle gun	Immature embryo	pGA1.8/pGA4.2	<i>Bar</i>	Hogg <i>et al.</i> (2004)
Particle gun	Immature embryo		<i>Bar</i>	Zuther <i>et al.</i> (2004)
Particle gun	Immature inflorescence	pAHC25	<i>Bar</i>	Tosi <i>et al.</i> (2004)
Particle gun	Immature embryo			Yan <i>et al.</i> (2004)
Particle gun	Immature embryo	pJF <i>npII</i>	<i>npII</i>	Altpeter <i>et al.</i> (2005)
Particle gun	Immature embryo	pAB5	<i>Bar</i>	Bahieldin <i>et al.</i> (2005a)
Particle gun	Immature embryo	P _{Ubi} <i>Bar</i>	<i>Bar</i>	Loukoianov <i>et al.</i> (2005)
Particle gun	Immature embryo	<i>pUbi::Ace-AMPI/pAct::bar</i>	<i>Bar</i>	Roy-Barman <i>et al.</i> (2006)
Particle gun	Immature embryo	pAHC25	<i>Bar</i>	Mackintosh <i>et al.</i> (2006)
Particle gun	Immature embryo	pAHC20	<i>Bar</i>	Khanna and Daggard (2006)
Particle gun	Immature embryo	pAHC20	<i>Bar</i>	Martin <i>et al.</i> (2006)
Particle gun	Immature embryo	pAHC25/pAHC20	<i>Bar</i>	Yao <i>et al.</i> (2006)
<i>Agrobacterium</i>	Many	pMON	<i>npII</i>	Cheng <i>et al.</i> (1997)
<i>Agrobacterium</i>	Immature inflorescence	PAL156	<i>Bar</i>	Amoah <i>et al.</i> (2001)
<i>Agrobacterium</i>	Developing ear	pBI-P5CS	<i>npII</i>	Sawahel and Hassan (2002)
<i>Agrobacterium</i>	Mature/immature embryo	p35SG/USINT	<i>npII</i>	Khurana <i>et al.</i> (2002)
Agro/particle gun	Leaf basal segment calli	pCambiaIA3301/pAHC25	<i>Bar</i>	Chugh and Khurana (2003)
Agro/particle gun	Immature embryo	pMON	<i>Bar</i>	Hu <i>et al.</i> (2003)
<i>Agrobacterium</i>	Immature embryo	pAL154	<i>Bar</i>	Wu <i>et al.</i> (2003)
<i>Agrobacterium</i>	Immature embryo	pHK21/pHK22	<i>Bar</i>	Khanna and Daggard (2003)
<i>Agrobacterium</i>	Immature embryo	pTOK233/pDM805/	<i>bar/hpt/npII</i>	Przetakiewicz <i>et al.</i> (2004)
<i>Agrobacterium</i>	Mature embryo calli	pCambiaIA3301	<i>bar</i>	Vishnudasani <i>et al.</i> (2005)
<i>Agrobacterium</i>	Basal segment	PBLG	<i>npII</i>	Zhao <i>et al.</i> (2006)
<i>Agrobacterium</i>	Mature embryos	Many	<i>bar/npII</i>	Patnaik <i>et al.</i> (2006)

Picloram and AgNO_3 . *Agrobacterium* hyper virulent strain AGL1 with plasmid pAL156 and pAL186 were used for transformation by liquid media inoculation, as well as by sonication and vacuum infiltration. Presence of acetosyringone (200 μM) in inoculation and co-cultivation media was necessary as no GUS spots were visible in the absence on the same. Moreover, acetosyringone was found to be effective in a dose-dependent and time-scale manner. A lower concentration (100 μM) can be complimented by longer time inoculation and vice versa, while a higher concentration of acetosyringone (400 μM) was found to be less responsive and somewhat bacteriostatic in action. Another factor influencing transient gene expression was the time of preculture, i.e., time between when inflorescence was first isolated and cultured to when the explants were incubated with *Agrobacterium*. A period of 21-day preculture was most responsive (76% GUS expression) but, 7-day preculture is necessary for GUS expression (if any). Preculture length beyond 21 days resulted in decrease in the number of explants showing *uidA* expression. A similar trend was observed in *uidA* expression in terms of number of spots per explant. In the line of other workers, Amoah *et al.* (2001) found a bacterial density of $A_{660} = 1.5$ most responsive. They also found beneficial effects of vacuum infiltration of *Agrobacterium* during inoculation both in terms of explants showing *uidA* expression as well as spots per responding explants. In a separate experiment, sonication was done for different time periods (2–6 s) during inoculation and positive effect was seen in terms of explants producing GUS expression, but number of spots per explants reduced as compared to the control.

Wu *et al.* (2003) studied and optimized factors affecting transfer-DNA (T-DNA) delivery and regeneration, namely, embryo size and duration of preculture, inoculation, co-cultivation, and presence of acetosyringone and Silwett L-77 in the media for two winter and two spring wheat genotypes. Among all the genotypes tested, embryo size of 1.5 mm was found to be best responsive in terms of GUS expression. However, the response in tissue culture showed opposite trend with the smaller size calli showing significantly higher regeneration frequency. In accordance with previous studies (Cheng *et al.*, 1997; Amoah *et al.*, 2001), presence of acetosyringone (200 μM) enhanced GUS expression significantly, both in

terms of percent explant showing GUS expression as well as spots per explant. Contrary to the study carried out with immature inflorescence by Amoah *et al.* (2001) where longer preculture (21 days) enhanced transformation frequency, a reverse phenomenon was observed by Wu *et al.* (2003) in their study. Transformation efficiency reduced greatly even if explants were precultured for more than 1 h.

In an augmented approach toward overcoming the recalcitrance for transformation and regeneration in commercial wheat genotypes, Khanna and Daggard (2003) used super binary vector carrying extra set of *vir* genes for transformation of Australian spring wheat genotype “veery 5.” *Agrobacterium* strain LBA4404 with super binary vector pHK21 and binary vector pHK22 were used. They found that normal binary vector pHK22 when used gave no transient GUS expression in 587 3-week-old calli, while super binary vector pHK21 gave 17 stable transgenic plants out of 658 calli used in agroinfection. To gain maximum regeneration during selection after agroinfection, they used 0.1 M spermidine in the media after 3 weeks of callus development on selection. Addition of spermidine in the media resulted in three times more regeneration of transgenic plants (24% from 7%).

In their attempt to produce commercial GM wheat, Hu *et al.* (2003) at Monsanto carried out large-scale *Agrobacterium*-mediated transformation with an array of vectors carrying 5-enol-pyruvyl shikimate-3-phosphate synthase gene (governed by different promoters and transient peptides) from the *Agrobacterium* strain CP4. This CP4 confers resistance to glyphosate and serves as a selection marker besides being a gene of interest. More than 3000 transformation events were produced with an average transformation efficiency of 4.4%. The entire procedure was completed in 80 days and first Roundup Ready (glyphosate tolerant) commercial wheat was produced for field trail. However, the genotype used in the study was Bobwhite, which is of little agronomic importance. The comparison with biolistic transformation was also done simultaneously and it was found that *Agrobacterium*-mediated transformation resulted in more single copy transgenics (66%) and around half of them showed Mendelian inheritance of 3:1. Moreover, transgene expression was stable up to T_9 generation.

Toward developing transgenics of Polish winter wheat, Przetakiewicz *et al.* (2004) used three combinations of *Agrobacterium tumefaciens* strains and vectors. They used two hypervirulent strains, AGL1, containing the pDM805 binary plasmid, and EHA101, containing pGAH and the common *Agrobacterium* strain LBA4404, harboring the super binary pTOK233 vector. The pDM805 contained *bar* under the control of *Ubi1* promoter, pGAH had *nptII* under *nos*, and pTOK233 had *hpt* under CaMV 35S. Additionally, pDM805 and pTOK233 carried the *gus* reporter gene under the *Act1* promoter or 35S promoter, respectively. They obtained 12.6% transformation efficiency (highest reported) with EHA101 (pGAH) on a kanamycin-containing medium. The second best combination was LBA4404 (pTOK233) with kanamycin selection, which gave an average transformation rate of 2.3%. Phosphinothricin selection gave 1.0% transformation efficiency, while hygromycin, depending on the strain/vector used, gave from 0.2% to 0.4%. PCR analysis of T₁ revealed that 67% of the lines showed a 3:1 segregation ratio, and 11% a 15:1 ratio, while in 22%, segregation was non-Mendelian.

In a nonconventional approach through *Agrobacterium*-mediated genetic transformation in wheat, Sawahel and Hassan (2002) injected *Agrobacterium* inoculum directly into spikelets just before anthesis. The strain used in the study was LBA4404 and the vector was pBI-P5CS carrying *nptII* and *Vigna aconitifolia* Δ' -pyroline-5-carboxylate synthetase complementary DNA (cDNA), for proline biosynthesis. A transformation frequency of 0.9% was achieved and the transgenics were analyzed by Southern, Northern, and Western analysis of P5CS product. However, this method has not been exploited since by other workers.

Besides bread wheat (*Triticum aestivum* L.), *Agrobacterium*-mediated transformation was extended to tetraploid emmer wheat (*Triticum dicoccum* Schuble) by Khurana *et al.* (2002). Two commercial *dicoccum* wheat cultivars, namely DDK1001 and DDK1009, were transformed with LBA4404 with two different vector constructs (p35SGUSINT and pBI101:act1) using a variety of explants. Both the constructs had *nptII* as selection marker and *gus* as the reporter gene. Mature embryo-derived calli were found to be more effective than any other explant and

it was concluded that the monocot promoter *act1* is four times more effective in terms of transient GUS expression than CaMV 35S promoter. In a subsequent study, Chugh and Khurana (2003) made a comparison between particle bombardment and *Agrobacterium*-mediated transformation of bread and emmer wheat using basal segments as explants. LBA4404 carrying pCambia3301 and vector pAHC25 were used for *Agrobacterium*-mediated and direct gene transfer through particle bombardment. Both vectors had *bar* gene for selection and *uidA* as reporter gene. Transformation efficiency in the range of 4% was obtained with particle bombardment, whereas it was 7.5% using *Agrobacterium*. More recently, Patnaik *et al.* (2006) successfully transformed six different commercial Indian cultivars of bread and *durum* wheat with a variety of constructs and optimized several key factors such as explants, efficiency of selection gene, and performance of different promoters in effecting expression of transgene. In this study, direct transformation of mature embryo explants was done to minimize the loss of regeneration potential due to prolonged culture procedures, thus opening the possibility for mature embryo explants as they are not only easily available but large numbers can also be handled simultaneously.

2.1.3 Other approaches toward wheat transformation

For the first time, Sautter *et al.* (1991) constructed a novel microprojectile accelerating system for efficient gene transfer into cells *in situ* that avoids binding DNA to microprojectiles and keeps the DNA in solution. Instead of a macroprojectile (or the equivalent), the particles were accelerated in a Bernoulli air stream. This micro-targeting approach directed highly dispersed particles to sites with diameters as little as 0.15 mm, allowing precise aiming to restricted tissues. Transient expression of the *Escherichia coli* β -glucuronidase gene in immature wheat embryo scutella was obtained at a frequency of up to 3% of the treated cells in the surface layer. Zhu *et al.* (1993) reported transformation and regeneration of wheat protoplasts by plasmid pCGN1055 containing *hpt* gene under control of a plant promoter by cationic liposomes (lipofectin).

Transformation frequency of 6.0% and 8.8% was reported based upon hygromycin resistance and DNA molecular hybridization studies. However, the plants regenerated were albinos.

Exogenous DNA uptake and transient expression of the *gus* reporter gene was studied in wheat zygotic embryos employing a simple procedure of cellular permeabilization by Mahalakshmi *et al.* (2000). Use of membrane inactive agents like saponin and toluene resulted in uptake of expression vectors in mature as well as immature embryos. Variation of GUS activity was not different among the three genotypes tested in the study suggesting relative genotype independence of the method. Initially developed by Kaeppler *et al.* (1992), silicon carbide whisker-mediated stable transformation system was adopted by Brisibe *et al.* (2000) for different lines of wheat anther culture and compared with microprojectile bombardment technology. They used a variety of vectors with different selection markers and found that after 2 months of selection pressure, silicon carbide whisker-mediated transformed cell lines had 28–64% recovery while recovery for microprojectile bombarded cell lines was 15–36% only.

In an alternative approach for particle bombardment, electroporation was used effectively to deliver DNA into the tissue of intact but immature embryos by Sorokin *et al.* (2000). Transformed plantlets were recovered after electroporation using field strengths of 275 and 750 Vcm⁻¹, 960-μF capacitor, and 50 μgml⁻¹ of linear plasmid DNA, containing *bar* and *uidA* genes. A field strength of 750 Vcm⁻¹ proved to be more effective for DNA delivery (estimated by transient GUS expression) and for recovery of transformed plants (two transgenic plants were recovered with an efficiency of 0.4%). After application of field strength of 275 Vcm⁻¹ there was no visual evidence of transient GUS expression, but one transgenic plant was recovered with an efficiency of 0.2%, based on the number of electroporated embryos. Southern blot hybridization revealed a low copy number of transgene integration with some rearrangements at the integrated loci. None of the transgenic plants showed any visible GUS expression, however, RT-PCR analysis showed presence of *uidA* gene. This may indicate that suppression of *uidA* expression occurred at the post-transcriptional level, as also suggested by Bahieldin *et al.* (2005a). Use of electroporation to

effectively deliver DNA into the tissue of intact wheat immature embryos was also reported by Liang *et al.* (2005). Several physical parameters were optimized such as field strength, capacitor strength, and amount of plasmid. Electroporation was carried out by a portable and permanent ring electrode that can be fitted into the wells of 24-well plates and samples pulsed three times. Integration of the introduced genes into the genome of transgenic plants was shown by PCR and Southern analysis. Transformation frequency was 7.5% much higher than 4.2% by microprojectile bombardment.

A modified, nondamaging protocol for the production of fertile transgenic wheat (*T. aestivum* L. cv. Giza 164) plants by laser micropuncture was reported by Badr *et al.* (2005). The new indigenous setup secured transformation of as many as 60 immature embryo-derived calli (10 000 cells each) in less than 1 h using a UV excimer laser with two-dimensional translation stages, a suitable computer program and a proper optical system. Five-day-old calli were irradiated by a focused laser microbeam to puncture momentarily made self-healing holes (approximately 0.5 μm) in the cell wall and membrane to allow uptake of the exogenous DNA. The plant expression vector pAB6 containing *bar* gene as a selectable marker and GUS (*uidA*) gene as a reporter gene were used for transformation. Induced calli without any selection pressure were transferred to a modified MS medium with 1 mg l⁻¹ Bialaphos for regeneration followed by more stringent selection on 2 mg l⁻¹ Bialaphos for rooting. Three regenerated transgenic events were evaluated for the integration and stable expression of both genes.

2.2 Wheat Transgenics for Functional Genomics Analysis

Takumi *et al.* (1999) for the first time used wheat as a system to investigate the excision of a maize transposable element in wheat cells. The *uidA* gene expression indicated that the *Ds* excision was observed only in the transgenic callus lines stably expressing the *Ac* transposase gene. Drakakaki *et al.* (2000) employing particle bombardment produced transgenic wheat and rice plants expressing recombinant soybean ferritin, a protein that can store large amounts of iron,

expressed using the constitutive maize *ubiquitin-1* promoter, and showed that iron levels increased in vegetative tissues by 50% in R₂ generation of transgenic plants.

Stoger *et al.* (2001) used heterologous cereal system of wheat endosperm to produce, isolate, and crystallize 11S legume globulins for structural analysis and characterization of other important seed storage proteins of legumes. To study a single type of homogenous legumin they produced pea legumin A in transgenic wheat (*T. aestivum*) endosperm where prolamins are predominant, confirming that high-level production of a single type of legumin polypeptide also results in spontaneous crystal formation of *in vivo*. Kluth *et al.* (2002) undertook a detailed analysis of granule bound starch synthase 1 (GBSS1) promoter with the chimeric constructs of 5' deletions of *gbss1* gene with β -glucuronidase gene. They found that promoter activity was decreased with deletions below -4 kb. Beecher *et al.* (2002) showed that the pinB-D1b alteration, common in hard textured wheat, can be complemented by the expression of wild-type pinB-D1a in transformed plants. These results indicate that the pinB-D1b alteration is most likely the causative Ha mutation in the majority of hard wheat.

Laurie *et al.* (2002) successfully transformed wheat with both sense and antisense constructs of high affinity potassium transporter HKT1 and characterized it with different magnitudes of salt stress suggesting its role in the roots of wheat plants and the Na⁺:K⁺ ratios were reduced in salt stressed transgenic tissue when compared with controls. Rasco-Gaunt *et al.* (2003) characterized the constitutive maize Histone H2B promoter in both wheat and maize tissues using the *gusA* reporter gene and two synthetic versions of the *pat* (phosphinothricin acetyl transferase) selectable marker gene, namely mopat and popat. Analyses of transgenic plants showed that the H2B promoter is able to drive the expression of *gusA* to strong, constitutive levels in wheat and maize tissues. However, expression in the tissues was particularly strong in the floral tissues, in the young parts of the leaf and root tissues and in the meristems.

Huang *et al.* (2003) reported map-based cloning of the leaf rust resistance gene *Lr21*, previously mapped to a gene-rich region at the distal end of chromosome arm 1DS of bread wheat (*T. aestivum*

L.). Cloning of *Lr21* was confirmed by genetic transformation and by a stably inherited resistance phenotype in transgenic plants of susceptible genotype "Fielder." Yong *et al.* (2003) isolated a vernalization-related gene *VER2* from winter wheat (*T. aestivum* L.) using a differential screening approach and characterized it through transgenic approach involving antisense technology. Antisense inhibition of *VER2* in transgenic wheat showed that heading and maturation time were delayed up to 6 weeks. Tissue degeneration at the top of the spike was also noticed in the antisense-inhibited transgenic wheat suggesting that *VER2* plays an important role in vernalization signaling and spike development in winter wheat. In another interesting report, Yan *et al.* (2004) explored molecular difference between winter and spring wheat. They reported positional cloning of wheat vernalization gene *VRN2*, a dominant repressor of flowering that is down-regulated by vernalization. Through RNA interference (RNAi) they obtained transgenic winter wheat plants that have reduced flowering time of at least 1 month less than control wild type. Later, Loukoianov *et al.* (2005) characterized another vernalization gene of wheat *VRN1* and showed its interaction with *VRN2*, involving transgenic approach. They showed that the level of *VRN1* transcripts in early developmental stages is critical for flowering initiation. A reduction of *VRN1* transcript levels by RNAi delayed apex transition to the reproductive stage, increasing the number of leaves, and delayed heading time by 2–3 weeks.

Zuther *et al.* (2004) through transgenic approach in wheat made a detailed analysis of acetyl-CoA carboxylase (ACC) gene and confirmed presence of nested promoter governing differential response in cell organelles. The internal promoter is located in an intron removed from transcripts originating at the first promoter. These complex promoters, which are different for the cytosolic and plastid *ACC* gene control tissue-specific expression of the enzymatic activity supplying cytosolic, plastid, and mitochondrial pools of Melonyl-Co A. Wang *et al.* (2005) cloned and characterized a Ca²⁺ permeable channel gene *TaTPC1* of wheat. *TaTPC1* transgenic plants exhibited more stomatal closing in the presence of Ca²⁺ than the control, supporting a role for the calcium channel in regulating plant responses to environmental change.

Global comparative gene expression analysis is potentially a very powerful tool in the safety assessment of transgenic plants, since it allows for the detection of differences in gene expression patterns between a transgenic line and the mother variety. Gregersen *et al.* (2005) undertook global gene expression analysis of developing seeds of transgenic and nontransgenic wheat plants. Comparisons and statistical analyses of the gene expression profiles, however, revealed only slight differences at the three developmental stages. First complementation study through transgenic technology in wheat was done by Martin *et al.* (2006) when they restored wild-type (soft) phenotype in a *Pin a* null mutant having hard phenotype, additionally suggesting that both *Pin a* and *Pin b* genes need to be present for friabilin formation and soft grain.

The *Q* gene in wheat pleiotropically influences many other domestication-related traits such as glume shape and tenacity, rachis fragility, spike length, plant height, and spike emergence time. Simons *et al.* (2006) isolated the *Q* gene and verified its identity by analysis of knock-out mutants and transformation. The *Q* allele is more abundantly transcribed than *q*, and differs in a single amino acid. Rachis fragility, glume shape, and glume tenacity mimicked the *q* phenotype in transgenic plants exhibiting post-transcriptional silencing of the transgene and the endogenous *Q* gene. Variation in spike compactness and plant height was associated with the level of transgene transcription suggesting dosage effects of *Q*.

2.3 Wheat Transgenics for Biotic Stress Tolerance

2.3.1 Fungal resistance

Clausen *et al.* (2000) cloned a cDNA encoding the antifungal protein KP4 (killing protein) from double-stranded RNA virus, infecting *Ustilago maydis* and this cDNA was inserted behind the ubiquitin promoter of maize and genetically transferred to wheat varieties particularly susceptible to stinking smut (*T. tritici*) disease. The transgene was integrated and inherited over several generations. Of seven transgenic lines, three showed antifungal activity against *U. maydis*. The antifungal activity

correlated with the presence of the *KP4* transgene. *KP4*-transgenic, soil-grown wheat plants exhibit increased endogenous resistance against stinking smut. Similarly Oldach *et al.* (2001) introduced three cDNAs encoding the antifungal protein Ag-AFP from the fungus *Aspergillus giganteus*, a barley class II chitinase and a barley type I RIP, all regulated by the constitutive *Ubiquitin1* promoter from maize, in transgenic wheat. Heterologous expression of the fungal *afp* gene and the barley *chitinase* II gene in wheat demonstrated that colony formation and, thereby, spreading of two important biotrophic fungal diseases was inhibited approximately 40–50% at an inoculum density of 80–100 spores/cm².

Altpeter *et al.* (2005) developed a novel epidermis-specific promoter to enhance resistance of wheat to the powdery mildew fungus *Blumeria graminis* f. sp. *tritici*, with two defense-related genes. A 2.3 kb fragment of the wheat epidermis-specific *GstA1* promoter was used for overexpression of oxalate oxidase 9f-2.8 and *TaPERO* peroxidase, two defense-related wheat genes expressed in inner leaf tissues. Transient as well as stable overexpression of the *TaPERO* peroxidase gene in wheat epidermis under the control of the *GstA1* promoter resulted in enhanced resistance against *B. graminis* f. sp. *tritici*, whereas oxalate-oxidase overexpression had no effect in either system. To increase resistance against fungal pathogens through phytoalexins, two resveratrol synthase genes *vst1* and *vst2* from grapevine (*Vitis vinifera* L.) and the pinosylvin synthase gene *pss* from pine (*Pinus sylvestris* L.) under stress-inducible grapevine promoters were stably transformed into bread wheat by Serazetdinova *et al.* (2005). The *vst1* and *vst2* promoters were functional in wheat. Upon inoculation with the biotrophic pathogen *Puccinia recondita* f. sp. *tritici* several *vst* expressing wheat lines showed a significant reduction of disease symptoms ($19 \pm 9\%$ to $27 \pm 8\%$) compared to wild-type plants.

Mackintosh *et al.* (2006) evaluated the feasibility of using the rapid-maturing dwarf wheat cultivar Apogee as an alternative genotype for transgenic *Fusarium* head blight (FHB) resistance research. With comparable transformation efficiency (1.33%), Apogee was also found to exhibit high FHB susceptibility and reached anthesis within 4 weeks. A microsatellite marker haplotype analysis

of the chromosome 3BS FHB resistant QTL) region indicated that this region may be deleted in Apogee, indicating that Apogee is particularly well suited for accelerating transgenic FHB resistance research and transgenic wheat research in general.

Genes encoding pathogenesis-related (PR) proteins (chitinase and β -1,3-glucanase) were transformed into spring wheat, “Bobwhite” using a biolistic transformation protocol, with the goal of enhancing levels of resistance against scab (Anand *et al.*, 2003a). Twenty-four putative transgenic lines expressing either a single PR-protein gene or combinations thereof were regenerated. Transgene expression in a majority of these lines (20) was completely silenced in the T₁ or T₂ generations. Transgenic plants when bioassayed against scab showed a delay in the spread of the infection (type II resistance) under greenhouse conditions. However, none of the transgenic lines had resistance to scab in the field under conditions of strong pathogen, suggesting these plants lacked effective resistance to initial infection (type I resistance) under these conditions (Anand *et al.*, 2003b).

To enhance fungal disease resistance, wheat plants (cv. Bobwhite) were engineered to constitutively express the potent antimicrobial protein Ace-AMP1 (a lipid transfer protein from *A. cepa*), driven by a maize *ubiquitin* promoter along with its first intron by Roy-Barman *et al.* (2006). The levels of Ace-AMP1 in different transgenic lines correlated with the transcript levels of the transgene. Up to 50% increase in resistance to *B. graminis* f. sp. *tritici* was detected in detached leaf assays. In ears of transgenic wheat inoculated with *Neovossia indica*, *Ace-AMP1* intensified expression of defense-related genes and elevated levels of salicylic acid and of transcripts of phenylalanine ammonia lyase (PAL), glucanase (PR2), and chitinase (PR3) in the transgenic plants indicated manifestation of systemic acquired resistance (SAR). Makandar *et al.* (2006) showed that the *Arabidopsis thaliana* *NPR1* gene (*AtNPR1*), which regulates the activation of SAR, when expressed in the FHB-susceptible wheat cv. Bobwhite, confers a heritable, type II resistance to FHB caused by *Fusarium graminearum*. The enhanced FHB resistance in the transgenic *AtNPR1*-expressing wheat is associated

with the faster activation of defense response when challenged by the fungus, suggesting that *NPR1* is an effective candidate for controlling FHB.

Zhao *et al.* (2006) addressed two common issues of wheat biotechnology, to improve the transformation efficiency of wheat (*T. aestivum* L.) mediated by *Agrobacterium tumefaciens* and to make transgenics with higher level of fungal resistance. They explored the possibility of employing the basal portion of wheat seedling (shoot apical meristem) as the explants in three genotypes of wheat and transformed by *A. tumefaciens* carrying β -1, 3-glucanase gene. A total of 27 T₀ transgenic plants were obtained, and the average transformation efficiency was as high as 9.82%. Investigation of the T₂ plants revealed that some transformed plants had higher resistance to powdery mildew than the controls.

2.3.2 Viral resistance

Wheat (*T. aestivum* L. cv. Hi-Line) immature embryos were transformed with the replicase gene (*NIb*) of wheat streak mosaic virus (WSMV) by Sivamani *et al.* (2000b) through biolistic method. Transgenic plants showed various degree of resistance to WSMV. Two lines displayed higher resistance with very mild virus symptoms after inoculation and the new growth of 72% and 32% plants from these lines, respectively, were asymptomatic and had no detectable virus throughout the plant life cycle. In a later study, the same group stably transformed wheat plants with the coat protein (CP) gene of WSMV (Sivamani *et al.*, 2002). One transgenic line showed high resistance to inoculations of two WSMV strains. This line had milder symptoms and lower virus titer than control plants after inoculation. Northern hybridization indicated a high level of degraded CP-mRNA expression. However, when field evaluation was done for the plants from above the two experiments by Sharp *et al.* (2002), none of the transgenics performed better than the wild types under both virus-challenged and control conditions.

To produce virus resistant transgenic wheat plants, an *rnc70* gene encoding a mutant bacterial ribonuclease III (RNase III) was introduced by Zhang *et al.* (2001) through microprojectile

bombardment. T₁, T₂, and T₃ plants regenerated from three callus lines were challenged with barley stripe mosaic virus, and plants expressing RNase III exhibited a high level of resistance to the virus infection as evidenced by the absence of virus symptoms and reduced accumulation of virions in these plants.

2.3.3 Nematode resistance

Serine proteinase inhibitors (PIs) are proteins found naturally in a wide range of plants with a significant role in the natural defense system of plants against herbivores. Vishnudasana *et al.* (2005) engineered a plant serine PI (*pin2*) gene into *T. durum* cv. PDW215 by *Agrobacterium*-mediated transformation to combat cereal cyst nematode (*Heterodera avenae*) infestation with a transformation efficiency of 3% using mature embryo as explant. The *PIN2* systemic expression confers satisfactory nematode resistance. The correlation analysis suggests that at $p < 0.05$ level of significance the relative PI values show a direct positive correlation *vis-à-vis* plant height, plant seed weight, and also the seed number.

2.4 Wheat Transgenics for Abiotic Stress Tolerance

To obtain resistance against abiotic stress, Sivamani *et al.* (2000a) introduced the ABA-responsive barley gene *HVA1*, a member of group 3 late embryogenesis abundant (LEA) protein genes, into spring wheat (*T. aestivum* L.) cv. Hi-Line using the biolistic bombardment method. High levels of expression of the *HVA1* gene, regulated by the maize *ubi1* promoter, were observed in leaves and roots of independent transgenic wheat plants and inherited to offspring generations. When tested in control environmental conditions, the transgenic plants had higher water use efficiency values, significantly greater total dry mass, root fresh and dry weights, and shoot dry weight compared to the controls under soil water deficit conditions. Four selected transgenic lines were tested in nine field experiments over six cropping seasons and one promising line was selected for commercialization (Bahieldin *et al.*, 2005b).

Biosynthesis of mannitol improves tolerance to water stress and salinity. Abebe *et al.* (2003) showed ectopic expression of the *mtlD* gene in wheat (*T. aestivum* L. cv. Bobwhite) and tolerance to water stress and salinity was evaluated using calli and T₂ plants transformed with or without *mtlD*. However, the amount of mannitol accumulated in the callus and mature leaf was not in accordance with the performance of transgenics over wild type, concluding that the improved growth performance of mannitol-accumulating calli and mature leaves was probably due to other stress-protective functions of mannitol, although possibility of osmotic effects in growing regions of the plant cannot be ruled out.

Khanna and Daggard (2006) produced transgenic wheat lines through microprojectile bombardment of immature embryos of spring wheat cultivar Seri 82 with a recombinant antifreeze gene, *rAFPI*, targeted to the apoplast using a Murine leader peptide sequence from the mAb24 light chain for retention in the endoplasmic reticulum using a C-terminus KDEL sequence. Transgenic wheat line T-8 with apoplast-targeted antifreeze protein exhibited the highest levels of antifreeze activity and provided significant freezing protection even at temperatures as low as -7°C .

2.5 Wheat Transformation for End Product Use

Although several cases of successful and stable wheat transformation were reported till 1996, Bleschl and Anderson (1996) for the first time transformed wheat to change the amount and composition of high-molecular-weight glutenin subunits (HMW-GS), a class of seed storage proteins via genetic engineering. A gene encoding a novel hybrid subunit under the control of native *HMW-GS* regulatory sequences was inserted into wheat. Of 26 independent transgenic lines identified by Bialaphos selection, 18 expressed the co-transformed hybrid *HMW-GS* gene in their seed. The hybrid subunit accumulated to levels comparable to those of the native *HMW-GS*. Transgene expression was stable for at least three seed generations in the majority of lines and results showed that a native *HMW-GS* gene promoter can be used to obtain high levels of expression

of seed storage and, potentially, other proteins in wheat endosperm. Shimoni *et al.* (1997) studied the structural requirements for the assembly of HMW-GS, a recombinant protein between two cognate x- and y-type subunits expressed in transgenic wheat. In contrast to the naturally polymerized x- and y-type HMW-GS, a significant amount of the recombinant subunit remained monomeric, showing the possibility of modifying gluten assembly by expressing recombinant HMW-GS in transgenic wheat, thus having major implications for the improvement of wheat bread-making quality adjustment for end product need.

Altpeeter *et al.* (1996) transformed wheat with another gene of interest when they introduced the HMW-GS *1Ax1* gene, known to be associated with good bread-making quality into the Bobwhite cultivar of wheat (*T. aestivum* L.), in which it is not present naturally. Of the 21 independent transformed lines selected, 20 expressed the selectable *bar* gene, and nine the *1Ax1* gene. The amount of HMW-GS *1Ax1* protein produced in the different transgenic lines varied from 0.6% to 2.3% of the total protein, resulting in an increase of up to 71% in total HMW-GS proteins. The transgenic plants were normal, fertile, and showed Mendelian segregation of the transgenes. It has been found that the accumulation of HMW-GS *1Ax1* was consistent and stable up to the R₃ seed generation. Popineau *et al.* (2001) studied the effect of transgenes coding for subunits *1Ax1* and *1Dx5* in two near-isogenic wheat lines differing in their HMW subunit compositions and mixing properties. Overexpression of *1Ax1* and *1Dx5* subunits modified glutenin aggregation, but glutenin properties were much more affected by expression of the *1Dx5* transgene. This resulted in increased cross-linking of glutenin polymers. In dynamic assay, the storage and loss moduli of hydrated glutes containing *1Dx5* transgene subunits were considerably enhanced, whereas expression of the *1Ax1* transgene had a limited effect.

Agronomic traits and yield components were studied in seven transgenic lines of wheat in which four lines contained the transgenes for β -glucuronidase (*uidA*), herbicide resistance (*bar*), and for one HMW subunit, and three lines contained a transgene for one HMW glutenin subunit with no marker genes, and compared with the nontransgenic parent and null segregant

lines under field conditions during two agronomic years (Barro *et al.*, 2002). All transgenic lines had a longer heading date than the parents and no other differences were found between the lines constitutively expressing the *uidA* and *bar* genes from those which expressed only the HMW genes. Among the traits studied such as number of flowers per spike, flower fertility, harvest index, test weight, and grain protein content differences between transgenic lines and the parents were small, and could be eliminated by backcrossing transgenic lines with their parents and selecting for the wanted genotypes.

Yield in cereals is a function of seed number and weight; both parameters are largely controlled by seed sink strength. The allosteric enzyme ADP-glucose pyrophosphorylase (AGP) plays a key role in regulating starch biosynthesis in cereal seeds and is most likely the important determinant of seed sink strength. Toward overcoming the yield barrier during stress environments, Smidansky *et al.* (2002) transformed wheat with a modified form of the maize (*Zea mays* L.) *Shrunken2* gene (*Sh2r6hs*), which encodes an altered AGP large subunit. The *Sh2r6hs* transgene was functional after five generations in wheat. Developing seeds from *Sh2r6hs* transgenic wheat exhibited increased AGP activity in the presence of a range of orthophosphate concentrations *in vitro*. Transgenic *Sh2r6hs* wheat lines produced on average 38% more seed weight per plant. Total plant biomass was increased by 31% in *Sh2r6hs* plants.

Although the wheat grain is an excellent source of energy in the form of metabolizable carbohydrates and also provides protein, it has an important nutritional shortcoming with respect to phosphate, as phosphate is stored in the form of phytic acid, which is largely indigestible to humans and other monogastric animals. For a plausible solution wheat transformation was done by Brinch-pedersen *et al.* (2000) with an *Aspergillus niger* phytase encoding gene (*phyA*) under maize *ubiquitin1* promoter and wheat α -amylase signal peptide (Ubi-SP-Phy) for endosperm localization. The Ubi-SP-Phy transgenic seeds showed up to fourfold increase of phytase activity. Later on, transgenic wheat materials possessing wheat endogenous 6-phytase and *Aspergillus* 3-phytase activities under the control of the maize *ubiquitin1* promoter and the wheat HMW-GS

IDX5 promoter, respectively were comparatively analyzed (Brinch-Pedersen *et al.*, 2003) and it was found that heterologous phytase synthesized in the endosperm efficiently hydrolyzed inositol phosphates in flour of transgenic seeds. To explore the role of subunits of low-molecular weight (LMW)-glutenins on quality parameters of complex gluten system in starchy endosperm of wheat, Tosi *et al.* (2004) transformed pasta (*T. durum*) wheat cultivars. In this work, a range of transgene expressions was observed, and analysis carried out in independent transgenic lines showed that the transgenic protein was incorporated in the glutenin polymers.

Recently, Shewry *et al.* (2006) analyzed a series of transgenic wheat lines expressing additional HMW subunit genes produced by Barro *et al.* (1997) and the corresponding control lines in replicated field trials at two sites in UK over 3 years with successive generations of the transgenic lines being planted. Detailed statistical analyses showed that the transgenic and nontransgenic lines did not differ in terms of stability of HMW subunit gene expression or in stability of grain nitrogen, dry weight, or dough strength, either between the 3 years or between sites and plots indicating that the transgenic and control lines can be regarded as substantially equivalent in terms of stability of gene expression between generations and environments.

3. FUTURE OUTLOOK

The obvious merits of *Agrobacterium*-mediated wheat transformations are becoming more apparent and emerge as the method of choice due to its ease of use, low costs, and the unique ability to introduce single or low copy numbers of the intact transgene. Presently, attempts have been made to develop transgenic wheat without the aid of either a selection pressure or a selectable marker. Successful transformation in wheat still remains a multidisciplinary approach that is mainly dependent on efficient and reliable tissue culture techniques for regeneration of viable transgenic plants. The advances in molecular markers and mapping technologies along with the recent techniques to construct large DNA insert libraries can speed up the discovery and character-

ization of genes for complex traits. The genetic transformation combined with the advances in genomics, proteomics, and bioinformatics opens up tremendous possibilities to genetically engineer ideotype with desirable complex traits to satisfy producers and consumers of wheat.

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Barley

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1. INTRODUCTION

1.1 Barley Taxonomy and Systematics

Barley, generic name *Hordeum*, is classified within the tribe Triticeae of the family Gramineae. The tribe Triticeae also encompasses some of the other economically important crops such as wheat, rye, and triticale. Barley has been regarded as the third founder cereal of Old World Neolithic agriculture; the first two being einkorn wheat (*Triticum monococcum*) and emmer wheat (*Triticum dicoccoides*) (Harlan and Zohary, 1966; Zohary, 1973; Zohary and Hopf, 2000). It is believed that domesticated barley had its beginnings about 10 000 years ago in the Fertile Crescent (Zohary and Hopf, 2000). Two-row types with brittle rachis, bearing similarity to *Hordeum spontaneum*, were considered to be the progenitors for the domestication of barley (Zohary and Hopf, 1988). *H. spontaneum* as such is thought to be the wild ancestor of present day cultivated barley, *Hordeum vulgare* L. (Harlan and Zohary, 1966; Zohary, 1969).

The genus *Hordeum*, although considered simpler than the other temperate cereals, has been disputed as to its species level delineation and it has been suggested that it be subdivided into sections based on genomic variation, with the recognition

of 32 species, totaling 45 taxa (Bothmer *et al.*, 1995; Table 1). Across these 45 taxa, the basic chromosome number of $x = 7$ is represented as diploid ($2n = 2x = 14$), tetraploid ($2n = 4x = 28$), and hexaploid ($2n = 6x = 42$) (Table 1).

Besides cytotype delimitation, systematics of the genus *Hordeum* has included studies based on morphology, distribution, cytogenetics, isozymes, and DNA markers. It would be prudent to state that a combination of all these approaches is likely to lead to a more comprehensive understanding of the genus *Hordeum*. However, aside from the semantics of *Hordeum* systematics, the subsequent purpose for an understanding of systematics is to allow use of exotic germplasm for improvement of cultivated barley through wide hybridization (see Section 1.4.3). Morphologically, the *Hordeum* species are characterized by three one-flowered spikelets positioned at the rachis node, with all three spikelets being fertile in six-row barleys and the central spikelet being fertile in two-rowed barleys (Bothmer and Jacobsen, 1985; Bothmer *et al.*, 1995). The most determinant delimiting morphological traits for distinguishing the various *Hordeum* species are based on the entire spike as well as on the spikelets, although an assessment of different criteria has been suggested to more likely give proper characterization of the species (Bothmer and Jacobsen, 1985).

Table 1 Subdivision of the genus *Hordeum* into sections and species delineation^(a)

Section	Species	Ploidy status	Origin
Hordeum	<i>H. bulbosum</i> L.	2x, 4x	Mediterranean region, eastward to Afghanistan, and south Tadjikistan
	<i>H. murinum</i> ssp. <i>glaucum</i> (Steudel) Tzvelev	2x	Southern Mediterranean, eastward to Iran, Afghanistan, and Kashmir
	<i>H. murinum</i> ssp. <i>leporinum</i> (Link) Arcangeli	4x, 6x	Mediterranean region, eastward to Afghanistan, Turkey, and eastward to Iran
	<i>H. murinum</i> ssp. <i>murinum</i> L.	2x, 4x, 6x	Northern and western Europe
	<i>H. vulgare</i> ssp. <i>vulgare</i>	2x	Mediterranean region, Ethiopia, south-west Asia eastward to China and Japan, now cultivated in all temperate agricultural zones
	<i>H. vulgare</i> ssp. <i>spontaneum</i>	2x	Greece, Egypt, south-west Asia eastward through Iran, Afghanistan, western Pakistan, and southern Tadjikistan
Anisolepis	<i>H. chilense</i> Roemer and Schultes	2x	Central Chile and western-most parts of Neuquen and Rio Negro provinces of Argentina
	<i>H. cordobense</i> Bothmer, Jacobsen, and Nicora	2x	Central and northern Argentina
	<i>H. euclaston</i> Steudel	2x	Central Argentina, Uruguay, and southern Brazil
	<i>H. flexuosum</i>	2x	Province of Buenos Aires, Argentina and dispersed around adjacent areas including Uruguay
	<i>H. intercedens</i> Nevski	2x	Endemic to southwestern California, adjacent Santa Barbara Islands and north-west Baja California of Mexico
	<i>H. muticum</i> Presl	2x	Northwestern Argentina, northeastern Chile, Bolivia, Peru, Ecuador, and Colombia
	<i>H. pusillum</i> Nuttall	2x	Most of United States except western-most region, few records from Canada and Mexico
	<i>H. stenostachys</i> Gordon	2x	Central and northern Argentina, Uruguay, and southern-most Brazil
Critiesion	<i>H. arizonicum</i> Covas	6x	Primarily in southern Arizona, few locations in south-east California and northern Mexico
	<i>H. comosum</i> Presl	2x	Restricted to Andean areas in Chile and north Argentina to the province of Mendoza
	<i>H. halophilum</i> Grisebach	2x	From Tierra del Fuego northward on both sides of the Andes through Argentina and Chile, dispersed in Bolivia and Peru
	<i>H. jubatum</i> L.	4x	Native from Mexico, northward through the United States, Canada, and Alaska to eastern Siberia
	<i>H. lechleri</i> (Steudel) Schenck	4x	Restricted to Chile and Argentina from the province of Mendoza and southward to Tierra del Fuego; also on Falkland Islands
	<i>H. procerum</i> Nevski	4x	Mainly in the provinces of Buenos Aires, Rio Negro, and La Pampa of Central Argentina; single record from Uruguay
	<i>H. pubiflorum</i> Hooker F.	2x	Mainly in Tierra del Fuego and the Magellanes, the province Santa Cruz in Argentina, single record from Nuble in Chile
Stenostachys	<i>H. bogdanii</i> Wilensky	2x	Central Asia, western Iran, Afghansitan, north and west Pakistan, north India, south Siberia, Mongolia, and north China
	<i>H. brachyantherum</i> ssp. <i>brachyantherum</i> Nevski	4x, 6x	Western North America, from Baja California in Mexico northward to Alaska and the Aleutian islands
	<i>H. brachyantherum</i> ssp. <i>californicum</i> (Covas and Stebbins) Bothmer, Jacobsen, and Seberg	2x	Endemic to southwestern California up to north of Bay area

<i>H. brevisubulatum</i> ssp. <i>brevisubulatum</i> (Trinius) Link	2x, 4x	South-eastern Siberia, Mongolia, and northern China
<i>H. brevisubulatum</i> ssp. <i>iranicum</i> Bothmer	4x, 6x	Western and southern Iran in the Zagros and the Alborz Mountains
<i>H. brevisubulatum</i> ssp. <i>nevskianum</i> (Nevski) Tzvelev	2x, 4x	Nepal, northern Kashmir in India, Chitral in Pakistan, western-most China, western Siberia, and north-eastern Afghanistan
<i>H. brevisubulatum</i> ssp. <i>turkestanicum</i> (Nevski) Tzvelev	4x, 6x	In restricted areas of central and north-eastern Afghanistan, Chitral (Pakistan), south Tadjikistan, and western-most China
<i>H. brevisubulatum</i> ssp. <i>violaceum</i> (Boissier and Hohenacker) Tzvelev	2x, 4x	Central Turkey, Caucasus, northern parts of east and west Azarbaijan in Iran, Alborz Mountains
<i>H. capense</i> Thunberg	4x	Republic of South Africa and Lesotho
<i>H. depressum</i> (Scribner and Smith) Rydberg	4x	Western United States
<i>H. erectifolium</i> Bothmer, Jacobsen, and Jørgensen	2x	Known only a single location in western part of Buenos Aires province of Argentina
<i>H. fuegianum</i> Bothmer, Jacobsen, and Jørgensen	4x	Tierra del Fuego and a few areas in Magellanes region of southern Chile
<i>H. guatemalense</i> Bothmer, Jacobsen, and Jørgensen	4x	Mountain region of Cuchumatanes in northern Guatemala
<i>H. marinum</i> ssp. <i>gussoneanum</i> (syn. <i>geniculatum</i>) (Parlatore) Thellung	2x, 4x	Eastern Mediterranean to south-west Asia (diploid); Turkey to Afghanistan (tetraploid)
<i>H. marinum</i> ssp. <i>marinum</i> Hudson	2x, 4x	Native in Mediterranean region
<i>H. parodii</i> Covas	6x	From northern part of Mendoza southward to Santa Cruz provinces of Argentina. Few dispersed locations in the province of Buenos Aires, in the region of Magellanes in south Chile, and Chilean side of Tierra del Fuego
<i>H. patagonicum</i> ssp. <i>magellanicum</i> (Parodi and Nicora) Bothmer, Giles, and Jacobsen	2x	From southern-most Patagonia with several localities inland to Tierra del Fuego
<i>H. patagonicum</i> ssp. <i>mustersii</i> (Nicora) Bothmer, Giles, and Jacobsen	2x	From two locations in Santa Cruz province
<i>H. patagonicum</i> ssp. <i>patagonicum</i> (Haumann) Covas	2x	Southern-most part of Chubut province and along the coast of Santa Cruz province
<i>H. patagonicum</i> ssp. <i>santacrucense</i> (Parodi and Nicora) Bothmer, Giles, and Jacobsen	2x	Southern part of Santa Cruz province, southward to Strait of Magellan
<i>H. patagonicum</i> ssp. <i>setifolium</i> (Parodi and Nicora) Bothmer, Giles, and Jacobsen	2x	Western part of Chubut province to north-west part of Santa Cruz province
<i>H. roshevitzii</i> Bowden	2x	Southern Siberia, Mongolia, and north-central China
<i>H. secalinum</i> Schreber	4x	From southern-most Sweden and central Denmark along Atlantic coast of Europe to Spain; scattered areas in Mediterranean and inland Europe; few locations in North Africa
<i>H. tetraploidum</i> Covas	4x	From province of Mendoza to province of Santa Cruz in Argentina

^(a)Reproduced from Bothmer *et al.* (1995)

Other distinguishing features have been based on geographic distribution, ecological niche, and reproductive habits (Bothmer and Jacobsen, 1985). Characterization at the genome level, including cytological studies is also valuable in evaluating *Hordeum* species relationships. Based on genome identity and meiotic pairing, Bothmer *et al.* (1983) were able to establish geographic distinctions among some of the diploid *Hordeum* species.

1.2 Barley Botany

The vegetative and reproductive attributes of the barley plant have changed considerably over the course of its evolution/domestication as has been the case for all the agriculturally important crops. Improvement, be it conscious or unconscious, has led to morphological changes suited to the needs and environments. There has been a range of

varietal distinctions based on the level of selection pressure applied, especially due to accelerated breeding efforts over the last 50 years to develop high performance crops with respect to yield as well as biotic and abiotic adversities. Thus, a generalized description of the barley plant would be adequate to address its botanical features in this section.

From a botanical perspective, growth and development of the barley plant from seed to maturity marks the fulfillment of its life cycle, as with all flowering plants. However, on a producer perspective, the grain must fulfill two important requirements, namely, efficient germination to ensure maximum crop stand and high grain yield to ensure profitability. Thus, knowledge of the components of the barley grain prior to the onset of the germination process *per se* would iterate the importance of some of its underlying features. A generalized mature barley grain consists of the husk (palea and lemma), pericarp (mature ovary wall), testa (seed coat), aleurone layer, starchy endosperm, and embryo (Figure 1). A distinguishing feature of commercial varieties of barley is the adherence and nonadherence of the husks to the grain, giving rise to hulled barley varieties and hullless or naked barley, respectively. Hullless barley is desirable as feed, while hulled barley is desirable for malting. The

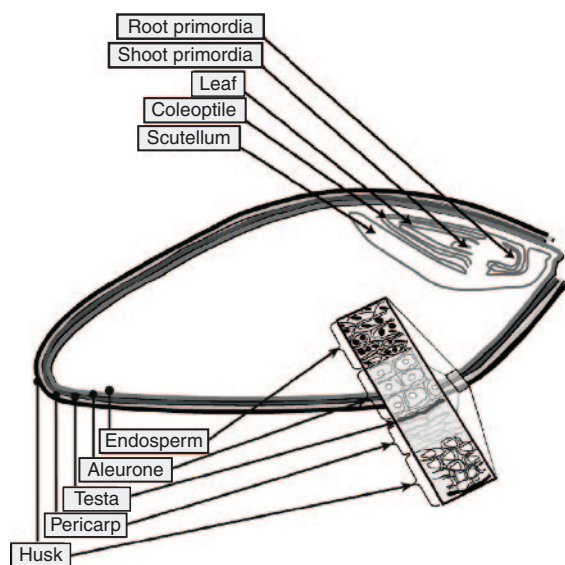


Figure 1 Diagrammatic representation of a longitudinal section through a barley grain

barley grain without the husk is referred to as the caryopsis—a fruit with fused testa and pericarp. These two layers enclose the barley endosperm, which consists of the aleurone layer and the major starchy endosperm tissue. The aleurone layer is generally 2–4 cells thick. The basal part of the grain bears the embryo and consists of the root and shoot primordia, apical meristems, coleoptile, and scutellum.

The onset of germination of the barley grain is marked by the appearance of the coleorhiza (which encloses the primary and secondary roots) upon water imbibition. The importance of the germination process in barley is not only relevant from a crop stand establishment in the field, but also in the malting process wherein a uniform germination is desired for optimal malt production. Most of the physiological, biochemical, and molecular events during germination have been elucidated and documented (Briggs, 1978, 1992) and culminate into the emergence of the primary leaf above the soil surface, after breaking through the coleoptilar tip. The growth and development of the seedling proceed with further elongation of the “stem”, production of leaves, and emergence of tillers. The latter are generally predetermined axillary shoots. The number of tillers produced, although genetically determined, is influenced by growth conditions and often growth and development of the tillers are not fully realized under field conditions. Mature embryos induced to produce multiple shoots under *in vitro* culture conditions have been shown to be a result of prolific induction of axillary meristems in wheat and barley (Ganeshan *et al.*, 2003, 2006).

The transition from the vegetative to reproductive phase marks another important stage in the development of the barley plant. The spikelet primordia are generally initiated from the vegetative shoot apical meristem after the production of a certain number of leaves. This reproductive transition is marked by the double-ridge formation, wherein the apical meristem morphs from a single ridge (which represents the leaf primordia) to double ridge (which represents the spikelet primordia) (Briggs, 1978). Thereafter the development of the spike proceeds, enclosed within the flag leaf sheath during the course of its development. The spike subsequently emerges out of the sheath and anthesis generally occurs while the spike is still within the sheath (boot).

1.3 Cultivation and Economic Importance

Since the dawn of agriculture, barley has steadily gained value and its importance, in terms of economic and consumer attributes, is constantly being re-evaluated. Novel uses for nutraceutical, pharmaceutical, and industrial applications are being explored to diversify its cultivation. But to date barley has primarily been of great economic importance for the malting and brewing industry and as animal feed. Barley has worldwide growing regions extending from temperate climatic zones to high altitudes. Versatile climatic regimens often provide necessary impetus for cultivation of barley. The estimated world production of barley in 2005 was around 140 million metric tons (FAO, 2005) and the production generally fluctuates based on global market trends and demands. Of the total production, the Russian Federation produces 12%, followed by Canada and Germany (9% each), France (8%), Ukraine and Turkey (7% each), Australia (5%), UK (4%), US and Spain (3% each), and the remaining from the other countries around the world (FAO, 2005). Barley is generally used as malt for the production of alcoholic beverages and as animal feed. It is also consumed as food in some parts of the world. There is a lot of interest in developing barley for consumption as food and attributes such as starch quality and protein composition and content are being studied. There has also been interest in specialty barleys for industrial applications.

1.4 Traditional Approaches of Crop Improvement

The flowering plants appeared in the middle of the Mesozoic era of the lower Cretaceous period about 150 million years ago, predating the *Homo sapiens*, whose appearance trace back to the quaternary period of the Cenozoic era about 1.8 million years ago. Thus, the flowering plants already had a long evolutionary history and improvement strategies in plant agriculture would date back to the dawn of civilization, when primitive gatherers started identifying quality attributes in plant products and began intentionally propagating them. In fact, besides influencing plant product palatability and ease of harvest, human beings did not have a major

impact on the overall structure of crop plants (Hancock, 2004). Therefore, the improvement brought about by early humans, although based on intuitiveness, could be conceived as the beginnings of some form of conventional plant breeding, which became more organized as a result of more colonial life styles and establishment of homesteads. Fairly well blended into this scheme of plant agriculture evolution is barley as one of the ancient crops. Its share of “directed evolution” from this deliberate propagation of desirable traits is exemplified by a change from brittle rachis forms to nonbrittle forms. It is believed that about 19 000 years ago ancestral humans gathered wild two-rowed barleys with spikes showing shattering disposition and nonshattering forms had started to be cultivated about 9500 years ago in areas such as Netiv Hagdud, north of Jericho, and Abu Hureya (present day Syria) (Zohary and Hopf, 2000). The available gene pool in barley has been fairly wide and sources of genetic variability are potentially available in wild species and landraces. There have been extensive efforts directed toward identification of traits of interest for incorporation into new varieties.

1.4.1 Conventional breeding

Improvement of barley varieties through breeding programs has been multifaceted and has to a great extent been geared toward producer and consumer. However, environmental adaptation for adequate growth and yield has also been given due consideration. Besides development of high yielding varieties as their main objective (Anderson and Reinbergs, 1985), plant breeders have also targeted other characters that affect quality and yield indirectly. With barley, the breeding objectives have been somewhat more elaborate due to the existence of the malting market niche and the feed market, the former being more profitable and therefore, receiving more incentive for improvement by way of funding from malting and brewing industries. In recent years, another dimension has been added to barley breeding efforts to cater to food quality barley of nutritional value. The realization that barley can offer nutraceutical alternatives has had health initiative groups and industry to take advantage of this new market value for barley.

Breeding objectives have thus evolved over the years, with characteristics such as malting quality, protein content, disease resistance, days to maturity, plant height, straw strength, etc. all being considered in some form or the other in addition to keeping up with the dynamic nature of the barley market demands. Breeding methods have, therefore, been structured to meet the intended goals and plant breeders have very successfully adopted a number of the conventional breeding strategies. Further details regarding barley breeding can be found elsewhere (Anderson and Reinbergs, 1985). A number of parameters such as resistance to lodging and disease, reduced height and increase in harvest index have significantly contributed to yield increases (Busch and Stuthman, 1990). Considering the economic returns on malting barley, improved grain quality attributes have targeted increases in grain plumpness, higher malt extract, α -amylase activity, and diastatic power (Wych and Rasmusson, 1983). There has been a general trend among breeders to aim for high malting quality barley varieties during breeding, with a notion that the newly developed variety will also be valuable as feed barley (Kling *et al.*, 2004). The progress in breeding for value-added barley has gained further impetus in recent years with the advent of a variety of DNA-based markers, heralding the concept of marker-assisted breeding. Incorporation of a desirable trait, qualitative or quantitative, into new varieties can now be accomplished reasonably quickly in breeding programs that have embraced a parallel molecular marker-based program.

1.4.2 Mutagenic approaches

Even though breeding efforts have contributed significantly to the release of countless improved varieties, a gradual erosion of genetic diversity has led to a need for the identification of new sources of variability, pre-existing as well as *de novo* generated. Therefore, plant breeders have had recourse to screening for mutations for incorporation of new traits into their breeding programs—a practice eventually termed mutation breeding, which has been around for almost a century. The tobacco cultivar, Chlorina, was the first variety derived from mutagenic x-ray irradiation treatments and was commercially

released in 1934 (Tollenaar, 1934, cited in van Harten, 1998). It was almost two decades later, in 1950 that another commercial variety of mustard, cv. Primex, was released also as a result of x-ray-induced mutations. It should, however, be noted that plant breeders have also used naturally occurring mutants successfully. The green revolution was heralded by the use of dwarfing genes found in a Japanese wheat cv. Norin-10. High yielding dwarf rice varieties with strong straw were derived from spontaneous semi-dwarf mutation phenotype in a rice cv. Dee-geo-woo-gen from Taiwan.

Naturally occurring variability in barley has generally not been an issue for incorporation in breeding programs. However, with the ever-changing demands on barley breeding programs and priorities, alternative sources of variation need to be identified. This need for variability could not be more emphasized than with breeding for resistance to diseases such as the *Fusarium* head blight of barley. Mutation breeding in barley has nonetheless been practiced and a first mutant resistant to powdery mildew was produced by Freisleben and Lein in 1942. As of February 2007, 2454 mutant plant varieties were listed in the FAO/IAEA Mutant Varieties Database, of which 256 were barley varieties (FAO, 2007). Induced mutations in barley were generally aimed toward the development of disease resistant and high grain protein varieties in the past (Gupta, 1998). Traits pertaining to lodging resistance (FAO/IAEA, 1984a, 1988), days to maturity (Gottschalk and Wolff, 1983; Donini *et al.*, 1984; Konzak, 1984), and grain protein content (FAO/IAEA, 1979, 1984b; Micke, 1983; Müeller, 1984) have been modified by mutation breeding. Development of barley resistant to diseases can best be exemplified by the cultivar Heine's Haisa, which is resistant to *Erysiphe graminis* f. sp. *hordei*, the causal agent of powdery mildew and was produced by x-ray irradiation (Micke, 1992). Mutants with improved brewing and malting quality or improved grain protein content have also been produced (FAO/IAEA, 1996). Using barley mutants Lundqvist and Lundqvist (1998) elucidated genetics of spikelet rows. They were able to show that at least 12 gene loci (*hex-v* and *11 int*) were involved in the spike development including the lateral florets, kernel size, awns, and fertility. Considering the number of barley varieties

released as a result of mutation breeding, it is conceivable that barley improvement is likely to be more significantly influenced by mutagenesis in the future. Even though mutation can generate a lot of variability, a large number of nonuseful mutants are also produced. Therefore, a more targeted and controlled method of producing useful mutants would be of value.

Induced mutations can now be specifically targeted for alteration of known genes. This mutation strategy termed TILLING (targeting induced local lesions in genomes) (McCallum *et al.*, 2000) has become an important tool in genomics studies. TILLING is combination of classical chemical mutagenesis and high-throughput molecular biology techniques for the identification of desired mutants. With the TILLING strategy single base pair changes can be identified in a gene of interest (Till *et al.*, 2003). It involves mutagenesis of seeds with ethylmethane sulfonate (EMS), growing plants (M_1), allowing selfing, planting individual M_2 seeds for DNA extraction, and cataloging M_3 seeds (Figure 2). DNA from the M_2 plants is pooled for polymerase chain reaction (PCR) amplification of the gene of interest. PCR products are denatured and upon reannealing heteroduplexes are formed. Originally denaturing high-performance liquid chromatography (HPLC) was used to analyze heteroduplexes for mutations (McCallum *et al.*, 2000). Subsequent modifications included treatment of the heteroduplexes with an endonuclease, *CELI*, which specifically cleaves

mismatches between mutated and nonmutated variant heteroduplexes (Colbert *et al.*, 2001). *CELI*, which was identified in celery, recognizes single base mismatches and cleaves on the 3'-side of the mismatch (Oleykowski *et al.*, 1998). Cleaved heteroduplexes are resolved by electrophoresis for the identification of mutations. The mutant plant is then identified by screening DNA from individual samples constituting the pool. EMS treatment primarily leads to G/C to A/T transitions and such randomly distributed G/C to A/T transitions in *Arabidopsis thaliana* account for up to 99.5% of mutations (Greene *et al.*, 2003). TILLING populations have been produced for *A. thaliana* (Till *et al.*, 2003), *Lotus japonica* (Perry *et al.*, 2003), maize (Till *et al.*, 2004), barley (Caldwell *et al.*, 2004), and wheat (Slade *et al.*, 2005). The wheat TILLING population has led to the identification of a range of *waxy* phenotypes (Slade *et al.*, 2005) and similar barley starch variants could be identified for specialty end uses. Using Eco-TILLING, which is a high-throughput TILLING strategy (Comai *et al.*, 2004), Mejlhede *et al.* (2006) were able to identify point mutations and deletions in the powdery mildew resistance genes *mlo* and *Mla* of barley. Thus, a large number of variants were generated, offering the possibility of combining these novel alleles for studying powdery mildew resistance.

Another approach to targeted mutagenesis is the use of transposable elements. Transposable elements have certain advantages over other approaches for identifying and determining gene function in large genome cereals, like barley. Different strategies have been used to exploit the maize *Activator (Ac)/Dissociator (Ds)* transposon system for targeted mutagenesis in heterologous species. Either large numbers of independent *Ds* insertion lines or transposants (TNPs) are generated and screened for biochemical or physiological phenotypes, or smaller numbers of TNPs are produced, *Ds* locations mapped and the element remobilized for localized gene targeting. Barley cv. Golden Promise was engineered with a *Ds-bar* element and *Ac* transposase (Koprek *et al.*, 2000). The two elements were brought together in the same cell, resulting in transposition of *Ds-bar* to a new location; the reactivation of *Ds* occurred at frequencies of 11.8–17.1% (Singh *et al.*, 2006). Sequence flanking the *Ds* insertion was determined and BLAST searches using a

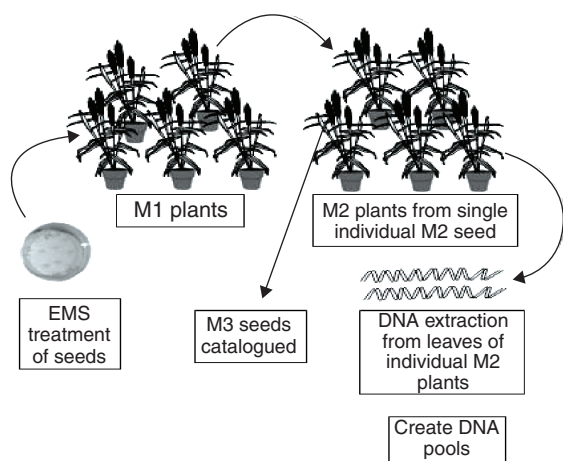


Figure 2 Schematic of TILLING strategy

gene prediction program demonstrated that 86% of the TNP flanking sequence matched either known or putative genes. Mapping of insertion sites (e.g., Cooper *et al.*, 2004; Singh *et al.*, 2006), coupled with the observed remobilization frequencies of primary, secondary, tertiary, and quaternary TNPs and the tendency for localized *Ds* transposition (~75% frequency), validate a saturation mutagenesis approach using *Ds* to tag and characterize genes linked to *Ds*.

1.4.3 Interspecific and intergeneric crosses

The use of wild species and genera for wide crosses has appealed to plant breeders due to the possibility of identifying traits of value for incorporation in new cultivars. Identification and introgression of genes from wild species of *Hordeum* into cultivated barley have been attempted. The crossability of *Hordeum* species with barley has been very low (Bothmer *et al.*, 1983) and chromosome pairing in the interspecific hybrids has generally been poor, except for hybrids derived from *Hordeum bulbosum* and *H. spontaneum* crosses with barley (Fedak, 1989). Difficulties associated with the production of interspecific hybrids have been reviewed recently and include incompatibility, chromosome elimination, endosperm degeneration, instability of hybrids and chromosome pairing, infertile hybrids, reduced recombination, and linkage drag (Pickering and Johnston, 2005). However, in spite of all barriers, agronomic traits for disease resistance and stress tolerance have been expressed in hybrids or backcross progenies (Fedak, 1985). Intergeneric hybrids have also been possible for barley. The number of intergeneric hybrids produced in combinations of *Secale*, *Agropyron*, or *Elymus* with *Hordeum* species has far exceeded those produced with *H. vulgare* (Fedak, 1989, 1992), and could be due to closer phylogenetic relationships among wild genera within the Triticeae tribe than to domesticated barley. With more research it is likely that many of the innate barriers with regards to wide hybridization would be overcome. Baum *et al.* (1992) have reviewed some of the strategies to overcome these barriers in wide crosses in cereals. One of the contributing breakthroughs in recovering interspecific or intergeneric hybrids has been the use of *in vitro* cultures (see Section 2.1.1).

2. DEVELOPMENT OF TRANSGENIC BARLEY

2.1 Technology-driven Improvement

The worldwide importance of barley for food, feed, and malt demands that efficient methods be developed for the improvement of barley cultivars for enhanced agronomic performance, pest resistance, yield, and quality traits. Such methods can also be applied for diversifying attributes of barley for alternative uses. The use of genomics tools, marker-assisted selection, doubled haploid production, and genetic engineering approaches are all contributing toward this technology-focused improvement. The introduction of information into barley through genetic transformation technologies, as with other crop species, utilizes methods based on the delivery, integration, and expression of specific genes of interest into plant cells that are grown *in vitro*. The success of this approach is dependent on the efficiency of generating independently and stably transformed, fertile, green plants that maintain the important characteristics of the starting germplasm.

Biotechnology has unequivocally become an integral part of barley improvement programs. The access to additional gene pools has become a possibility due to the development of tissue culture and transgene technologies. For a long time, barley was considered to be recalcitrant to both technologies; however, major obstacles have now been overcome. DNA-based markers provided the breeder with another tool to select for desirable traits, thereby enabling more effective selection and also expediting the breeding process. The use of *in vitro* culture for barley improvement has been three pronged, namely, induction of variation, doubled haploid production, and genetic transformation.

2.1.1 *In vitro* culture and somaclonal variation

The availability of an efficient regeneration system is essential for successful tissue culture. Even though barley was fairly recalcitrant to tissue culture, significant progress has been made. The explants used for culture have included immature tissues such as embryos, scutella, inflorescences, ovaries and ovules, anthers, microspores, apical

meristems, mature embryos, and leaf bases. Immature tissues as explants have generally responded better in culture than mature tissues. In recent years, there has been interest in the use of meristematic tissues and mature embryos for culture and these have been shown to efficiently produce multiple shoots directly without an intervening callus phase in response to the plant growth regulator, thidiazuron (Ganeshan *et al.*, 2003, 2006).

The *in vitro* culture system was thought of as a novel approach at identifying variability by subjecting the cell cultures to selection pressure in a manner similar to that employed by breeders. Tissue culture-induced variation referred to as somaclonal variation (Larkin and Scowcroft, 1981) led to further speculation that *in vitro* cultures could generate more genetic variability. As mentioned earlier, the generation of new chromosomal combinations and mutations by way of interspecific and intergeneric hybrids was well recognized. The occurrence of chromosomal variability in callus and plants derived from ovary tissue cultures of a *H. vulgare* x *H. jubatum* hybrid was also observed (Orton, 1980). Thus tissue cultures had started to be perceived as a way for overcoming barriers inherent in conventional gene introgression in breeding. A similar approach was explored with hybrid plants recovered by embryo rescue from a cross between *H. vulgare* x *Elymus canadensis* (Canada wild rye) (Dahleen and Joppa, 1992). Canada wild rye possesses winter-hardy attributes, drought tolerance, and resistance to barley yellow dwarf virus, but F₁ hybrids with barley are sterile and the *in vitro* culture rescued hybrids showed chromosomal recombination. Other earlier studies have reported cultured barley tissues producing plants showing chromosomal rearrangements, aneuploids and polyploids (e.g., Lupotto, 1984; Singh, 1986; Gaponenko *et al.*, 1988; Lühns and Lörz, 1988), and several studies have attempted to capitalize on this tissue culture-induced variation.

In *H. spontaneum*, heritable somaclonal variation was observed, but no aberrant chromosome structures or numbers were observed (Breiman *et al.*, 1987). Barley plants regenerated on medium containing a toxin from the net blotch pathogen of barley were shown to have improved resistance to the pathogen (Hunold *et al.*, 1992). Control plants regenerated from media containing no toxins, however, also exhibited resistance and were most

likely a result of somaclonal variation. Partially purified toxins from *Helminthosporium sativum* (causal agent of spot blotch) were also used in culture media on barley callus and plants resistant to the pathogen were produced (Kole and Chawla, 1993). Barley plants derived from cultures have also been shown to exhibit tolerance to herbicides such as chlorsulfuron (Baillie *et al.*, 1993) and glyphosate (Concepcion-Escorial *et al.*, 1996) in response to culture media containing the respective herbicides.

Even though somaclonal variation has had positive effects, there have been negative effects associated with it. For example, tissue culture-derived malting barley plants showed poor malting quality (Bregitzer *et al.*, 1995). Furthermore there were differences in agronomic performances in a genotype-dependent manner from tissue culture-derived lines (Bregitzer and Poulson, 1995). In the case of protoplast-derived barley plants, evaluation of the progeny in the field did not show any abnormal phenotypes, but some agronomic characters were affected (Kihara *et al.*, 1998). The adverse effects of somaclonal variation have led to the suggestion that identification of useful transformants might be precluded (Bushnell *et al.*, 1998; see also Section 2.1.2.2).

The lack of consensus with regard to the occurrence or nonoccurrence of somaclonal variation indicates a need to reassess perspectives on somaclonal variation in barley. The culture-induced variation could be due to the components of the specialized media being constantly developed to improve efficiency of regeneration from barley. A highly efficient regeneration system may not always lead to production of all normal plants. Due to the low regeneration frequencies in earlier studies it may have been possible that undesirable regenerants did not develop. Many studies have demonstrated that *in vitro* culture-regenerated barley plants were normal, with very low frequencies of somaclonal variants (Karp *et al.*, 1987; Pickering, 1989; Ruiz *et al.*, 1992). It is believed that the barley genome is tolerant to tissue culture conditions or it may be that due to a competitive disadvantage during the regeneration process, aberrant cell lines fail to develop and therefore fewer somaclonal variants are obtained (Karp and Lazzeri, 1992). More recent data involving the *in vitro* culture of barley cv. Golden Promise indicate that callus and plants derived from nontransgenic and transgenic callus

of approximately the same age had varying levels of ploidy variation after performing chromosomal analysis. Nontransgenic plants regenerated after *in vitro* culture had a lower percentage of ploidy variation (0–4.3%), compared to transgenics of which 46% were tetraploid or aneuploid around the tetraploid level (Choi *et al.*, 2000); tetraploid plants had abnormal morphological features. Ploidy determinations were also made on randomly selected cells from callus of immature embryos and the number of tetraploid cells in 1-day and 7-day-old cultures was 2–4%. However, in callus comparable in age to that used for regeneration the percentage of tetraploid cells was 23%, a percentage lower than the number of tetraploid plants regenerated from transformed lines.

2.1.2 Genetic transformation

The first report of recovery of stably transformed fertile plants utilized a model cultivar, Golden Promise, and microparticle bombardment (Wan and Lemaux, 1994). Organized tissues, like immature embryos, were utilized, rather than embryogenic callus or suspension cells, because of problems encountered in barley transformation, which were more marked than those encountered with other cereals, for example, rapid loss of regenerability of *in vitro*-cultured tissue and the increase in the frequency of albinism over time. The ability to introduce DNA directly into organized scutellar cells at the inception of callus formation enabled identification of transgenic callus and the regeneration of plants before regenerability was lost. Despite successful development of transformation methods for model barley cultivars, their utility for improvement of commercial barley cultivars was hampered by certain problems: (a) lack of reproducible transformation systems for commercial cultivars, (b) potential for introducing somatic mutation and heritable changes during *in vitro* culture, and (c) transgene and transgene expression instability.

2.1.2.1 Lack of reproducible transformation systems for commercial cultivars

Initial reports of successful transformation were highly dependent on using particular cultivars,

like Golden Promise, that were amenable to *in vitro* growth and regeneration of fertile, green plants. Despite their utility for developing transformation strategies, the ability to deploy commercial germplasm with useful novel genes to meet urgent needs is hindered by the use of this germplasm. In fact, the barley germplasm forming the foundation of North American breeding programs is different from Golden Promise in virtually every aspect important to commercial production and utilization. The obligatory transfer of transgenes from Golden Promise to unrelated six-rowed germplasm that forms the basis for malting cultivars in the mid-western United States would require multiple cycles of hybridization and selection prior to the utility of the germplasm for commercial deployment. Such a lengthy and costly process is necessary to preserve allelic combinations critical to commercial use.

Problems arose with the transformation of commercial cultivars because frequencies of callus induction were often low with certain cultivars and long-term cultures of barley lose regenerability quickly. To address this difficulty, efforts were made to develop novel tissue culture systems. In general, callus-maintenance medium containing auxin and no cytokinin was used for the long-term tissue culture periods needed for barley transformation (Wan and Lemaux, 1994). This frequently led to tissues with low regenerability. The use of medium containing 2,4-D, 6-benzyl aminopurine (BAP) and higher levels of copper for callus initiation stimulated the formation and maintenance of green, regenerative tissues with long-term regenerability characteristics (Cho *et al.*, 1998). A callus maintenance medium with 2,4-D, BAP and higher levels of copper (DBC2 or DBC3, depending on the genotype) was developed and this medium was used as an intermediate step between callus maintenance and regeneration.

The use of the intermediate step appeared to improve regeneration by allowing the small number of green, totipotent cells to continue proliferating and ultimately to convert the tissue from an embryogenic state into a state that more closely resembled the morphology of a shoot meristem culture. The latter tissue could be triggered to produce multiple shoots. Such tissue was used, with other alterations in the transformation procedure, to transform both Harrington and Galena (Lemaux *et al.*, 1999).

Green, regenerative tissues of Galena, Golden Promise, Harrington, and Salome, were maintained for more than a year on DBC2 or DBC3 and gave rise to fertile, green plants. Morphologically, green tissues look strikingly similar to those derived from excised shoot apices of barley cultured on 2,4-D and BAP (Zhang *et al.*, 1998). Molecular analysis using immunolocalization confirmed visual observations about the meristematic state of the barley tissues and was different from the state of tissue maintained on auxin alone (2,4-D or dicamba). Green regenerative tissues generated and maintained on DBC2 or DBC3 medium were used successfully as transformation targets for Galena, Golden Promise, and Harrington (Lemaux *et al.*, 1999).

Another possible target tissue, similar in some respects to the shoot apical meristem, is *in vitro*-derived shoot meristems. These cultures derive from shoot apices of barley (Zhang *et al.*, 1998) and other cereals and they arise from the shoot apex by culturing the tissue on auxin- and cytokinin-containing media. This medium arrests the development of the shoot apical meristem and allows proliferation of axillary meristems from which adventitious meristems develop. DNA is introduced into the cells of the proliferating adventitious meristems and by culturing under selection nonchimeric, transformed plants were generated from barley (Zhang *et al.*, 1999). *In vitro* shoot meristematic cells are fundamentally different because they proliferate from pre-existing meristematic cells in the axillary meristems (Zhang *et al.*, 1998), which undergo little or no dedifferentiation. Proliferation appears to require only a simple redirection of cells in the shoot meristem, not a dedifferentiation as occurs with cells in the immature scutellum or microspore.

2.1.2.2 Potential for introducing somatic mutation and heritable changes during *in vitro* culture

Plants from *in vitro* culture frequently accumulate heritable genetic changes, which can result in moderate to severe negative changes in important agronomic and biochemical characteristics. The elements of the *in vitro* environment causing these changes correlate with specific facets of the transformation process (Choi *et al.*, 2001)—the

most important of which is the selection process. The means by which the *in vitro*-cultured tissue is established also has an impact on regenerability but is minor relative to the other factors. Somaclonal variation, the accumulation of genetic and epigenetic changes, hinders the regeneration of fertile, green plants and their subsequent use as parents in a breeding program.

In barley, analysis of the agronomic performance of nontransgenic tissue culture-generated plants and transgenic barley plants showed significant somaclonal variation for important determinants of agronomic performance and the impact varied with the tissue culture and transformation methods (Bregitzer *et al.*, 1998). Although backcrossing can usually eliminate these problems, this requires additional time and effort and will be problematic if the induced mutations are closely linked to the transgene(s). An unexpectedly high percentage of somaclonal variants (14–60%) were shown to be present among progeny of transgenic barley plants evaluated in the field (Bregitzer *et al.*, 1998). The report also shows that somaclonal variation was accentuated by the transformation procedure. This supposition is based on chromosomal analyses of callus and plants from *in vitro*-cultured nontransgenic and transgenic barley lines (Choi *et al.*, 2000; see also Section 2.1.1). A higher percentage (78%) of karyotypically abnormal cells was observed in transgenic barley callus cultures, compared with 15% of cytologically abnormal cells in nontransgenic callus and aspects of the transformation process, i.e., osmotic treatment and selection pressure, appeared to have triggered the cytological abnormalities (Choi *et al.*, 2001).

2.1.2.3 Transgene and transgene expression instability

One limitation of current transformation methods is that insertions occur randomly and insertion location might not be optimal for gene expression. This has been noted in plants derived from *Agrobacterium*-mediated methods as well as microparticle delivery. Another problem, more common with microparticle methods, is that introduced DNA is usually present in multiple, tandemly arrayed copies. Having multiple copies of the introduced genes, closely linked and in

inverted orientation can lead to gene inactivation and genetic instability. While position effects and multicopy-induced gene silencing occur, they are not the only mechanisms of transgene silencing. For example, neither the presence of tandemly arrayed copies of the transgene nor position effects completely explain variability in expression among progeny derived from the same parent (e.g., Meng *et al.*, 2006) (see Section 2.4).

2.1.3 Doubled haploid technology

Doubled haploid breeding technology has been widely adapted in barley breeding programs. The fact that homozygosity can be achieved in one generation by chromosome doubling after haploid plant production allows for advanced generation breeding within a short time. The doubled haploid breeding approach allows breeders to decide on the number of cycles of recombination to allow before homozygosity is attained, since doubled haploid plant can be initiated at any stage during the breeding process (Snape and Simpson, 1981; Choo, 1985).

Methods for producing haploids in barley include the bulbosum technique, haploid initiator gene method, ovary, anther, and microspore culture. Several reviews have been published on haploid production and use in barley breeding (e.g., Choo, 1985; Kasha and Ziauddin, 1990; Pickering and Devaux, 1992; Forster and Powell, 1997). The bulbosum method was a result of wide hybrids between *H. vulgare* x *H. bulbosum*, wherein upon embryo rescue the regenerated plant was found to be haploid (Kasha and Kao, 1970). Since this method has been adaptable to a number of barley genotypes, its use has been widespread in the past (Kasha and Reinbergs, 1981). An induced mutation in barley has led to the identification of a haploid initiator gene (*hap*), which leads to haploid barley plants (Hagberg and Hagberg, 1980). The advantage of the *hap* gene is that recourse to a tissue culture system is not necessary, but there has been difficulty in distinguishing between spontaneous doubled haploids and hybrid embryos (Forster and Powell, 1997). Furthermore only genotypes possessing the *hap* gene can be used with this method (Forster and Powell, 1997).

Due to the potentially large numbers of plants that can be regenerated using the anther and microspore culture techniques, the latter

have become very popular. However, androgenic cultures require very delicately optimized conditions for high regeneration efficiency. The growth conditions of the donor barley plants and physiological state of the anthers play important roles in responsiveness. Plants obtained from androgenic cultures have shown genetic instability and genotype dependency, but due to extensive research efforts these drawbacks have been overcome. Segregation distortion ratios observed in doubled haploids from androgenic cultures still need to be understood (Foroughi-Wehr and Friedt, 1984; Powell *et al.*, 1986; Thompson *et al.*, 1991). Irrespective of these problems, doubled haploids derived from androgenic cultures have been extensively used in barley breeding programs, surpassing the bulbosum method (Forster and Powell, 1997).

The doubled haploid barley breeding program has been very successful and has led to the release of a number of improved varieties. It has been suggested that true-to-the-type mutants could be generated using mutagenesis in conjunction with haploid production (Maluszynski *et al.*, 1995). Several doubled haploid barley mutants have been obtained using this approach (Umba *et al.*, 1991). The barley haploid production scheme can also be used for genetic transformation and microspores had been suggested to be attractive targets for this purpose (Ziauddin *et al.*, 1990), and there are a number of reports pertaining to this possibility. Microspores transformed by electroporation have been shown to transiently express a gene of interest (Kuhlman *et al.*, 1991). Transient *GUS* gene expression was also demonstrated in barley microspores transformed by particle bombardment (Yao and Kasha, 1997). Stable transformants derived from microspore transformation have also been obtained (e.g., Harwood *et al.*, 1995). More recently Kumlehn *et al.* (2006) successfully produced stable barley transformants by infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*.

2.1.4 Spike culture and antisense oligodeoxynucleotide technology

This culture system was recently optimized specifically for following the starch biosynthesis pathway during grain development in cereals from preanthesis to maturity under defined and

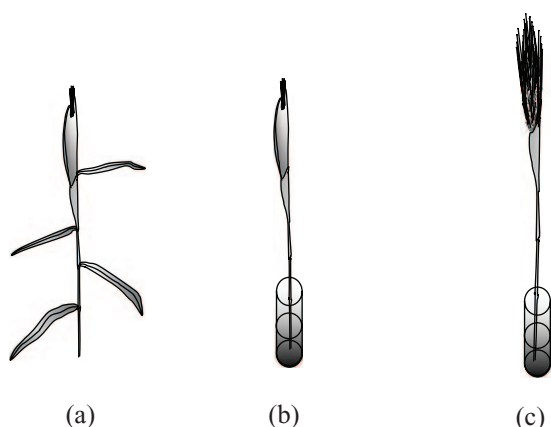


Figure 3 Diagrammatic representation of cereal spike culture establishment. (a) Immature spike is excised just above the soil surface when about a centimeter of the awns is visible. (b) All the leaves are removed and the spike is placed in a sterile culture medium after cutting a few millimeters from its base. The liquid medium consists of sucrose and L-glutamine and is replaced every 4 days, at which time a few millimeters are cut from the base of the spike before placing it back into the fresh medium. (c) Anthesis occurs after about 7 days in culture and maturity is attained in about 30 days

controlled conditions (Figure 3) (Chibbar *et al.*, 2005). Grain development studies have generally been conducted in growth chambers or field. However, due to a number of uncontrollable variables under these conditions, changes in starch accumulation patterns can be confounding. It is, therefore, difficult to assign cause and effect. The immature spike culture is established at a stage prior to anthesis and thus, postanthesis changes can be followed up to maturity, which is attained within about 30 days from culture initiation. The culture medium being simple, allows for targeted supplement of desired chemical stimuli or other factors for monitoring their effects on starch biosynthesis. Using quantitative real-time PCR the expression patterns of the genes involved in starch biosynthesis can be studied. The antisense oligodeoxynucleotide (ODN) technology can also be used with the spike culture system for the suppression of specific starch biosynthetic genes and to observe the effects on starch accumulation (Jansson *et al.*, 2007). The possibility of suppressing expression of genes using short antisense ODNs was first demonstrated in 1978 (Stephenson and Zamencnik, 1978; Zamencnik and Stephenson, 1978). Naturally occurring antisense ODN suppression of gene expression has

also been reported in prokaryotes and eukaryotes as a defense mechanism against viral infection (Vanhee-Brossollet and Vaquero, 1998; Lehner *et al.*, 2002). The antisense ODN technology has been extensively explored for therapeutics as a means of drug delivery and also for fundamental gene silencing studies in humans and animals (Shi and Hoekstra, 2004). The use of the antisense ODN technology in plants has not received the same fervor as with mammalian systems. The earliest report of antisense ODN in plants was demonstrated using barley aleurone to suppress the expression of α -amylase (Tsutsumi *et al.*, 1992). The other report used excised barley leaves to understand the role of a sugar-inducible transcription factor, SUSIBA2, in the regulation of starch synthesis (Sun *et al.*, 2005).

2.1.5 Genetic engineering approaches

The success of transformation technology in barley relies upon the availability of a suitable DNA delivery system, transformation-competent target cells, tissues or organs, and an efficient *in vitro* regeneration system. Ideally, an *in planta* transformation system would be desirable as it would eliminate the tissue culture regeneration steps. For example, injection of DNA from a two-row barley plant into developing seeds of a six-row waxy barley was attempted (Soyfer, 1980) and some plants with normal starch composition and two-row phenotype were observed, but there was no Mendelian segregation. Mendel *et al.* (1990) injected plasmids carrying the *NPTII* gene into floral tillers of barley and applied plasmid DNA to stigma, but were not successful. Brettschneider *et al.* (1990) inoculated florets at anthesis with *Agrobacterium* with no success. Attempts at DNA uptake by germinating seeds (Ledoux and Huart, 1969) and imbibition of embryos in DNA suspensions (Töpfer *et al.*, 1989) have also been unsuccessful.

Attempts have also been made at delivering DNA by microinjection and electroporation. Microinjection appears to be a very suitable system, but requires highly specialized and expensive resources, besides skills. Furthermore, the delivery of the DNA has to be precise to minimize damage to the cells. The low number of cells that can be injected implies that regeneration frequency needs to be high with respect to

the microinjected cells to generate putative transformants. Cells that would be ideal targets for microinjection include proembryos from zygotes and those derived from cultured microspores (Karp and Lazzeri, 1992). However, with recent successes in high regeneration frequencies from barley microspores, microinjection could become a potentially useful transformation approach again. As an alternative to microinjection, electroporation had gained widespread attention. Although initially barley protoplasts were thought as ideal targets for electroporation and used for transient expression assays (e.g., Teeri *et al.*, 1989; Salmenkallio *et al.*, 1990), cells and tissues have also been used subsequently. For example, Gürel and Gözükmizi (2000) used mature barley embryos for electroporation and produced stably transformed plants. Many of the studies using protoplasts also employed PEG to enable DNA uptake (e.g., Junker *et al.*, 1987; Lee *et al.*, 1991). The limitations of barley protoplasts as targets for transformation include viability of protoplasts for culture (especially after transformation), genotypic dependency, labor-intensive protoplast isolation procedure, and loss of morphogenic potential after extended culture duration (Lütticke *et al.*, 1996). Protoplasts are also prone to somaclonal variation and it has been suggested that barley protoplasts be isolated from primary callus tissue obtained from immature young scutella, microspores, or anthers to avoid regeneration problems (Stödt *et al.*, 1996).

The method of choice for the transformation of barley has been the microprojectile bombardment, in spite of its harsh nature. Since the first report of stably transformed barley plants using this method (Wan and Lemaux, 1994), there have been many reports and reviews regarding principles and parameters for barley transformation (Lemaux *et al.*, 1999; Klein and Jones, 1999; Horvath *et al.*, 2002). Optimization efforts are continually being conducted in various laboratories to improve the barley transformation efficiency further. The random insertion of the transgene into the genome has been a subject of interest, with issues such as transgene integration at multiple sites, rearrangements, truncations, gene stability, and expression (Båga *et al.*, 1999). In order to simplify integration patterns, remove selectable marker genes and decrease the amount of *in vitro* culturing needed to generate large numbers of

independent events, the *Ds* transposable element system was used as a gene delivery vehicle (Koprek *et al.*, 2000). A gene of choice was engineered between *Ds* inverted-repeat ends, introduced into barley by bombardment, reactivated by exposure to *Ac* transposase, and the transposed element carrying the gene of choice segregated away from transposase to stabilize its chromosomal location. Gene delivery via *Ds* was shown to dramatically increase gene expression stability (Koprek *et al.*, 2001; see also Section 2.4).

While the use of the microprojectile bombardment for barley transformation continues to be used and debated, in recent years the ability to use *Agrobacterium* for barley transformation has become a reality (Tingay *et al.*, 1997; Guo *et al.*, 1998; Wu *et al.*, 1998). Due to its simplicity, cost effectiveness, and fewer manipulative steps, this method of transformation has wide interest. However, optimization for routine transformation is still time consuming.

In the preceding paragraphs, transformation methods were alluded to with little emphasis on the tissue culture regeneration systems. As important as the DNA delivery system is for efficient barley transformation, the recipient cell or tissue is also important, specifically with regards to regeneration efficiency. There have been numerous reports on optimization approaches for improving the regeneration frequency in barley. Immature scutella have generally been used due to their responsiveness in *in vitro* culture. However, due to the time-consuming and labor-intensive isolation process, alternative sources of explants have been sought. Furthermore, the donor plants have to be grown under optimal conditions in carefully controlled growth chambers to ensure optimum response in culture. Callus derived from young leaf tissues has also been tested (Mohanty and Ghosh, 1988; Pasternak *et al.*, 1999), but due to limited success there has been a lack of widespread interest. More recently, Ganeshan *et al.* (2003) have reported the use of leaf-base/apical meristems with limited regeneration efficiency, but showed the use of mature embryo as more suitable explants for efficient regeneration from barley. The use of mature embryos and apical meristems as explants for *in vitro* regeneration is very appealing due to the ease of isolation. Callus induction and regeneration from mature embryos of barley was reported as early as 1984 (Lupotto, 1984) and from

isolated apical meristems as early as 1975 (Cheng and Smith, 1975). Circumvention of a callus phase and direct induction of shoots from such mature embryos and apical meristems have a definite advantage since somaclonal variation associated with the callus phase could be obviated. Using such meristematic tissues, transformed barley plants have been produced (Zhang *et al.*, 1998, 1999). More recently mature embryos have also been induced to produce multiple shoots directly and shown to be transformation competent (Ganeshan *et al.*, 2003, 2006; Ganeshan and Chibbar, unpublished). It is the development of specialized media that has led to the high frequency of regeneration in a fairly genotype-independent manner from mature embryos. The mature embryo also has the advantage that there is no requirement for the growth and maintenance of donor plants for explant isolation. Mature embryos can be readily isolated from mature seeds, including those obtained from field-grown plants. With respect to winter cereals, the need for vernalization prior to obtaining immature explants is also eliminated. Thus, the mature embryo culture offers a simple, efficient, and expedited culture system. Explants such as immature ovaries, immature inflorescences, and anthers/microspores have also been explored for use as targets for barley transformation. Transgenic plants from barley microspores have been reported using microprojectile bombardment (Jähne *et al.*, 1994; Yao *et al.*, 1996).

2.1.6 RNAi technology

The production of loss-of-function mutants by induced mutations, insertional knockout, and antisense down-regulation has been achieved in many plants, with *A. thaliana* being a classical example. Generally, production of such mutants is time consuming, resource demanding, and often leads to undesirable changes, especially in plants with complex genomes. More recently, the RNA interference (RNAi) technology has been viewed as a viable alternative for gene silencing. Unlike the antisense ODN, RNAi technology can allow stable silencing of genes. RNAi-mediated gene silencing occurs post-transcriptionally as a result of double-stranded RNA. It was first reported in the nematode worm, *Caenorhabditis elegans* (Fire *et al.*, 1998) and was also demonstrated in

plants (Waterhouse *et al.*, 1998). RNAi has down-regulatory potential which can be directed to the gene of interest and would be valuable in complex genomes like barley and wheat for studying both genes involved in biosynthetic processes as well as for targeting changes in *in planta* product synthesis.

Requirements for the establishment of RNAi technology in plants include availability of a transformation system and design of transgene constructs so that transgenes produce hairpin RNAs (hpRNA) with double-stranded RNA (dsRNA) regions (Waterhouse and Helliwell, 2002). Studies have been conducted to determine if RNAi technology would be useful for reverse genetics or for alteration of starch in model plants. In tobacco plants, Chen *et al.* (2005) showed that transgenic tobacco lines produced by RNAi accumulated 3–5 times more starch than nontransformed plants when the gene coding for sucrose-6-phosphate phosphatase was silenced. Sucrose-6-phosphate phosphatase converts sucrose-6-phosphate to sucrose. In transgenic *Arabidopsis* plants, RNAi-mediated silencing of β -amylase8 led to starch accumulation in the leaves, in the presence or absence of a cold treatment (Kaplan and Guy, 2005). Temperature stress induces β -amylase accumulation, which leads to an increase in maltose content and is a protective mechanism for membrane proteins and the electron transport chain (Kaplan and Guy, 2004). More recently by RNAi-mediated silencing of two starch branching enzyme II isoforms (SBEIIa and SBEIIb), Regina *et al.* (2006) produced high-amylose wheat, with some lines showing more than 70% amylose content. The resistant wheat starch produced in this manner was shown to improve large bowel indices in rats and can potentially be of use for designing healthy starches for human consumption (Regina *et al.*, 2006). The RNAi technology therefore opens new possibilities for tailoring starch in barley as well. Due to the diploid nature of the barley genome, application of RNAi-mediated gene silencing should be attainable. In transient assays in barley aleurone cells, Zentella *et al.* (2002) demonstrated that the RNAi silencing of the transcription factor, GAMyb led to a significant reduction in gibberellin (GA)-induced α -amylase. In another transient RNAi assay, Douchkov *et al.* (2005) assessed functionality of defense-related genes in barley epidermal cells. Thus, silencing

of desired traits in barley by RNAi technology is possible and is likely to contribute tremendously both in gene function analysis and gene targeting in the near future.

2.2 Altered Traits in Barley

2.2.1 Quality

There are many aspects to barley quality. Barley grain is used for human food and animal feed. Barley malt is used for brewing and human food. Barley straw has many uses in animal production and several novel applications in controlling odors and algal production in urban and municipal ponds. Transgenic research to improve barley quality has focused on enzymes needed to breakdown endosperm components, to make more nutrients available for yeast in brewing and for monogastric animals. Research to improve amino acid and mineral composition has just begun and is likely to expand as more genes become available for important nutritional components (Table 2).

2.2.1.1 Malting

Much of the transgenic research for malting barley has emphasized the enzymes involved in malting that help to modify the endosperm. These enzymes are generally irreversibly inactivated at higher temperatures ($>55^{\circ}\text{C}$), which can result in incomplete degradation of the targeted cell components. Three papers describe the insertion and testing of thermotolerant glucanases to help break down glucans and cellulose in the germinating seed. Jensen *et al.* (1996) examined the effects of a hybrid (1,3-1,4)- β -glucanase from *Bacillus* species that had been codon optimized based on preferences found in the native barley version of the gene. Stably transformed plants and their progeny expressed the thermotolerant enzyme. Horvath *et al.* (2000) expanded on this work by placing the hybrid gene and the codon-optimized gene under the control of the **D**-hordein (*Hor3-1*) gene promoter. The highest (1,3-1,4)- β -glucanase expression was obtained with the codon-optimized gene, the **D**-hordein promoter and a signal peptide targeting the enzyme to the vacuoles.

The increased thermostability of the enzyme may not only be useful for malting but the enzyme also survives processing into feed pellets, allowing barley to be fed to poultry by reducing the viscosity of the feed in the intestine and increasing nutrient availability. In the third study, Nuutila *et al.* (1999) report the insertion of a thermotolerant endo-1,4- β -glucanase from *Trichoderma reesei*, under the control of a hybrid high-pI α -amylase promoter. The transgene was expressed during germination and the enzyme retained activity after 2 h at 65°C . The enzyme was produced in quantities that should be sufficient to reduce wort viscosities.

Another important group of enzymes in malting barley is the amylases, which help in break down of starches, providing simple sugars for brewing. These enzymes show similar thermoinactivation as the glucanases. Yoshigi *et al.* (1995) created multiple recombinant barley β -amylases with individual amino acid replacements. One mutant with seven replacements showed an 11.6°C increase in thermostability. Kihara *et al.* (2000) transformed barley with this thermostable β -amylase gene under the control of the native gene promoter and intron. Progeny tested for β -amylase showed transgene enzyme activity was not inactivated until treated at 70°C , while the native enzyme was inactivated at 60°C . Stable transgene expression was confirmed through the T₄ without suppression of the native β -amylase gene. Another malt enzyme manipulated in transgenic barley is α -amylase. A thermostable *Bacillus* α -amylase alkBA, under the control of the barley high-pI α -amylase promoter was used by Tull *et al.* (2003) to develop transformed plants. Overall α -amylase activity was increased 30–100% in T₁ grain but transient expression studies of this enzyme showed reduced thermostability in protoplasts, decreasing its utility in improving malt modification. Increased α -amylase activity may have implications in preharvest sprouting.

One transformation study has attempted to manipulate grain hardness, as softer grains generally have better malting quality and harder grains may provide better feed nutrition. Zhang *et al.* (2003) expressed the maize (*Zea mays* L.) γ -zein protein under the control of the endosperm-specific wheat Glu-1D-1 promoter in transgenic barley. The transgenic grain was heavier and more plump, containing more nitrogen in the form of increased prolamine protein. Approximately 4% of

Table 2 Attempted trait modification by genetic transformation in barley

Trait	Gene	Source	Promoter ^(a)	Genotype	Effects	References
Malting quality	(1,3-1,4)- β -glucanase	Modified hybrid <i>Bacillus</i> gene	<i>Hor3-1</i> or high-pI α -amylase	Golden Promise	Thermostable glucanase expression	Jensen <i>et al.</i> (1996)
	(1,4)- β -glucanase	<i>Trichoderma reesei</i>	High-pI α -amylase	Kymppi and Golden Promise	Thermostable glucanase activity	Nuutila <i>et al.</i> (1999)
	(1,3-1,4)- β -glucanase	Modified hybrid <i>Bacillus</i> gene	<i>Hor3-1</i>	Golden Promise	Thermostable glucanase activity	Horvath <i>et al.</i> (2000)
	β -amylase	Modified <i>Hordeum vulgare</i> gene	β -amylase	Igri	Thermostable β -amylase activity	Kihara <i>et al.</i> (2000)
	Alkaline α -amylase	<i>Bacillus</i> ssp	High-pI α -amylase	Golden Promise	Increased α -amylase activity	Tull <i>et al.</i> (2003)
Nutritional quality	γ zein	<i>Zea mays</i>	<i>Glu-1D-1</i>	Golden Promise	No change in grain hardness	Zhang <i>et al.</i> (2003)
	Lysine feedback insensitive aspartate kinase and dihydropicolinate synthase	<i>E. coli</i>	35S	Golden Promise	Increased free lysine and methionine	Brinch-Pedersen <i>et al.</i> (1996)
	Xylanase	<i>Neocallimastix patriciarum</i>	<i>GluB-1</i> or <i>Hor2-4</i>	Golden Promise	Fungal xylanase activity	Patel <i>et al.</i> (2000)
	Cellulase (1,4- β -glucanase)	<i>Neocallimastix patriciarum</i> and <i>Pirromyces</i>	<i>GluB-1</i>	Golden Promise	Cellulase for potential commercial production	Xue <i>et al.</i> (2003)
	Zinc transporter <i>AtZIP1</i>	<i>Arabidopsis thaliana</i>	<i>Ubi</i>	Golden Promise	Increased short-term zinc uptake	Ramesh <i>et al.</i> (2004)

(continued)

Table 2 Attempted trait modification by genetic transformation in barley (*Continued*)

Trait	Gene	Source	Promoter ^(a)	Genotype	Effects	References
Disease resistance	BYDV coat protein	PAV and p-PAV serotypes	35S	Golden Promise	Moderate to high resistance to P-PAV	Wan and Lemaux (1994), McGrath <i>et al.</i> (1997)
	Stilbene synthase <i>Vst1</i>	<i>Vitis vinifera</i>	<i>Vst1</i>	Igri	Resistance to <i>Botrytis cinerea</i>	Leckband and Lorz (1998)
	C-terminal polymerase	BYDV-PAV and CYDV-RPV	35S or <i>Ubi</i>	Schooner	Reduced BYDV	Wang <i>et al.</i> (2001)
	Stem rust resistance <i>Rpg1</i>	<i>Hordeum vulgare</i>	<i>Rpg1</i>	Golden Promise	Increased stem rust resistance	Horvath <i>et al.</i> (2003)
	Leaf rust resistance <i>Rpl-D</i>	<i>Zea mays</i>	<i>Ubi</i> or <i>Act1</i>	Golden Promise	Low expression, aberrant RNA processing	Ayliffe <i>et al.</i> (2004)
	Translation initiation factor <i>Hv-eIF4E</i>	<i>Hordeum vulgare</i>	<i>Ubi</i>	Igri NIL	Bymovirus resistance	Stein <i>et al.</i> (2005)
	Lipoxygenase <i>LOX2:Hv:1</i> Acetyl transferase <i>Tri101</i>	<i>Hordeum vulgare</i> <i>Fusarium sporotrichioides</i>	35S or <i>Ubi</i> <i>Ubi</i>	Salome Conlon	Increased senescence Did not reduce FHB or DON	Sharma <i>et al.</i> (2006) Manoharan <i>et al.</i> (2006)
Other	ABC transporter <i>PDR5</i>	<i>Saccaromyces cerevisiae</i>	<i>Ubi</i>	Conlon	Did not reduce FHB or DON	Manoharan and Dahleen (unpublished)
	Chitinase + thaumatin-like protein	<i>Oryza sativa</i>	<i>Ubi</i>	Conlon	Disease testing underway	Tobias <i>et al.</i> (2006)
	Thioredoxin h	<i>Triticum aestivum</i>	<i>Hor2-4</i>	Golden Promise	Faster germination/ α -amylase production; enhanced selenite resistance/ uptake	Cho <i>et al.</i> (1999), Kim <i>et al.</i> (2003)
	Antisense protein kinase <i>SnRK1</i>	<i>Hordeum vulgare</i>	<i>Glu-1D-1</i>	Golden Promise	Leaky promoter resulted in no pollen transmission	Zhang <i>et al.</i> (2001)
	Malate transporter <i>ALMT1</i>	<i>Triticum aestivum</i>	<i>Ubi</i>	Golden Promise	High levels of aluminum tolerance	Delhaize <i>et al.</i> (2004)

^(a)35S from CaMV; *Ubi* from *Zea mays*; *Act1*, *GluB-1* from *Oryza sativa*; *Glu-1D-1* from *Triticum aestivum*; *Hor3-1*, *Hor 2-4*, high pl α -amylase from *Hordeum vulgare*

the total prolamine consisted of the γ -zein trans-protein, but this did not significantly affect grain hardness or vitreousness. It is likely that much higher expression levels would be needed to see any effects in barley as soft maize cultivars contain 15–54% γ -zeins and hard maize contains 14–21% γ -zein.

2.2.1.2 Nutrition

Several studies have examined transformation technology to improve nutritional aspects of barley as food or feed. Barley, like most grains, is deficient in the amino acids lysine and threonine, reducing food and feed quality. Brinch-Pedersen *et al.* (1996) inserted lysine feedback insensitive forms of aspartate kinase and dihydrodipicolinate synthase into barley from *Escherichia coli*. Increased activity of these enzymes provided a twofold increase in lysine, arginine, and asparagines while reducing proline levels in half in T₀ and T₁ seeds. Leaves showed significant changes in amino acid composition with as much as a 16-fold increase in free lysine. These results show promise in manipulating barley amino acid content to meet specific nutritional requirements.

Improving mineral nutrition was tested by Ramesh *et al.* (2004) who inserted an *A. thaliana* zinc transporter (*AtZIP1*) into barley. T₂ lines showed an increase in zinc uptake and transport after zinc deprivation. Seeds from T₂ plants grown under standard conditions showed significantly increased zinc, iron, and magnesium, but seeds were smaller than those of wild-type Golden Promise. Individual transgenic seed still had higher zinc content than individual wild-type seed, showing the potential of transgenics to provide essential minerals.

Transformation efforts also have looked at improving feed use of barley. High levels of arabinoxylans and β -glucans can limit the use of barley as feed for monogastric animals. Feed enzymes, such as xylanases, are often added to feed at higher costs to producers. Patel *et al.* (2000) looked at the practicality of producing these enzymes in barley. They inserted a modified xylanase gene from the fungus *Neocallimastix patriciarum* controlled by the seed-specific promoters from the rice glutenin-B1 or barley B-1 hordein gene (*Hor2-4*). Transgenic

plants showed low levels of endosperm-specific expression through the T₂ and the protein was stable in the seed after storage for 3 months. A codon-optimized form of the xylanase showed increased expression in transient assays and would likely provide increased activity in stable transformants. The research by Horvath *et al.* (2000) described above in the malting quality section provides a thermotolerant glucanase that also would be useful in animal feed. Xue *et al.* (2003) generated transgenic barley expressing a hybrid cellulase (1,4- β -glucanase) that can degrade both cellulose and 1,3-1,4- β -glucans. The codon-optimized hybrid enzyme was expressed at high levels in the endosperm under the control of the rice GluB-1 seed storage protein promoter. The enzyme retained activity in T₂ transgenic grain after 1 year of storage, providing a stable source for this feed enzyme at much lower costs than producing the enzyme by traditional microbial fermentation techniques.

One study attempting to manipulate carbon metabolism examined the SnRK1 protein kinase sequence (Zhang *et al.*, 2001). This sequence has been shown to be involved in sucrose synthesis using antisense constructs in potato. A chimeric antisense construct was created combining sequences from barley seed SnRK1a and SnRK1b, with the transgene targeted to the endosperm by the wheat Glu-1D-1 promoter. Transgenic T₀ plants were recovered and confirmed by Southern blot hybridization, but none of the T₁ plants contained the transgene sequences. Further examination showed the Glu-1D-1 promoter allowed some antisense SnRK1 expression in anthers, causing abnormal pollen development. The 50% of the pollen containing the construct were nonviable, probably from inability to use sucrose, preventing transmission of the transgene. A promoter that does not allow expression in anthers will be needed to manipulate expression of SnRK1 in barley seed.

2.2.2 Disease resistance

Some of the first transformation efforts in plants involved inserting coat protein genes from viruses to induce resistance (Beachy *et al.*, 1990). This was also true in barley as Wan and Lemaux (1994) reported transformants containing the marker

genes *bar* and *uidA* in addition to the coat protein (*cp*) gene from barley yellow dwarf virus (BYDV) serotype PAV. Their success was expanded by McGrath *et al.* (1997) who transformed and tested plants with the coat protein gene from BYDV isolate P-PAV. T₁ plants and T₂ progeny containing the *cp* gene were identified as highly resistant after inoculation with viruliferous aphids. More recently, Wang *et al.* (2001) designed a hpRNA construct that contained BYDV-PAV C-terminal polymerase sequences. Nine of the barley lines developed with this construct showed extreme resistance to BYDV-PAV. Further tests of progeny of two lines showed single gene inheritance correlated with resistance.

A strategy being used to engineer fungal disease resistance is to use plant genes that have shown antifungal activity or that are induced by fungal attack. These can include phytoalexins and various pathogenesis-related (PR) genes like chitinases and glucanases. Leckband and Lörz (1998) transformed barley with the stilbene synthase gene (*Vst1*) from grape (*Vitis vinifera* L.) to produce the phytoalexin resveratrol. This gene is induced upon pathogen attack in several plant species, so the native promoter was used in addition to an enhancer sequence. They showed that the promoter was induced by rubbing leaves with sand and inoculation with the biotrophic fungus *E. graminis*. Inoculation of leaves with *Botrytis cinerea* showed resveratrol was effective in providing resistance. Chitinases and thaumatin-like proteins (tlp) are PR proteins with effects on fungal cell walls and membranes. They have been used successfully in many species to increase fungal resistance. Recently, barley was transformed with a combination of a chitinase and tlp from rice (*Oryza sativa* L.) and the co-expression examined (Tobias *et al.*, 2006). One line showed stable expression of both genes while two other lines lost the chitinase gene while still expressing the tlp. Disease testing of these lines is underway which will provide a comparison of overexpression of tlp alone and in combination with chitinase. Another gene implicated in the hypersensitive response and gene regulation during pathogenesis is lipoxygenase (LOX; Weber, 2002). Overexpression of *LOX2* in transgenic barley using constitutive promoters increased plant senescence, supporting a role for LOX in programmed cell death (Sharma *et al.*, 2006).

Two transformation projects have targeted mycotoxin production resulting from *Fusarium* head blight (FHB) in barley. The first of these (Manoharan *et al.*, 2006) used a *Fusarium sporotrichioides* gene *Tri101*, an acetyl transferase gene that modifies trichothecene mycotoxins to a less toxic form. Unfortunately expression of the enzyme had little effect on *Fusarium graminearum* infection in transgenic plants and the contamination of grain with its main mycotoxin, deoxynivalenol (DON). The second gene inserted was the *PDR5* gene from *Saccharomyces cerevisiae*, an ABC transporter gene that pumps trichothecene mycotoxins from cells (Manoharan and Dahleen, unpublished). Again, the gene had no effect on FHB or DON. While DNA and RNA were present in *PDR5* transformants, no effective activity assay could be found for this membrane-bound protein so it is not known if the enzyme was functional.

As isolation of resistance genes from plants expands, transformation is being used for functional verification of candidate resistance genes. One of the first of these was the stem rust resistance gene *Rpg1* (Brueggeman *et al.*, 2002), which has provided barley with durable resistance to *Puccinia graminis* f. sp. *tritici* for more than 60 years. Horvath *et al.* (2003) transformed the genomic clone of *Rpg1* from the resistant cultivar Morex into the susceptible cultivar Golden Promise. Interestingly, the transgenic Golden Promise lines showed a higher level of resistance than found in the original sources of *Rpg1*, Chevron, and Peatland. Only one copy of the gene was necessary for resistance. When the maize gene *Rp1-D* for resistance to common leaf rust (*Puccinia sorghi*) was inserted into barley (Ayliffe *et al.*, 2004), no novel resistance was acquired. Instead, this maize gene showed aberrant RNA processing, with transcripts lacking the gene's leucine-rich repeat. The resulting truncated transcripts were ineffective, containing only the nucleotide-binding site. Another candidate gene verified by transformation was an eukaryotic translation initiation factor Hv-*elF4E* for *rym4* barley yellow mosaic virus resistance (Stein *et al.*, 2005). This recessive resistance gene was overcome when Hv-*elF4E* sequences from a susceptible cultivar were transformed into a *rym4* resistant near-isogenic line. The transgenic line had dominant susceptibility to barley mild mosaic virus, showing

that variation in Hv-eIF4E sequences control the response to these viruses.

2.2.3 Other traits

Several other genes have been inserted into barley for various purposes. Using the naturally occurring redox protein, *thioredoxin h*, transformants were developed to examine its role in regulating seed storage protein degradation. The wheat *trxh* gene was expressed in endosperm under the control of the B₁-hordein promoter, with highest expression levels when targeted to the protein bodies using a signal peptide sequence (Cho *et al.*, 1999). Transgenic lines over-expressing *thioredoxin h* up to 30-fold showed increased pullulanase activity by an unknown mechanism. Pullulanase is a starch debranching enzyme that hydrolyzes α -1,6-linkages in amylopectin during germination and seedling development. In addition, Wong *et al.* (2002) showed that these same transgenic lines, relative to null segregant lines, had (i) an accelerated germination rate by one day, (ii) an accelerated appearance of α -amylase activity by 1 day, (iii) a 35% increase in the abundance of -SH groups in the hordein I fraction, and (iv) an increase in extractable and soluble proteins—resulting in a shift of protein from the insoluble to the soluble fraction. Kim *et al.* (2003) examined the same transgenic plants for their effects on germination in high selenite concentrations. Not only was germination of seed expressing *thioredoxin h* higher on 2 mM selenite than in null control plants, the transgenic plants converted the selenite to selenomethionine, selenate, and elemental selenium. It is possible that the capability to grow and incorporate selenium in these plants could be exploited to clean up selenium-polluted environments.

Other chemicals besides selenium pollute soils worldwide. One of these is aluminum, which is often solubilized in acid soils, inhibiting plant growth. Delhaize *et al.* (2004) engineered barley to express the wheat *ALMT1* gene, a malate transporter that provides aluminum tolerance. High levels of constitutive expression conferred by the maize ubiquitin promoter allowed robust root growth in the presence of aluminum concentrations that severely inhibited nontransgenic root growth, in both hydroponic culture and in

acid soils. Aluminum tolerance in these transgenic lines was comparable to the tolerance in the wheat cultivar that was the source of *ALMT1*.

Transformation has been used to examine the expression of the maize homeobox gene *knotted1* (*kn1*) in barley (Williams-Carrier *et al.*, 1997). The *kn1* gene, which is required for maintaining indeterminacy and preventing cellular differentiation, is homologous to the barley *Hooded* locus, which causes ectopic meristems to form on the upper lemma and awn. Ectopic expression of *kn1* in the barley awn caused the development of ectopic meristems that formed inflorescence-like structures. Patterns of gene expression in the transgenic lines constitutively expressing *kn1* were very similar to the expression of the native *hvknox3* in awns of *Hooded* mutants. Transformation has been a useful tool to examine expression of potential gene homologs and to compare the underlying mechanisms in monocots and dicots.

2.3 Gene Source and Constructs

With extensive research having been done on producing efficient barley transformation systems, there was eventually an apparent void in deciding genes of specific value for use in barley transformation and also the necessary construct design for temporal or spatial expression of the transgenes. This void appears not to be due to a lack of knowledge base in these areas, but rather due to ill defined or uncertain priorities when it came to barley transformation. Furthermore, with the polemic surrounding genetically modified plants (see Section 2.8) there is a hesitance to investing in genetically modified barley, especially for malting purposes due to fear of nonacceptance. To compound matters further, barley genome sequence information available was limited until recently. While plant genomes like those of *Arabidopsis* and rice have been fully sequenced, such sequence information for barley is now only starting to become available. This has mostly been due to the large size of the barley genome and the presence repetitive sequences. The size of the barley genome is about 5.5×10^{-12} g (Bennett and Leitch, 1995) and contains about 76% of repetitive DNA (Flavell *et al.*, 1974). Nonetheless extensive progress has been made, mainly as a result of more high-throughput genome analysis tools such as

Table 3 Barley genes isolated by map-based cloning [Reproduced from Stein and Graner (2004)]

Gene	Function	References
<i>Mlo</i>	Transmembrane protein	Buschges <i>et al.</i> (1997), Devoto <i>et al.</i> (2003)
<i>Rar1</i>	Zinc-binding protein	Lahaye <i>et al.</i> (1998), Shirasu <i>et al.</i> (1999)
<i>Ror2</i>	Syntaxin	Collins <i>et al.</i> (2003)
<i>Mla1 Mla6 Mla7 Mla10 Mla12 Mla13</i>	CC-NBS-LRR	Wei <i>et al.</i> (1999, 2002), Halterman <i>et al.</i> (2001, 2003), Zhou <i>et al.</i> (2001), Shen <i>et al.</i> (2003), Halterman and Wise (2004)
<i>Rpg1</i>	Receptor kinase	Brueggeman <i>et al.</i> (2002)

large insert libraries, automated sequencing, and data mining being available. The United States Department of Agriculture (USDA) CSREES-sponsored Barley Coordinated Agriculture Project (Barley CAP; <http://www.barleycap.org>) is developing a 3000 single nucleotide polymorphism (SNP)-based genetic/physical/expression map of the barley genome. The project will develop The *Hordeum* Toolbox, which will serve as a public resource for the dissemination of large SNP data sets that relate to genetic, phenotypic, and trait data, and utilize resulting information to develop elite barley germplasm using high-throughput marker-assisted selection.

2.3.1 Gene isolation

Forward genetics and reverse genetics are essentially the two approaches available for gene isolation, i.e., eventually leading to assignment of function to DNA sequence information. While forward genetics proceeds from phenotype to gene following the classical Mendelian patterns of segregation, reverse genetics relies on available sequence information, be it genomic DNA, cDNA (complementary DNA), or protein, and proceeds toward phenotype/function assignment. Forward genetics has evolved to encompass molecular markers for the generation of saturated maps, enabling “tagging” of candidate genes of interest. Further narrowing the gaps between the candidate genes and the markers by fine mapping followed by physical mapping, subsequently leads to the possibility of isolating the candidate gene. This gene isolation strategy by map-based cloning has been successful in a number of plants (see Zhang, 2007 for a review). In barley, it has been very useful in the isolation of disease resistance gene analogs (RGAs). For example, by

positional cloning based on barley–rice synteny mapping, Collins *et al.* (2003) were able to isolate the *ror2* gene, which confers resistance to powdery mildew. Recently a translation initiation factor 4E conferring resistance to Bymovirus was also isolated by map-based cloning (Stein *et al.*, 2005). It is interesting to note that most of the map-based cloning approaches in barley have targeted disease resistance genes (Table 3).

The generation of mutant phenotypes by T-DNA insertional mutagenesis or transposon tagging or gene silencing can be of value for mapping and map-based cloning. The availability of TILLING and *Ds* tagging populations in barley could further expedite map-based cloning approaches. Furthermore, in addition to the isolation of genes, there is a need to isolate regulatory sequences to better understand control of gene expression. For example, in barley understanding the temporal and spatial expression patterns of key genes involved in germination would lead to better control of germination for production of good quality malt. It is known that in malting barley, breeding for high enzyme production is highly desired to obtain good malt and by overexpressing the *HvGAMYB* gene in barley, it was shown that the GA-induced amounts of hydrolytic enzymes were increased in germinating grains (Murray *et al.*, 2006). This study iterates the importance of understanding gene function and with regards to barley, besides disease resistance gene understanding, functions of those involved in quality attributes need to be elucidated.

2.3.2 Chimeric vector construct and special designs

The construction of chimeric vectors with specific promoters, enhancer elements, intronic sequences,

and matrix-attachment regions (MARs) has been explored extensively in plants for gene expression enhancement in transgenic plants, if not in barley. However, with the genetic transformation of barley, either by microprojectile bombardment or *Agrobacterium*, now being routine in many laboratories, there is a need to shift the focus from single gene transfer and function to multiple transgene transfer and function. The relevance of multiple transgene integration has a direct bearing on complex quality traits, such as starch biosynthesis. The transfer of large inserts carrying genes involved in starch biosynthesis would be valuable for *in planta* studies of starch modification. The possibility for transferring large fragments of DNA to plant genomes via *Agrobacterium* and specialized vectors has been demonstrated. Binary bacterial artificial chromosomes (BIBAC) vectors have been developed for plant transformation (Hamilton *et al.*, 1996; Hamilton, 1997). Transformation-competent artificial chromosome (TAC) vectors (Liu *et al.*, 1999) carrying large-insert genomic DNA of hexaploid wheat have also been developed (Liu *et al.*, 2000). Using these TAC vectors in *Agrobacterium*, transgenic rice carrying an 80kb insert was recovered (Liu *et al.*, 2002). Even though stability of these large inserts is still questionable, progress has been made in elucidating factors causing such instability and efficiency for transformation of tomato (Frery and Hamilton, 2001), potato (Song *et al.*, 2003), and rice (Nakano *et al.*, 2005). The availability of vectors carrying large inserts would enable a multi-gene linking approach to tandemly join a number of the cloned starch biosynthetic genes, for example, and study their effects on changes in starch biosynthesis. Lin *et al.* (2003) used such an approach to stack multiple genes (including 10 genes and functional DNA elements) into a TAC vector and successfully produced transgenic rice plants. Furthermore, gene interactions can be studied by pyramiding only those suspected to be involved in the concerted metabolism of starch.

2.4 Transgene Expression Inheritance

Instability of transgene expression in plants is often associated with complex multicopy patterns of transgene integration at the same locus that

results in inverted repeats and a variety of other position effects due to random integration. Inactivation of transgene expression is often observed in plants and is especially problematic in cereal crops. Using biolistic transformation methods, there are often complex, multicopy transgene integration patterns, where it has been observed that more than 50% of T₁ plants exhibit transgene silencing (Wan and Lemaux, 1994; Pawlowski *et al.*, 1998) and the number of plants with a tendency to silence continues to increase during generation advance (Bregitzer *et al.*, 1998). The majority of cases of transgene silencing are correlated with the presence of multicopy inserts, sequence rearrangements, and random integration into the genome. The insertion of single copies of transgenes increases the probability of transgene expression stability; however, silencing is also observed in plants with single, simple-pattern transgene inserts, even *Agrobacterium*-generated transgenic plants with single copies of the transgene.

Based on the maize transposable elements *Ac* and *Ds* a method was generated that led to large numbers of transgenic barley plants, each carrying a single transgene copy at a different location (Koprek *et al.*, 2000). The plants were developed by crossing barley plants expressing *Ac* transposase (*AcTPase*) with plants containing one or more copies of another transgene expressing a herbicide resistance gene, *bar*, which was contained between inverted repeat *Ds* ends. In the F₂, derived from selfing of F₁ plants, plants were identified in which transposed *Ds-bar* copies had segregated away from the original location that included the selection gene and plasmid backbone and from *AcTPase*.

Of the transposed elements, an estimated 25% had transposed to unlinked locations and 75% to linked sites. Transgene expression from *bar* was 100% stable in F₂ plants, while only 23% of F₂ plants with *Ds-bar* at the original site (introduced by bombardment) expressed stably from the transgene. In F₃ and F₄ populations, transgene expression in 81.5% of plants from plants with single copy transposed *Ds-bar* remained completely stable. Analysis of the integration site in the single copy plants indicated that *Ds-bar* transposed into single or low copy regions of the genome, whereas silenced *Ds-bar* elements at their original location were in redundant or highly

repetitive genomic regions. Methylation of the nontransposed transgene and its promoter, as well as a higher condensation of the chromatin around the original site of integration, was observed in plants exhibiting transgene silencing (Koprek *et al.*, 2001; Meng *et al.*, 2006).

A factor that might play a decisive role in transgene expression stability is the nature of the chromosomal location of the insertion, which varies from line to line. By analyzing the sequences flanking *Ds-bar*, it was determined that most transposed elements reinserted into single copy genomic DNA regions; 87% into genic regions (Singh *et al.*, 2006).

2.5 Tailoring Specialty Products

Barley is mostly recognized for its malting or feed uses. In recent years, there has been an interest to develop barley for other uses. Barley has been incorporated to some extent in wheat bread, soups, porridge, breakfast cereals, stews, baked goods, and production of maltose syrups. Other uses of barley for purposes such as the production of enzymes, oral vaccines, antibodies, pharmaceuticals, and vitamins would be the result of genetic engineering. The large-scale production of xylanase in barley grains (Patel *et al.*, 2000) is a good example of possible industrial application. Uses specifically related to grain composition could be achieved by traditional breeding and selection approaches. The production of high amylose or high amylopectin barley and varying percentages of these two components may be achieved without genetic engineering. The ratio of amylose:amylopectin has been implicated among other factors to play a role in industrial starch films (Koskinen *et al.*, 1996). It has been reported that a high percentage of amylopectin leads to reduction in flexibility, burst resistance, and tensile strengths of films (Wolff *et al.*, 1951) and therefore, barley grains with starch composition suited for this purpose could be developed. The bioplastics properties of starches from potato, corn, wheat, and rice have been studied (van Soest *et al.*, 1996) and similar studies could be initiated for barley starches. The novel uses for barley are still in the early stages of conception. This is due to its high economic value as malt and therefore, more resources are being allocated to that end.

2.6 Intellectual Property and Patent Landscape

Issues relating to intellectual property and patents have become highly debated and publicized in the recent years in plant biotechnology. This is mainly due to the perceived market value of biotechnology-derived products. There are number of patents related to barley for biotechnological purposes. Table 4 shows some of the patents that have relevance to barley transformation. The two main methods for transformation, i.e., microprojectile bombardment and *Agrobacterium* mediated, are covered under their own respective patents. With regards to cereals relevant patents that are likely to be of value in terms of transformation include the one employing vacuum infiltration and *Agrobacterium* (Kloti, 2001) and the other using particle bombardment of germinating barley mature embryos (Wu and Rodriguez, 2000) (Table 4). Furthermore, several of the genes (e.g., *phosphomannose isomerase*, *bar*, *hyg*) used for selection of transformation events are also patented. There is, however, a patented barley green regenerative tissue method (Lemaux and Cho, 2003; Table 4) that is available for nonexclusive licensing. Recently, to bypass patent-related issues governing *Agrobacterium*-mediated transformation, gene transfer to plants by other bacteria besides *Agrobacterium* has been explored (Broothaerts *et al.*, 2005), wherein *Rhizobium* sp., *Sinorhizobium meliloti* and *Mesorhizobium loti* carrying disarmed Ti plasmids were shown to be competent for transformation and are available as open-source-modeled licenses and are not commercialized. Details of this technology are available under the Bioforge project (<http://www.bioforge.net>) and Biological Innovation for Open Society (BIOS) at <http://www.bios.net>.

2.7 Risk Assessment

Regulation of the products of biotechnology is a very dynamic issue. Not only does nearly every developed country have very different approaches to regulations, but many underdeveloped nations also have yet to define how they will regulate these products or how they will deal with import and export issues. For current country details, see USDA Foreign Agricultural Service Global

Table 4 Some barley-specific biotechnology patents

Title	Inventors	Patent #	Date
Construction of barley with reduced gel protein	Hirota, N., Kihara, M. and Ito, K.	USP 7 074 986	July 11, 2006
Transposon tagging and gene delivery in small grain cereals	Lemaux, P.G., McElroy, D. and Koprek, T.	USP 6 951 972	October 04, 2005
Modified barley α -glucosidase	Henson, C.A., Muslin, E.H. and Clark, S.E.	USP 6 849 439	February 01, 2005
Methods and means for producing barley yellow dwarf virus resistant cereal plants	Waterhouse, P. and Wang, M.-B.	USP 6 777 588	August 17, 2004
Barley gene for thioredoxin and NADP-thioredoxin reductase	Cho, M.-J., del Val, G., Caillau, M., Lemaux, P.G. and Buchanan B.B.	USP 6 833 493	December 21, 2004
Method for preparing barley green regenerative tissue	Lemaux, P.G. and Cho, M.-J.	USP 6 541 257	April 01, 2003
Low lipoxxygenase 1 barley	Douma, A.C., Doderer, A., Cameron-Mills, V., Skadhauge, B., Bech, L.M., Schmitt, N., Heistek, J.C. and van Mechelen, J.R.	USP 6 660 915	December 09, 2003
Barley gene for thioredoxin and NADP-thioredoxin reductase	Cho, M.-J., del Val, G., Caillau, M., Lemaux, P.G. and Buchanan B.B.	USP 6 380372	April 30, 2002
Seed-preferred promoter from barley	Jiao, S., Habben, J.E., Niu, X. and Olsen, O.-A.	USP 6 407 315	June 18, 2002
Methods and apparatus for transformation of monocotyledonous plants using <i>Agrobacterium</i> in combination with vacuum filtration	Kloti, A.S.	WO/2001/012828	January 22, 2001
Methods for producing transformed cells of barley	Rikiishi, K., Noda, K. and Kihara, M.	USP 6 291 244	September 18, 2001
Methods for rapidly producing transgenic monocotyledonous plants	Alpeter, F. and Popelka, J.-C.	WO/2001/073084	October 04, 2001
Method for barley transformation	Wu, L. and Rodriguez, R.L.	USP 6 100 447	August 08, 2000
A methods for producing transformed cells of barley	Rikiishi, K., Noda, K. and Kihara, M.	WO/1999/004618	February 04, 1999
Tolerance of trichothecene mycotoxins in plants and animals through the modification of the ribosomal protein l3 gene	Harris, L., Gledlie, S.C. and Simmonds, J.A.	WO/1999/009173	February 25, 1999
Barley β -amylase structural gene	Yoshigi, N. and Okada Y.,	USP 5 726 057	March 10, 1998
Cloned α -glucosidase from barley	Skadsen, R.W. and Tibbot, B.K.	USP 5 763 252	June 09, 1998
Tissue-specific promoter	Okada, Y., Yoshigi, N., Ito, K. and Kihara, M.	WO/1997/002353	January 23, 1997

Agriculture Information Network (GAIN) reports (<http://www.fas.usda.gov/scriptsw/AttacheRep/default.asp>).

When developing regulations, one must first define all the risks associated with the new technology or product of the technology. Assessment of the risks associated with the production of transgenic barley, or for that matter any other transgenic plant event, may involve factors ranging from health and environmental safety to market acceptance to socio-economic issues. Many countries have chosen to regulate on a science-based regime involving

a thorough evaluation of the safety to humans, animals, and the environment. Other countries have given greater weight to socio-economic issues and market acceptance. In this section, we will discuss the scientific risk as it pertains to human, animal, and environmental safety.

2.7.1 Human and animal health

To date, no corroborated ill effects on human health have been attributed to the consumption

of biotechnology-derived foods. There have been numerous studies evaluating the safety of foods derived from genetically engineered crops conducted by several national regulatory authorities using procedures consistent with internationally agreed principles (ICSU, 2003), but they have yet to find any nutritionally deleterious effects as a result of the consumption of commercially available genetically engineered foods. This is not to say that there are no risks, as this cannot be said of foods developed from conventionally bred crops. To truly evaluate long-term human safety, two large, culturally diverse subsets of the population—one eating biotechnology derived foods and one not—would have to be monitored through their entire life span. As this approach is currently unrealistic, food safety risk assessment must attempt to identify probable risks associated with the consumption of these foods by humans and animals. These risks may include the unintended presence of allergens and toxins, the development of antibiotic resistance, and unintentional changes in food composition caused by the genetic engineering process. It is difficult if not impossible to test food safety of whole foods and feeds with animal tests. Despite what nonexperts commonly think, animal tests are not the gold standard. Compositional analysis and toxicity testing of individual components is much more sensitive than whole foods testing (International Life Sciences Institute, 2004). Preventing adverse health effects requires the application of appropriate scientific methods to predict and identify unintended compositional changes that may result from genetic modification of plants, animals, and microbes. However, it is the final product, rather than the modification method or process that is more likely to result in unintended adverse effects (National Academy of Sciences, 2004).

On an international basis, the WHO and FAO through the Codex Alimentarius Commission *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnologies have developed and continue to develop standards, guidelines, and recommendations for foods derived from biotechnology. However, each member government has the responsibility to formulate and implement its own policies. The regulatory approval process in each of the 21 countries currently growing transgenic crops (James, 2005) is distinct, but

most of them regulate on the basis of process, or in other words conducts risk assessments based on the fact that the product was created by genetic engineering. Table 5 outlines the main countries currently growing transgenic crops and the regulatory agencies overseeing their risk assessment.

Canada is currently the only country that regulates based on the concept of “novelty”. Plants with novel traits (PNTs) are defined “*as any plant containing a trait not present in plants of the same species already existing as stable, cultivated populations in Canada, or is present at a level significantly outside the range of that trait in stable, cultivated populations of that plant species in Canada*” (CFIA Directive 94-08). This essentially means that any new trait, regardless of how it was introduced [e.g., ribosomal DNA (rDNA), mutagenesis, or traditional breeding techniques], may be considered novel. This definition of novelty also translates to animal feed derived from plants if “*the feed is not substantially equivalent in terms of the use and safety of an approved feed ingredient*” (Feeds Section, CFIA).

2.7.2 Environmental concerns

When it comes to protecting the environment, primary concerns are transfer of genes to nontarget species, the potential for invasiveness or weediness, effects of traits on nontarget organisms and, depending on the trait, the potential to create weeds or diseases with resistance to the introduced trait, such as herbicide resistance or disease resistance, respectively.

On an international basis of risk assessment for living modified organisms (LMOs), over 138 countries, including Canada and members of the European Union, have signed the Cartagena Protocol on Biosafety. This document lays out general principles and steps regarding scientific risk assessments for the intentional introduction of LMOs into the environment. The Protocol, while requiring signed parties to adopt measures and strategies to manage and controlling any risks identified, does not develop these policies. They are developed by the individual country and its appropriate regulatory agency (Table 5). Several countries, such as the United States and Australia, have chosen not to sign the

Table 5 Regulatory agencies responsible for food, feed, and environmental safety of transgenic crops^(a)

Select countries growing GM Crops	GM crop area (million hectares)	Main regulatory agency(s) responsible for food/food ingredient/feed safety evaluation	Main regulatory body responsible for environmental safety evaluation
USA	49.8	Food and Drug Administration (FDA)/United States Department of Agriculture (USDA)	Environmental Protection Agency (EPA)
Argentina	17.1	National Agrifood Health and Quality Service (SENASA)	National Advisory Commission on Agricultural Biotechnology (CONABIA)
Brazil	9.4	National Bio-safety Council Technical Commission (CTNBio)	
Canada	5.8	Health Canada/ CFIA (Canadian Food Inspection Agency, 2008)	CFIA, Plant Biosafety Office (PBO)
China	3.3	Ministry of Agriculture (MOA)—Biosafety Administration Office	
Paraguay	1.8	Biosecurity Commission	
India	1.3	Ministry of Environment and Forests (MEF)—Genetic Approval Committee (GEAC)	
South Africa	0.5	Department for Agriculture, Executive Council	Department for Agriculture, Executive Council
Australia	0.3	Food Standards, Australia New Zealand (FSANZ),	Office of the Gene Technology Regulator (OGTR)
European Union Countries (Romania, Spain, Portugal, Germany, France, and Czech Republic)	Approximate total <0.2	European Food Safety Authority (EFSA)	European Commission—Directive 2001/18/EC

^(a)Reproduced from James (2005), Bulgaria Biotechnology Information Centre (2008), and Zarrilli (2005)

protocol but have policies in place to assess environmental risks and appropriate management practices.

As with traditional agriculture, associated risk can often be managed with good agricultural practices. The same can be said for potential risks associated with transgenic crops. Good farming and manufacturing practices for transgenic crops deemed to be safe for release into the environment must follow a strict regime to minimize the possibility of any of the identified risks. These practices may include the implementation of buffer crops, isolation distances, and proper crop rotation schedules.

Barley, in relation to gene flow, has been described as a low risk crop (Eastham and Sweet, 2002; Ritala *et al.*, 2002) due to its self-fertilizing nature, the small amounts of pollen produced and the low frequency of outcrossing. With the use

of adequate isolation distances and other best management practices, these risks can be managed but cannot achieve zero risk.

Volunteers from small grain, such as wheat and barley, can be issue. Typically management techniques such as proper rotations, preseed weed burn-off, and herbicide applications are used to control these issues. With a herbicide tolerant (HT) barley variety, management using chemicals can become a bit more difficult, but a variety of chemicals are available for use, making even the situation with HT transgenic barley crops manageable.

2.8 Public Acceptance and Awareness

Since the dawn of technological advances, public perception of newer technologies has generally

not been forthright. This dread for novelty is often due to a lack of information/education. This fear of technology could not be more apparent than by observing the “GMO (genetically modified organisms) polemic.” With regards to the important grain commodities, public awareness and consumer acceptance of biotechnology for grain value enhancement are likely to be key components for the success of further biotechnological developments. However, GM grains, although likely proven to be of potential value, are going to be a success story only as a result of improved consumer acceptance (Jones and Jones, 2004). It is therefore imperative that the benefits and risks of GM grain products be extensively discussed to allow consumers to make well-informed choices.

2.8.1 Measuring acceptance and awareness

In the past decade, numerous studies have been conducted to try to judge public perception, but interpreting results has not been an easy task.

For example, a recent survey (Eurobarometer 64.3, 2005) asked Europeans questions to gauge their opinion on GM foods. The result of this survey stated that 58% oppose the technology while 42% support it. A different study of Europeans was based on the concept of food safety (European Food Safety Authority, 2005). When they asked the public, “*What are all the things that come to mind when thinking about possible problems or risks associated with food?*” only 8% responded—GMOs.

Public acceptance and awareness of foods derived from biotechnology are generally low. These levels appear to have either stayed the same or only slightly improved over the last several years, according to a number of public surveys, as seen in Table 6. A positive association between acceptance and education has been identified (Hallman *et al.*, 2003), although the largest sector of the public remains undecided. Several studies have shown that support for these technologies typically increases after an explanation of the risk and benefits are discussed (McCormick, 2003; IFIC Food Biotechnology Report, 2006).

Table 6 Results of several recent opinion polls on public support for biotechnology^(a)

Organization	Country	Number of participants	Results
Awareness Biotechnology Australia (McCormick, 2003)	Australia	500	23% support GM foods 42% support GM after benefits and risks addressed
Pew (Pew Initiative on Food and Biotechnology, 2006)	United States	1000	Opposition declined from 58% to 46% since 2001—support has been stable at 27% in 2006
Eurobarometer (2006)	Europe	25 000	Over 50% of Europeans believe biotechnology will improve their quality of life—particularly for medicine and bio-based industrial applications 27% support GM foods
IFIC (2006)	United States	1000	72% of consumers say they are confident in the safety of the US food supply 32% of consumers have a positive view of biotechnology
Food Policy Institute (2003)	United States	1200	49% approve of plant-based GM foods (down from 58% in 2001)
Norfolk Genetic Information Network (2008)	United Kingdom, Denmark, Poland, Mexico, Brazil, Taiwan, Turkey, and United States		58% will not eat GM food 55% support GM food for developing countries 66% support GM crops for cheaper medicines
Genome Canada (Genome Canada Newsletter, 2003)	Canada	1500	56% find biotechnology useful

^(a) Reproduced from Pew Initiative on Food and Biotechnology (2006), McCormick (2003), Eurobarometer (2006), and Genome Canada Newsletter (2003)

It is important to note that surveys are intended only to give a representative view of the general audience. Caution must be exercised when analyzing any survey, as results can be swayed by the manner in which the questions are written or sampling of a nonrepresentative audience. In the end, the final results of consumer acceptance will only be seen when the consumers make their choice at the grocery store.

2.8.2 Information dissemination

The need to increase the general public's awareness of biotechnology is obvious, but the issue is to make the information available in a manner that is open, honest, and unbiased. According to the Special Eurobarometer 238 (European Food Safety Authority, 2005), people find consumer groups, physicians, and scientists as the most reliable sources of information, followed by public authorities. In the recent Pew Initiative Report, friends and family, farmers, and scientists were considered the most reliable sources of information, while trust in the government in the United States substantially dropped to fourth place from first place in 2001. Media in both reports were viewed as the least credible source of information, yet was the most widely reaching. Information, therefore, must come from a variety of sources and deliver messages the general public will find interesting and understandable.

Various organizations around the world are working to educate the public and bring an informed debate. At the primary and secondary school level, a number of innovative websites and programs are running with a goal of educating our youth. In the United States, a series of buses travel across the country which are in essence mobile instructional laboratories. Initially started by the Georgia State University, this concept has been copied across the globe with similar operations occurring in Australia, Canada, and Malaysia. "Celebrate ImaGENEnation" is the theme for Canada's National Biotech Week. A first of its kind, this week long series of events, debates, and lectures is aimed at informing politicians and the public on the innovative research happening across Canada. A number of US university-based educational websites also provide science-based information to end users and the public, for example, <http://www.nbiap.vt.edu>,

<http://www.ucbiotech.org>, <http://www.cls.casa.colostate.edu/TransgenicCrops/hotlabel.html>.

In the past, the benefits of biotechnology-derived crops have been mostly directed at producers. As new technologies are being developed which promise to bring greater direct benefits to the consumer, and increased efforts are aimed at public education, hopefully the public will become more engaged and better informed, allowing them to make more informed decisions.

3. CHALLENGES FOR BARLEY GENETIC MODIFICATION

Barley was generally considered to be recalcitrant to both *in vitro* culture and genetic transformation compared to other cereals like rice. Over the last decade, there have been a number of reports on the successful transformation of barley, including those with traits of value. With the possibility of using *Agrobacterium* for barley transformation, there is likely to be more reports of genetically engineered barley, but particle bombardment will continue to remain a method of choice due to better success to date. It is generally believed that *Agrobacterium* will allow for a more organized integration of the transgenes at low copy numbers with higher stable expression levels, although *Ds*-mediated gene delivery from bombardment events offers an alternative for generating single copy events. However, with *Agrobacterium* there may be DNA rearrangements, deletions, multiple copies, and insertion sites (for review, see Stam *et al.*, 1997). Integration of transgenes in multiple copies or at multiple sites leads to silencing as a result of hypermethylation. In barley, the stability of foreign DNA and the importance of methylation have been recognized and it was suggested that a certain methylation pattern might be necessary for stability of foreign DNA (Rogers and Rogers, 1992). Several subsequent studies have addressed transgene stability, silencing, and methylation in barley (e.g., Koprek *et al.*, 2001; Meng *et al.*, 2003, 2006; Singh *et al.*, 2006). Results suggest that the transposon-mediated DNA delivery system in barley (Singh *et al.*, 2005) will be useful for more successful transgenic events. However, in many other instances due to silencing occurring as a result of multiple copies, it has been suggested that only transgenic lines carrying a single copy of the gene of interest be selected

and advanced (Finnegan and McElroy, 1994; Demeke *et al.*, 1999). Furthermore, the presence of multiple and rearranged copies complicates regulatory approval. Endogenous genes can also be suppressed by transgenes and by using different promoters with the transgenes of interest this can be prevented (Båga *et al.*, 1999). Sequences derived from MARs used to flank transgenes have also been shown to influence stable integration and expression, possibly preventing the transgenes from being affected by surrounding chromatin (Liu and Tabe, 1998). Due to the presence of about 76% repeats in barley (Flavell *et al.*, 1974), use of the MARs flanking strategy should lead to efficient targeting of transgenes to the euchromatic regions thereby enhancing stable expression.

As mentioned earlier, *Agrobacterium*-mediated transformation could also be used to deliver more specialized vectors carrying large inserts such as bacterial artificial chromosomes (see Section 2.3.2). This approach will be particularly useful for transfer of large fragments of DNA with several genes. There are numerous reports indicating the existence of clusters of genes on specific chromosomal segments for specific traits. For example, the existence of gene clusters conferring disease resistance has been reported (Pryor, 1987; Crute and Pink, 1996; Micheltore and Myers, 1998). Clusters such as these have been identified in barley (Jørgensen, 1994; Seah *et al.*, 1998) and the potential for using a BIBAC system for transformation of barley does exist. Furthermore, with the availability of genomics data and quantitative trait loci (QTL) localization for quality traits in barley, transformation using large inserts will be of interest especially for an understanding of biosynthetic pathways such as those of starch. Research efforts also need to continue to address quality attributes in barley to diversify barley uses for food and industrial applications.

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Oat

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Reports on the time and place of oat domestication remain conflicting even though the processes leading to the domestication of oat are tightly linked to the history of the domestication and cultivation of wheat (*Triticum*) and barley (*Hordeum*) species, which were the primary domesticated cereals of Neolithic era agriculturalists in the Near East (Vavlov, 1926; Murphy and Hoffman, 1992). Weedy grasses, such as oat and rye (*Secale cereale* L.), were eventually developed into secondary crops thanks to the selection pressure that were accidentally imposed on admixtures of these weedy plants (Murphy and Hoffman, 1992). Conclusions about the center of origin and evolutionary history for oat are not easy to reach for several reasons. For example, many environmental and chromosomal changes may have taken place over time as well as evolutionary developments, such as modes of seed dispersal or reproductive isolation barriers (Rajhathy and Thomas, 1974; Wesenberg *et al.*, 1992). In addition, these authors indicated that what was once a natural habitat has been changed in recent times via cultivation and seed transport by humans. All or most of the oat species currently overlap in the western-most fringe of the native range (southern Spain, northern Morocco, and northern Algeria), suggesting that the center of origin for *Avena* spp. is probably located in those

areas. Oat was first used as a forage crop in southern Europe long before it was grown for grain (Coffman, 1977). It was first introduced in southern United States in the early to mid 16th century by the Spanish (Coffman, 1977). Oat is adapted to a wide range of soil types with a variety of chemical and physical properties, but it is best adapted to cool and moist climate (Sorrells and Simmons, 1992). Oat production is mostly concentrated between latitudes 35–65°N and 20–46°S. It is principally cultivated in Europe (Sweden, Finland, Russia, Poland, and Germany), the northeastern United States (North Dakota, Minnesota, Wisconsin, South Dakota, and Iowa), Canada, and Australia.

1.2 Taxonomy, Habit, and Cytological Features

The genus *Avena* consists of about 70 different species, but only a few are cultivated. Cultivated oat species consist of *Avena byzantina* C. Koch (red oat) and *Avena sativa* L. (white oat), with the latter being the primary cultivated species. Both these species are hexaploid. The ploidy levels of oats include diploid ($2n = 2x = 14$), tetraploid ($2n = 4x = 28$), and hexaploid ($2n = 6x = 42$) (Rajhathy and Thomas, 1974; Baum and Fedak, 1985; Legget and Thomas, 1995; Li *et al.*, 2000) (Table 1). Four *Avena* genomes, A, B, C, and D, have been identified, and *A. sativa* is a self-pollinated and natural

Table 1 A list of some *Avena* species^(a)

Section	Species	Ploidy	Genome formula
Avenotrichon (Holub) Baum	<i>A. macrostachya</i> Bal. ex Cross. et Dur.	$2n = 4x = 28$??
Ventricosa (Baum)	<i>A. clauda</i> Dur.	$2n = 2x = 14$	C_pC_p
	<i>A. eriantha</i> Dur.	$2n = 2x = 14$	C_pC_p
	<i>A. ventricosa</i> Bal. ex Cross	$2n = 2x = 14$	C_vC_v
Agraria (Baum)	<i>A. brevis</i> Roth.	$2n = 2x = 14$	A_sA_s
	<i>A. hispanica</i> Ard.	$2n = 2x = 14$	AA?
	<i>A. nuda</i> L.	$2n = 2x = 14$	A_sA_s
	<i>A. strigosa</i> Schreb	$2n = 2x = 14$	A_sA_s
Tenuicarpa (Baum)	<i>A. agadiriana</i> Baum et Fedak sp. Nov.	$2n = 4x = 28$	AABB?
	<i>A. atlantica</i> Baum et Fedak sp. Nov.	$2n = 2x = 14$	A_sA_s
	<i>A. barbata</i> Pott. Ex Link.	$2n = 4x = 28$	AABB
	<i>A. canariensis</i> Baum, Rajhathy et Simpson	$2n = 2x = 14$	A_cA_c
	<i>A. damascena</i> Rajhathy et Baum	$2n = 2x = 14$	A_dA_d
	<i>A. hirtula</i> Lag.	$2n = 2x = 14$	
	<i>A. longiglumis</i> Dur.	$2n = 2x = 14$	A_lA_l
	<i>A. lusitanica</i> (Tab. Mor.) Baum Comb. Nov. et Stat. Nov.	$2n = 2x = 14$	AA?
	<i>A. matritensis</i> Baum sp. Nov.	$2n = 2x = 14$??
	<i>A. prostrata</i> Ladiz.	$2n = 2x = 14$	A_pA_p
Ethiopica (Baum)	<i>A. wiestii</i> Steudel.	$2n = 2x = 14$	A_sA_s
	<i>A. abyssinica</i> Hochst.	$2n = 4x = 28$	AABB
	<i>A. vaviloviana</i> (Malz.) Mordv.	$2n = 4x = 28$	AABB
Pachycarpa (Baum)	<i>A. maroccana</i> Gdgr.	$2n = 4x = 28$	AACC
	<i>A. murphyi</i> Ladiz.	$2n = 4x = 28$	AACC
Avena	<i>A. atherantha</i> Presl.	$2n = 6x = 42$	AACCDD
	<i>A. byzantina</i> C. Koch.	$2n = 6x = 42$	AACCDD
	<i>A. fatua</i> L.	$2n = 6x = 42$	AACCDD
	<i>A. hybrida</i> Petrem.	$2n = 6x = 42$	AACCDD
	<i>A. occidentalis</i> Dur.	$2n = 6x = 42$	AACCDD
	<i>A. sativa</i> L.	$2n = 6x = 42$	AACCDD
	<i>A. sterilis</i> L.	$2n = 6x = 42$	AACCDD
	<i>A. trichophylla</i> C. Koch	$2n = 6x = 42$	AACCDD

^(a)Reproduced from Baum (1975, 1977), Legget (1992), and Thomas (1992)

allohexaploid containing A, C, and D genomes. In oat, three distinct types of juvenile growth are generally recognized, including prostrate, semiprostrate, and erect or upright (Etheridge, 1916; Stanton, 1955). The prostrate growth type develops more tillers and is winter resistant while the most upright early growth is associated with spring (Coffman, 1977). Strength of the culms or standing ability of the plant depends on the number and attachment of the roots as well as the structure of the culms (Hamilton, 1951). Coffman (1977) described two types of roots in oat, seminal and adventitious. The seminal roots originate during embryo development and consist of a jointed primary root (radical) and branches arising at the first node or joint. As for the adventitious roots, they arise at the nodes of the stem and tillers, just beneath the soil surface (Coffman, 1977).

1.3 Economic Importance

For the last 30–40 years, world oat production has markedly declined because of increased mechanization in farming (oats were the principal feed of work horses), competition with other crops (barley, sorghum, and maize) as animal feeds, and low yield gains. Oat yields have been stagnant in part because there is very little genetic diversity in oat germplasm (De Koeper *et al.*, 1999; Langer *et al.*, 1978). Decline in oat production has been particularly pronounced in countries such as the United States (Figure 1) where oat production has decreased about 80% in the last past 30 years. Oat is currently grown on about 13 million hectares, and for the period 2000–2006, the world production has stabilized at around 25 million metric tons. Oat is a multipurpose crop,

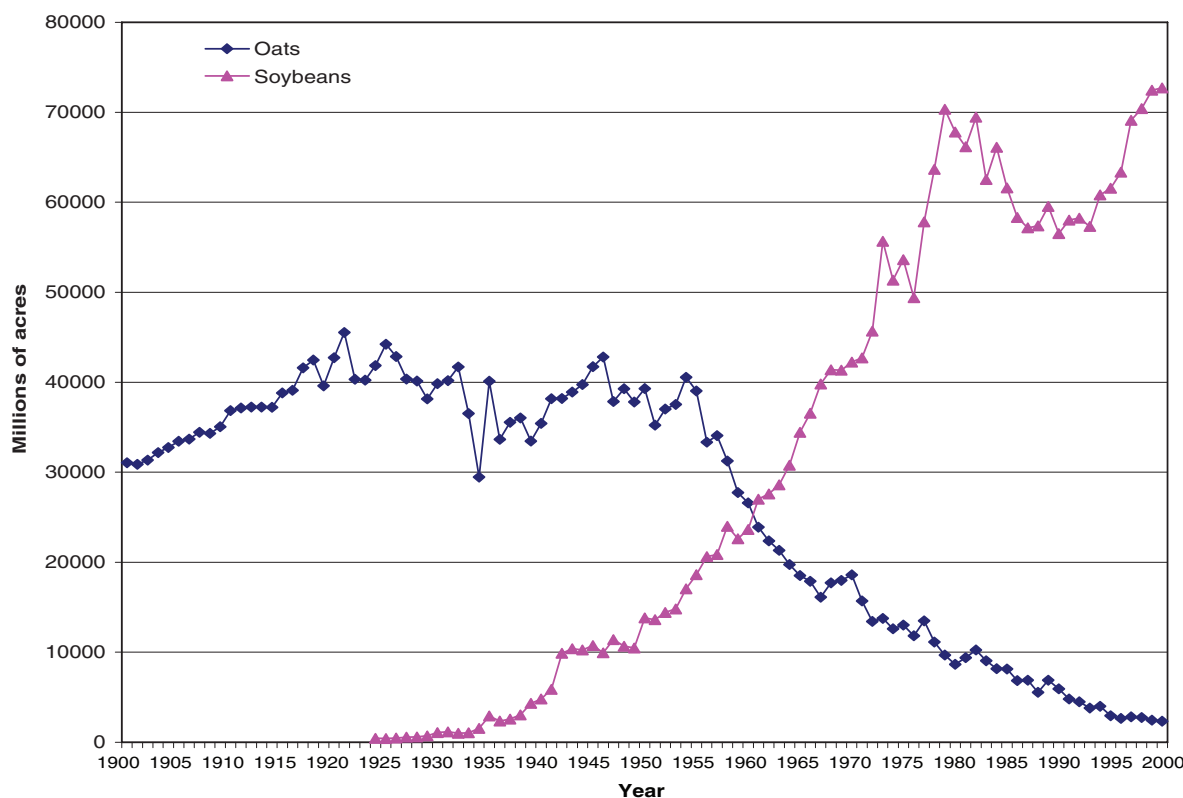


Figure 1 Farm mechanization and stiff competition from crops such as soybeans have led to a steady decline in oat production over the years, particularly in the United States [Source: USD-NA]

which is an important source of food for both humans and domesticated animals. It contains many compounds that exhibit antioxidant activity, including vitamin E, phytic acid, phenolic compounds, and avenanthramides (Peterson, 2001). In addition, oat contains the soluble fiber β -glucan, which is reported to be associated with specific health benefits, including lowering blood lipids, regulating blood glucose, protecting against tumor development in the colon, reducing risks of coronary disease. Industrially, oats are used in the production of plastics, pesticides, preservatives, cosmetics, synthetic rubber solvents, disinfectants, and papers as well as in the brewing industries.

1.4 Traditional Breeding Objectives of Oats

Most of the improvement in oat has been achieved using classical breeding methods, which rely mostly on hybridization followed by selection.

In many oat breeding programs, the focus was on improving agronomic characteristics such as yield improvement, suitable maturity, lodging resistance, disease resistance, and environmental stress tolerance. In the United States, disease resistance is the most critical aspect of oat germplasm enhancement and cultivar development, with particular emphasis on crown rust resistance, barley yellow dwarf virus resistance or tolerance, stem rust resistance, and smut resistance (Wesenberg, 2000). Lately, however, more focus has been put on developing oat varieties with improved grain quality, such as high milling yield and increased β -glucan content (Cervantes-Martinez *et al.*, 2001; McMullen *et al.*, 2005).

The most critical issue relative to oat improvement is the marked decline in oat acreage over time, especially in the United States, and the gradual decline in oat research effort in both the public and private sector in North America (Wesenberg, 2000).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Oat Breeding

Traditional breeding has been successfully used to develop hundreds of improved oat cultivars; however, the process is time consuming. Also, hybridization barriers could hamper the process. Therefore, attempts have been made to incorporate molecular breeding tools, such as genetic transformation to introduce certain genes of interest into the oat genome. In addition to speeding up the development of improved cultivars, genetic engineering can be very useful in a crop such as oat, which has a huge genome. Genetic engineering can be used to add new traits or improve existing traits in oat by introducing genes from any other organisms into oat or by manipulating oat genes for increased expression (Somers, 1998). Some of the goals of oat genetic engineering include identification of gene promoter sequences for high levels of tissue-specific transgene expression, virus and fungus disease resistance, modification of grain composition and agronomic traits using genes from other organisms and antisense suppression of endogenous homoeologous genes, and tolerance to abiotic stress (Koev *et al.*, 1998; Somers, 1998; Torbert *et al.*, 1998a; Kelley, 2001; Maqbool *et al.*, 2002; Oraby *et al.*, 2005). However, in order to reach these goals, two prerequisites must be met, namely a dependable regeneration system and an efficient transformation method.

2. DEVELOPMENT OF TRANSGENIC OATS

2.1 Tissue Culture in Oats

2.1.1 Callus- and meristem-based regeneration systems

Production of plants from transformed callus using regenerable oat genotypes (Table 2) in a callus-based regeneration system is one of the schemes applied in oat transformation. Monocots are generally recalcitrant in tissue culture and typically exhibit genotype-specific regenerability (Chowdhry *et al.*, 1993). *In vitro* plant regeneration from a callus-based system offers a great potential for production of larger numbers of plants.

However, with the exception of the derived genotype, Gaf/Park, and its derived selection, GP-1, most oat genotypes, in particular the elite cultivars, are recalcitrant to regeneration through somatic embryogenesis. Callus-based regeneration protocols have been developed for oats (Cummings *et al.*, 1976; Heyser and Nabors, 1982; Bregitzer *et al.*, 1991; Gana *et al.*, 1995; Hassan *et al.*, 1999; Kelley *et al.*, 2002a, b), but the apparent inability to de-differentiate reflects a fundamental property of oats, rather than simply a negative response to the traumatic and artificial conditions of tissue culture (Christou, 1995). Callus cultures of most oat cultivars either result in a low frequency of plant regeneration or fail to produce plants at all. In addition, with time, progressive callus subcultures result in loss of plant regenerability due to reduced somatic embryogenesis and other developmental barriers. Somaclonal variation also occurs often because of the length of time required by the callus culture process. Cellular and morphogenic events during somatic embryogenesis of oats appear to be controlled by an array of conditions, including medium composition, physical environment, and genetic effects (Altman *et al.*, 1990).

Despite the problems mentioned above, some progress has been achieved in oat callus-based regeneration during the last few decades (Table 2). For example, Torbert *et al.* (1998b) surveyed 16 elite oat genotypes to determine if they are capable of producing somatic embryos from mature embryos. Fifteen out of the 16 lines tested produced embryogenic callus, and only the line Milton failed to produce any callus at all. This group also succeeded in transforming the cultivar Belle via particle bombardment of mature embryo-derived embryogenic callus. In addition, cultivar Tibor (*A. nuda*) (low plant regeneration), experimental line GP-1 (*A. sativa*) (high plant regeneration), and their crosses, GP-1 x Tibor and Tibor x GP-1, were grown on putrescine-containing media to determine the effects of putrescine on somatic embryogenesis and plant regeneration (Kelley *et al.*, 2002b). Analysis of the effect of putrescine on each genotype revealed that the addition of 0.5 mM putrescine to a shoot proliferation medium enhanced the formation of somatic embryos and thus subsequent plant regeneration. Regeneration of plants from Tibor opens the possibility for the transformation of this agronomically important cultivar, and these

Table 2 List of oat genotypes tested for their regeneration potential using various explant sources and regenerations systems

Genotype	Explant source	Regeneration system	References
Dumont	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Gaf/Park-1	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998b, c), Cho <i>et al.</i> (1999), Kaeppler <i>et al.</i> (2000, 2001), Kelley <i>et al.</i> (2002a, b; 2004), Choi <i>et al.</i> (2001)
88Ab3073	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c), Hassan <i>et al.</i> (1999)
Robert	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Preakness	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Monida	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Belle	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
86Ab4582	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Calibre	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Dane	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Starter	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Derby	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Pacer	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Gem	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Jim	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
CDC Boyer	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Milton	Mature embryos	Somatic embryogenesis	Torbert <i>et al.</i> (1998c)
Tibor	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002b)
GP-1 x Tibor	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002b)
Tibor x GP-1	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002b)
Corbit/GP-1	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002a)
GP-1/Corbit	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002a)
GP-1/Corbit//GP-1	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002a)
GP-1/Corbit//Corbit	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002a)
Corbit/GP-1//GP-1	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002a)
Corbit/GP-1//Corbit	Mature embryos	Somatic embryogenesis	Kelley <i>et al.</i> (2002a)
Park	Mature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976), Heyser and Nabors (1982), Nabors <i>et al.</i> (1982, 1983), Hassan <i>et al.</i> (1999)
Gaf/Park	Mature embryos	Somatic embryogenesis	Hassan <i>et al.</i> (1999)
Flamingskrone	Mature embryos	Somatic embryogenesis	Lörz <i>et al.</i> (1976)
Arnold	Mature embryos	Somatic embryogenesis	Lörz <i>et al.</i> (1976)
Tiger	Mature embryos	Somatic embryogenesis	Lörz <i>et al.</i> (1976)
Garland	Mature embryos	Som. Emb/organogenesis	Kelley <i>et al.</i> (2004)
Park	Mature embryos	Som. Emb/organogenesis	Kelley <i>et al.</i> (2004)
Gaf/Park	Mature embryos	Som. Emb/organogenesis	Kelley <i>et al.</i> (2004)
Corbit	Mature embryos	Som. Emb/organogenesis	Kelley <i>et al.</i> (2004)
GP-1 x Corbit	Mature embryos	Som. Emb/organogenesis	Kelley <i>et al.</i> (2004)
Corbit x GP-1	Mature embryos	Som. Emb/organogenesis	Kelley <i>et al.</i> (2004)
Fuchs	Leaf bases	Somatic embryogenesis	Gless <i>et al.</i> (1998)
Jumbo	Leaf bases	Somatic embryogenesis	Gless <i>et al.</i> (1998)
Gramena	Leaf bases	Somatic embryogenesis	Gless <i>et al.</i> (1998)
Bonus	Leaf bases	Somatic embryogenesis	Gless <i>et al.</i> (1998)
Alfred	Leaf bases	Somatic embryogenesis	Gless <i>et al.</i> (1998)
Sanna	Leaf bases	Somatic embryogenesis	Chen <i>et al.</i> (1995)
Sang	Leaf bases	Somatic embryogenesis	Chen <i>et al.</i> (1995)
Vital	Leaf bases	Somatic embryogenesis	Chen <i>et al.</i> (1995)
Puhti	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997)
Foothill	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997)
OT 194	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997)
Pazano	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997)
Fulghum	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997)
Stout	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997), Kiviharju and Pehu (1998)
WW 18019	Anthers	Somatic embryogenesis	Kiviharju and Pehu (1998)
CAV 2648	Anthers	Somatic embryogenesis	Kiviharju <i>et al.</i> (1997), Kiviharju and Pehu (1998)

(Continued)

Table 2 List of oat genotypes tested for their regeneration potential using various explant sources and regenerations systems
(Continued)

Genotype	Explant source	Regeneration system	References
Garry	Vegetative shoots	Organogenesis	Zhang <i>et al.</i> (1999), Cho <i>et al.</i> (2003)
Ogle	Vegetative shoots	Organogenesis	Zhang <i>et al.</i> (1999)
Pacer	Vegetative shoots	Organogenesis	Zhang <i>et al.</i> (1999)
Porter	Vegetative shoots	Organogenesis	Zhang <i>et al.</i> (1999)
Prairie	Vegetative shoots	Organogenesis	Zhang <i>et al.</i> (1999)
Gaf	Immature embryos	Somatic embryogenesis	Rines <i>et al.</i> (1992)
Gaf/Park	Immature embryos	Somatic embryogenesis	Rines <i>et al.</i> (1992), Bregitzer <i>et al.</i> (1991)
Gaf-30/Park	Immature embryos	Somatic embryogenesis	Somers <i>et al.</i> (1992), Torbert <i>et al.</i> (1995, 1998a), Pawlowski and Somers (1998)
Melys	Immature embryos	Somatic embryogenesis	Kuai <i>et al.</i> (2001), Perret <i>et al.</i> (2003)
Bulwark	Immature embryos	Somatic embryogenesis	Perret <i>et al.</i> (2003)
Gaf/Park	Immature embryos	Somatic embryogenesis	Bregitzer <i>et al.</i> (1991), Bregitzer <i>et al.</i> (1995)
Lodi	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976), McCoy <i>et al.</i> (1982), Rines and McCoy (1980), Dahleen <i>et al.</i> (1991a, b)
Tippecanoe	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976), McCoy <i>et al.</i> (1982), Rines and McCoy (1980), Dahleen <i>et al.</i> (1991a, b)
Clintland 64	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Clinton	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Dal	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976), Rines and McCoy (1980)
Dodge	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Goodland	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Hulless HA 14	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Jaycee	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Otter	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Portal	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Putnam 61	Immature embryos	Somatic embryogenesis	Cummings <i>et al.</i> (1976)
Moore	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Lyon	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Benson	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Marathon	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Victorgrain	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Garry	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Hudson	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Terra	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Black Mesdag	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Victoria	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Selma	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
AJ 109/5	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
NP 3/4	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Rallus	Immature embryos	Somatic embryogenesis	Rines and McCoy (1980)
Park	Immature embryos	Somatic embryogenesis	Heyser and Nabors (1982), Nabors <i>et al.</i> (1983)
Victory	Seeds	Somatic embryogenesis	Carter <i>et al.</i> (1967)
Park	Roots	Somatic embryogenesis	Nabors <i>et al.</i> (1982)
Park	Mesocotyls	Somatic embryogenesis	Heyser and Nabors (1982), Bregitzer <i>et al.</i> (1989)
Gaf/Park	Hypocotyls	Somatic embryogenesis	Hassan <i>et al.</i> (1999)
Park	Hypocotyls	Somatic embryogenesis	Hassan <i>et al.</i> (1999)
88Ab3073	Hypocotyls	Somatic embryogenesis	Hassan <i>et al.</i> (1999)
Peniarth	Protoplasts	Somatic embryogenesis	Hahne <i>et al.</i> (1989)
Adamo	Protoplasts	Somatic embryogenesis	Hahne <i>et al.</i> (1989)
Alfred	Protoplasts	Somatic embryogenesis	Hahne <i>et al.</i> (1989)
Major	Protoplasts	Somatic embryogenesis	Hahne <i>et al.</i> (1989)
Phoenix	Protoplasts	Somatic embryogenesis	Hahne <i>et al.</i> (1989)

results suggest that putrescine may be used to induce or enhance somatic embryogenesis in more agronomically important oat cultivars.

Meristem-based regeneration systems are genotype independent and have the potential for producing a high number of fertile plants in a tissue culture environment (Kelley *et al.*, 2004). Because the culture time is shorter than that for the callus-based regeneration, meristem-based regenerated plants may be less prone to somaclonal variation.

2.1.2 Sources of explants

Several sources of explants, including leaf bases, anthers, vegetative shoots, roots, mesocotyls, hypocotyls, and protoplasts, have been used in oat tissue culture, but immature and mature embryos have been the most commonly used explant sources for establishment of regenerable tissue cultures of oat, and somatic embryogenesis is the most widely used regeneration pathway (Table 2). However, immature embryos do not lend themselves well to particle bombardment because callus initiation arises from the mesocotyl of the immature embryo, a very small target for biolistic transformation (Torbert *et al.*, 1998a). Regeneration and transformation systems based on shoot meristematic cultures have been developed (Zhang *et al.*, 1999; Maqbool *et al.*, 2002; Cho *et al.*, 2003; Kelley *et al.*, 2004). Compared to callus-based cultures, shoot meristematic cultures have several advantages, including generation of more vigorous plants and the potential for increased genetic stability (Zhang *et al.*, 1999). In addition, shoot meristematic cultures are genotype independent; therefore, the method can be extended to elite oat cultivars, which are generally recalcitrant to callus-based regeneration system.

2.2 Transformation of Oats

Plant transformation is predicated upon the availability of an efficient gene delivery method and a routine and reliable regeneration system. *Agrobacterium*-mediated transformation, particle bombardment, and protoplast transformation are the most widely used gene delivery methods. Most of the original experiments and breakthroughs in genetic transformation have been performed

with species belonging to the Dicotyledonae. The Poaceae genera, particularly *Avena*, have not been used in genetic engineering efforts commensurate with their economic importance nor to the extent that they are consumed as a caloric or protein source. One of the reasons for this is that the preferred plant transformation system has been *Agrobacterium*-mediated and until recently, monocots, traditionally, had not responded to the proper, efficient transfer of the transfer DNA (T-DNA) (Christou, 1995). More successful for monocot transformation has been biolistics (Hagio *et al.*, 1991), which employs high velocity metal particles to deliver biologically active DNA into plant cells (Christou, 1995).

2.2.1 Gene delivery methods

2.2.1.1 Particle bombardment-mediated transformation

Until recently, the method of predilection for transferring genes into monocot plant genomes was biolistics (biological ballistics), also referred to as “gene gun” or particle bombardment. The biolistic technique, first developed by Sanford *et al.* (1987), consists of introducing genetic material into intact cells and tissues through the use of high-velocity microprojectiles. DNA containing genes is coated onto microcarriers (tungsten or gold particles) and loaded onto macrocarriers, which are accelerated under slight vacuum into barriers, whereby the microcarriers are then accelerated into target tissues or cells. Particle bombardment is genotype independent, and it has virtually no inherent host range limitations and is not restricted to certain specific tissues or cells. Successful transformation of hexaploid oats was first achieved using the particle method to genetically transform friable embryogenic callus cultures (Somers *et al.*, 1992). Since then, the same approach has been employed to transform other oat explants, such as meristematic cultures (Zhang *et al.*, 1999). However, the percentage of cells that are stably transformed by this method remains low as compared to *Agrobacterium*- or protoplast-mediated transformation. In addition, the method is prone to high copy number insertion, which may lead to transgene inactivation. Particle bombardment was also reported to produce more transgene rearrangements (Songstad *et al.*,

1995; Pawlowski and Somers, 1998), but this assertion has been challenged by a recent study (Kohli *et al.*, 1999). Even the superiority of *Agrobacterium*-based DNA delivery techniques over direct delivery methods has been called into question (Repellin *et al.*, 2001).

2.2.1.2 *Agrobacterium*-mediated transformation

For the dicotyledonous species, *Agrobacterium*-mediated gene delivery system remains the method of choice because it is more reliable and efficient, and transformation efficiencies can be in the range of 70–80% (Wilkins *et al.*, 2000). The *Agrobacterium* gene transfer technique is based on the natural ability of the soil-dwelling *Agrobacterium tumefaciens*, containing a tumor-inducing Ti plasmid, to introduce a portion of its DNA into a plant cell as a part of its normal pathogenic process (Matzk *et al.*, 1996). It has been successfully used to transform many plant dicotyledonous species. However, monocotyledonous species traditionally had not responded well for the efficient transfer of the T-DNA (Christou, 1995) because they were thought to be out of the range of *Agrobacterium* transformation (Potrykus, 1990), although in nature, some monocotyledonous species are susceptible to *Agrobacterium* infection (De Cleene and De Ley, 1976). The first reports of transformed dicotyledonous plants via *Agrobacterium*-based technique appeared in the early 1980s. Since then, transgenic monocotyledonous species have also been produced using this method, including transgenic rice (Chan *et al.*, 1992; Hiei *et al.*, 1994; Dong *et al.*, 1996), wheat (Cheng *et al.*, 1997), barley (Tingay *et al.*, 1997; Fang *et al.*, 2002), rye (Popelka and Altpeter, 2003), and *Alstroemeria* (Akutsu *et al.*, 2003). The *Agrobacterium*-mediated transformation method appears to be best suited for lines that undergo somatic embryogenic regeneration, so its use would be mostly limited to a few oat genotypes because many elite oat genotypes remain recalcitrant to somatic embryogenesis. However, transformed rice plants have been obtained from isolated shoot apices following *Agrobacterium* inoculation (Park *et al.*, 1996). *Agrobacterium*-mediated transformation of monocots appears to be influenced by several

factors, including genotype, explant type, pretreatment, inoculation, and co-culture conditions, *Agrobacterium* strain, plasmid, and selectable marker (Cheng *et al.*, 2004). Currently, particle bombardment appears to be the method of choice for oat transformation, and several oat genotypes have been genetically engineered using this method. There seems to be no published reports of successful *Agrobacterium*-mediated transformation for oat (Table 3).

2.2.1.3 *Protoplast*-mediated transformation

Protoplast-mediated transformation was among the first methods of direct gene transfer in plants, particularly in cereals such as rice (Toriyama *et al.*, 1988; Zhang *et al.*, 1988) and maize (Golovkin *et al.*, 1993). Direct gene transfer to protoplasts is appealing because no biological vector is needed, and it has the potential to pave the way for oat somatic hybridization. Somatic hybridization can be particularly useful for the transfer of desirable traits that are controlled by polygenes or uncloned genes and for the generation of novel gene combinations by overcoming sexual crossing barriers (Binsfeld *et al.*, 2000). However, the isolation and culture of protoplasts is a difficult and time-consuming procedure. The lack of a dependable and routine regenerable protoplast system constitutes a major limitation to the greater use of *in vitro* genetic manipulations in oat (Rines *et al.*, 1992).

2.3 Reporter and Selectable Marker Genes Used in Oat Transformation

There are several reporter genes, including the *GUS* and *GFP* genes, used in plant biotechnology to monitor gene transfer and expression and help in the recovery of transformed plant cells, tissues, or organs. The *UidA* gene for β -glucuronidase (*GUS*) of *Escherichia coli* has been one of the most widely used genetic markers in the study of foreign gene expression in plants (Willmitzer, 1988; Walden and Schell, 1990). The *GUS* assay has several advantages. The procedure is fast, nonradioactive, and sensitive, and it allows for both quantitative (level of expression) and qualitative (specificity of expression in tissues and

Table 3 Several oat genotypes have been genetically modified using particle bombardment method

Genotype	Gene product	Method of gene transfer	Phenotype	References
GAF-30/Park	β -glucuronidase (GUS) and phosphinothricin (PPT)	Biolistics	Gus activity in the seed of fertile regenerated was detected; PPT resistance in progeny of transgenic plants co-segregated with bar and <i>uidA</i> sequences demonstrating stable inheritance of the transgenes	Somers <i>et al.</i> (1992)
GAF-30/Park	Gus and neomycin phosphotransferase II (NPTII)	Biolistics	Npt II and paromomycin provided efficient selection of transgenic oat tissue cultures	Torbert <i>et al.</i> (1995)
GAF/Park	RNA-dependent RNA polymerase (<i>RNA pol</i>)	Biolistics	Resistance to Barley yellow dwarf virus	Koev <i>et al.</i> (1998)
GAF30/Park	Coat protein	Biolistics	Resistance to Barley yellow dwarf virus isolates planthopper (<i>Nilaparvata lugens</i> STal)	McGarth <i>et al.</i> (1997)
Belle/Dane/Gem	NPTII	Biolistics	Seventeen independently genetically engineered tissue cultures were produced that regenerated fertile, transgenic plants	Torbert <i>et al.</i> (1998a)
GAF/Park-1	pNGI contained the uid A Gus and NPT II; pH24 contained the NPT II gene	Biolistics	Npt II and gus detected in transgenic oat	Torbert <i>et al.</i> (1998b)
GAF-30/Park	Gus and NPTII	Biolistics	CoYMC-Gus plants conferred expression in vascular cells of vegetative tissues	Torbert <i>et al.</i> (1998c)
GAF-30/Park	Gus and <i>bar</i> gene	Biolistics	All transgenic lines analyzed exhibited genomic interspersions of multiple clustered transgenes	Pawlowski and Somers (1998)
GAF/Park-1	<i>uidA</i> ; gus and hygromycin phosphotransferase (HPT)	Biolistics	Transgenes was stably transmitted to T ₁ and T ₂ progeny of transgenic oat	Cho <i>et al.</i> (1999)
Garry	Bar/NPTII and <i>uidA</i>	Biolistics	Transgene expression observed in T ₁ and T ₂ progeny of transgenic oat	Zhang <i>et al.</i> (1999)
GAF/Park-1	Green fluorescent protein (GFP)	Biolistics	Transgene integration and expression confirmed plants and progeny	Kaeppeler <i>et al.</i> (2000)
GAF/Park-1	Green fluorescent protein (GFP)	Biolistics	Transgene copy number and integration patterns were similar to those in transgenic plants derived from chemical-based selection systems	Kaeppeler <i>et al.</i> (2001)
GAF/Park-1	Gus and HPT	Biolistics	Some stresses imposed by the transformation process led to cytological variation in transgenic oat plants	Choi <i>et al.</i> (2001)
Melys	<i>bar</i> or <i>uidA</i>	Biolistics	Stable transmission and expression of the bar gene in T ₁ and T ₂ progenies	Kuai <i>et al.</i> (2001)
	pBY520 and pAct1-D containing <i>hval-bar</i> and Gus genes	Biolistics	Tolerance to salt and mannitol stresses	Maqbool <i>et al.</i> (2002)
Melys/Bulwark	Gus genes with rice actin, CaMV 35S or glutenin promoters	Biolistics	Cell and tissue-specific expression of Gus was evident; HMW glutenin promoter showed endosperm-specific expression	Perret <i>et al.</i> (2003)
Garry	Gus and GFP	Biolistics	Stable GFP expression was observed in T ₂ progeny from five independent GFP-expressing lines tested, and homozygous plants from two lines were obtained	Cho <i>et al.</i> (2003)

organs) information. In rare cases, the *GUS* gene has been used as a selectable marker (Jefferson, 1989). There was the general assumption that there was no detectable intrinsic *GUS* activity in higher plants, but studies have found that *GUS*-like activity was widely distributed in seed plants (Hu *et al.*, 1990). The *GUS* gene has been by far the most extensively utilized reporter gene in oat transformation (Table 4). However, there are several drawbacks associated with the *GUS* assay, including endogenous activity, background signal from microbial contaminants, instability of some fluorescent stain products, and toxicity to plant cells (Hodal *et al.*, 1992; Guivarc'h *et al.*, 1996; Kaeppler *et al.*, 2001). Because of these limitations, new reporter genes, such as the *GFP* reporter gene, have been developed to increase the efficiency and flexibility of cereal transformation (Kaeppler *et al.*, 2001). *GFP* does not interfere with cell growth and function; therefore, it can be used in both nonlethal selection and in co-transformation schemes where it is detected by irradiation with near ultraviolet (UV) light or blue light. Thus the damage caused by lethal selection of transformants using antibiotics or the sacrifice of valuable transformed tissue for *GUS* assay can be avoided.

Transformation systems require a selection system in an attempt to generate nonchimeric transgenic plants. Antibiotics and herbicides have served as the most popular selectable markers to generate genetically modified plants. Selectable marker genes encode proteins rendering the transformed cells resistant to an antibiotic or herbicide that is used for selection. Therefore, a medium containing a selectable toxin allows for the survival of the transformed cells, while the nontransformed cells are killed or lag in growth. The *bar* gene, isolated from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987), has been widely used, particularly in grasses and legumes. The recovery of transformed cells of cereals is generally achieved by using a positive selection system for cells that are resistant to herbicides or antibiotics. However, transformation of herbicide resistance is of great concern in oats because oat relatives are common weeds and readily cross with the cultivated *Avena*, possibly resulting in ecological and agricultural problems. Antibiotic selection was also of concern, but the common presence of already resistant bacteria lessens the risk of ecological disturbance. Therefore, antibiotics have

been the selection system of choice for oats and many other crop plants (Torbert *et al.*, 1995).

2.4 Value-Added Traits

Ongoing efforts to incorporate molecular breeding tools into breeding programs to improve oat germplasm include transgenics, molecular markers, and genomics. Plant transformation technology has made it possible to insert foreign genes of interest into plant genomes with more precision in overcoming sexual incompatibility as well as species barriers between organisms. Most of the genetic engineering in oat has involved the introduction of reporter and selectable marker genes into the oat genome of the modified oat genotypes (Tables 3 and 4). However, a few oat cultivars have been modified by introducing potentially value-added traits, such as the barley yellow dwarf virus (BYDV) resistance gene (Koev *et al.*, 1998) and the barley *hva1* gene for tolerance to osmotic stresses (Maqbool *et al.*, 2002; Oraby *et al.*, 2005).

BYDVs are the most serious and ubiquitous economically important viruses of several small grains, including wheat, barley, and oats. Koev *et al.* (1998) transformed oat plants with the 5' half of the BYDV strain PAV genome, which includes the RNA-dependent RNA polymerase gene by bombarding callus cultures of GAF/Park, a nonagronomic but highly regenerable oat line. Two gene constructs, p35S39K60K and p35S99K, which were used are described as follows (Koev *et al.*, 1998). The construct p35S39K60K consisted of the 5' untranslated region (UTR) of BYDV-PAV and wild type ORFs 1 and 2 preceded by the cauliflower mosaic virus double 35S promoter and followed by the nopaline synthase (*nos*) transcription termination signal. The construct p35S39K60K was developed in two steps: (1) The p35SPAV5' (bases 1–1596) construct was generated by polymerase chain reaction (PCR) using pPAV6 full-length BYDV-PAV plasmid as a template. The upstream primer (5'-ACGGGATCCGAGTGAAGATTGACCATCTTCACA-3') contained a *Bam*HI site and the first 23 nucleotides of the 5' end of the BYDV-PAV genomic RNA, and the downstream primer (5'-ATGAAGCTTGACATTGGCATCCTTCTGATG-3') was complementary to viral bases

Table 4 Commonly used reporter genes and selectable markers in oat transformation

Genotype	Reporter gene	Selectable marker	References
GAF-30/Park	β -glucuronidase (GUS)	Phosphinothricin (PPT)	Somers <i>et al.</i> (1992)
GAF-30/Park	Gus	Neomycin phosphotransferase II gene (NPTII)	Torbert <i>et al.</i> (1995)
GAF-30/Park	<i>UidA</i>	<i>bar</i> gene	McGarth <i>et al.</i> (1997)
Belle/Dane/Gem		NPTII	Torbert <i>et al.</i> (1998a)
GAF/Park-1	Gus	NPTII	Torbert <i>et al.</i> (1998b)
GAF/Park-1	<i>uidA</i> ;Gus	Hygromycin phosphotransferase (HPT)	Cho <i>et al.</i> (1999)
Garry	<i>uidA</i> ;Gus	<i>bar</i> /NPTII	Zhang <i>et al.</i> (1999)
GAF/Park-1	Green fluorescent protein (GFP)	GFP	Kaepler <i>et al.</i> (2000)
Melys	<i>UidA</i>	Phosphinothricin acetyl transferase (PAT)	Kuai <i>et al.</i> (2001)
GAF/Park-1	Gus	GFP	Kaepler <i>et al.</i> (2001)
Melys/Bulwark	Gus	PAT	Perret <i>et al.</i> (2003)
Garry	Gus	GFP	Cho <i>et al.</i> (2003)
GAF-30/Park	Gus	<i>bar</i> gene	Pawlowski and Somers (1998)
GAF-30/Park	Gus	NPTII	Torbert <i>et al.</i> (1998c)
GAF/Park-1	Gus	HPT	Choi <i>et al.</i> (2001)

1596–1569 including a *Hind*III site. The *Bam*HI-*Hind*III-cut PCR fragment was gel purified and cloned into pAGUS1, which had been cut with the same enzymes, resulting in plasmid p35SPAV5'. (2) The *Hind*III-*Bst*1107I (bases 1591–2787) fragment from pPAV6 was cloned into *Hind*III-*Eco*RI-cut p35SPAV5', resulting in p35S39K60K. As for the second gene construct, p35S99K, a stop codon of ORF 1 UAG, was mutated to UCAG, which brought ORFs 1 and 2 into the same ORF. If translated, this construct would produce only the 99-kDa protein and not the 39-kDa protein. This construct was generated by cloning the *Bgl*II-*Sac*I (bases 856–2453) fragment from pFLFSM4 into p35S39K60K, which had been cut with the same enzymes. The resulting genetically transformed T₂- and T₃- plants showed tolerance to BYDVs.

Water deficiency is a major environmental stress factor limiting crop production. Salt affected soils contribute to accentuating this abiotic stress by reducing the amount of usable water by the plants, thus reducing crop production in many parts of the world. Developing crop plants capable of withstanding abiotic stresses would help reduce water use (FAO, 1999). Maqbool *et al.* (2002) genetically engineered three oat (*A. sativa* L.) cultivars, “Ogle”, “Pacer”, and “Prairie”, by biolistic bombardment of multiple shoots derived from oat apices. Two plasmids were used: BY520 and Act1-D. The plasmid BY520 contained a selectable marker/herbicide resistant *Streptomyces hygroscopicus bar* (Phosphinothricin

acetyl transferase) gene and the barley *hva1* gene. The selectable marker *bar* is driven by CaMV 35S (cauliflower mosaic virus promoter) and the *nos* (*Agrobacterium* nopaline synthase gene) 3' noncoding region, while *hva1* is driven by *Act1* (5 region of the rice actin 1 gene) and terminated with the *pinII* (potato protease inhibitor II gene) 3 noncoding region. The plasmid Act1-D contained the *E. coli* β -glucuronidase (*gus*) gene flanked by the *Act1* promoter and the *nos* terminator. The resulting T₂- and T₃- generation plants showed very high levels of tolerance to osmotic stress (salt and mannitol) under *in vitro* conditions. Later, Oraby *et al.* (2005) investigated the third generation of the same transgenic oat (cv. Ogle) plants under greenhouse conditions and reported significant increase in tolerance to salt stress (200 mM NaCl) for traits such as number of days to heading, plant height, flag leaf area, root length, panicle length, number of spikelets/panicle, number of tillers/plant, number of kernels/panicle, 1000-kernel weight, and kernel yield/plant.

2.5 Challenges Facing Oat Transformation

Some of the challenges facing oat transformation are related to environmental and health concerns. The usefulness of antibiotics and herbicides as selectable markers has been demonstrated, but questions and concerns have been raised about the potential risks associated with their use in

transformation schemes. One concern often voiced is whether antibiotic resistance genes could be inadvertently transferred horizontally from plant cells to bacteria living in nature or in the guts of animals and humans. This would render the bacteria resistant to antibiotics, making these useless for treating bacterial diseases. As for the herbicide selectable markers, one of the main concerns is that the transgenic plant may cross with a weedy relative, thus making that weed resistant to herbicide. This is for example the case for oat. Also, the potential for the presence of herbicide residues in food products is a source of concern. Furthermore, even though the current scientific evidence indicates that there is no significant risk in consuming genetically modified food (Bouchie *et al.*, 2002), there remain concerns among the general public. The main concerns voiced include inadvertent introduction of toxins and allergens, change in the levels of essential nutrients, and the compromising of antibiotic therapies (Bouchie *et al.*, 2002). These concerns are sometimes violently expressed by some extremist organizations, such as the Earth Liberation Front, which destroyed 800 transgenic oat plants at the University of Minnesota in 2000. To alleviate some of these concerns, oat transformation systems based on visual selection using GFP as a selectable marker have been developed (Kaeppler *et al.*, 2000, 2001). In addition to environmental and health risk concerns, the dramatic decline in oat production, in particular in leading research countries such as the United States, constitutes a major challenge to oat biotechnology.

3. CONCLUSIONS AND FUTURE PROSPECTS

Recent advances in oat biotechnology have made it possible for breeders to investigate incorporation of molecular breeding tools in breeding programs. Ongoing efforts to incorporate molecular breeding tools into breeding programs to improve oat germplasm include transgenics, molecular markers, and genomics. Plant transformation technology has made it possible to insert foreign genes of interest into plant genomes with more precision in overcoming sexual incompatibility as

well as species barriers between organisms. Most of the genetic engineering in oat has involved the introduction of reporter and selectable marker genes into the oat genome of the modified oat genotypes. However, a few oat cultivars have been improved through the introduction of potentially agronomically useful traits, such as the *hva1* gene for tolerance to osmotic stresses and the BYDV resistance gene.

The major obstacle to oat transformation has been the recalcitrance of most oat genotypes, in particular the elite cultivars, to regeneration through somatic embryogenesis. However, modifications of the culture media have enabled somatic embryogenic regeneration of more oat genotypes, and several oat genotypes have been genetically engineered by particle bombardment (Table 3) using various explant sources, such as leaf bases, mature embryos, and shoot meristems, which have the potential to reduce genotypic effects on tissue culture response (Torbert *et al.*, 1998b). Stable *Agrobacterium* transformation has been achieved in several monocotyledonous crops, including rice, wheat, barley, and *Alstroemeria*. No published reports could be found on the susceptibility of the various oat tissue culture explants to *Agrobacterium* transformation. *Agrobacterium*-mediated gene transfer is reported to have several advantages over biolistics, including higher frequency of gene transfer (Smith and Hood, 1995; Park *et al.*, 1996) and insertion of a well-defined DNA fragment into host genome (Repellin *et al.*, 2001). For the last several decades, substantial progress has been made in oat tissue culture, with the regeneration of several genotypes using various explant types, including shoot meristems (Torbert *et al.*, 1998b; Kelley *et al.*, 2004). Regeneration via meristem is genotype independent, and genetically engineered rice plants have been obtained from shoot apices following *Agrobacterium*-mediated transformation (Park *et al.*, 1996). This technique could be extended to oat to allow for the transformation of more elite oat cultivars. Oat is a source of many compounds with antioxidant activity (Peterson, 2001); therefore, increasing the antioxidant activity of these compounds could be achieved using the improved transformation techniques. In addition, the development of oat transformation systems based on visual selection using GFP as a selectable marker can help alleviate some of the health and environmental

concerns associated with the use of antibiotics and herbicides as selectable markers.

Genetic markers consist of specific locations on a chromosome and serve as landmarks that are used for genome analysis (Staub and Serquen, 1996; Kumar, 1999). There are two basic genetic markers, morphological markers and molecular markers. Morphological markers can be monitored visually and are controlled by a single locus, but their expression can depend on environmental conditions and epistatic pleiotropic interactions (Kumar, 1999). Biochemical markers and DNA markers are molecular markers indicating polymorphisms at the protein and DNA level, respectively. DNA markers include hybridization-based markers, such as restriction fragment length polymorphisms (RFLPs) and PCR-based markers, such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). There are several types of genetic markers (see e.g., Mohan *et al.*, 1997; Kumar, 1999 for an in-depth analysis of genetic markers). DNA-based molecular markers are useful to plant breeders because they form the basis for marker-assisted selection. They can be tightly linked to the gene of interest, are unaffected by environmental conditions, and are detectable in all stages of plant growth (Mohan *et al.*, 1997). They can complement and speed up the classical breeding process. A cross between *A. byzantina* (cv. 'Kanota') and *A. sativa* (cv. "Ogle") was the basis of the first molecular linkage map of *A. sativa* (O'Donoughe *et al.*, 1995), and this map currently consists of more than 1000 molecular markers, including molecular marker genes linked to various oat traits of interest, such as genes for crown rust resistance (Penner *et al.*, 1993a; Bush and Wise, 1996, 1998), stem rust resistance (Penner *et al.*, 1993b), seed dormancy (Fennimore *et al.*, 1999), BYDV (Jin *et al.*, 1998), vernalization responses (Holland *et al.*, 1997), and a marker linked to dwarfing gene (Milach *et al.*, 1997).

Genomics consists of two main parts, structural and functional genomics. Structural genomics deals with mapping of genomes, sequencing, and determining where genes and regulatory regions are located on a chromosome while functional genomics studies the role, expression, and regulation of genes as well as their interactions. Attempts to sequence cultivated oat genomes are hampered by several obstacles. The cultivated oat

has a large genome size of about 1C DNA content of 1.4×10^{10} base pairs (Bennett and Smith, 1976), which is more than five times larger than human genome. This is partially due to the fact that cultivated oat is an allopolyploid, more specifically an allohexaploid containing A, C, and D genomes, as compared to rice which has a diploid genome of only 430 Mb per haploid cell (McCouch, 1998).

The amount of financial support allocated to research for a crop is often commensurate with the economic importance of that crop, and for that reason, oat research does not receive as much research dollar as do more agronomically important crops, such as rice, maize, wheat, or cotton. The scarcity of funds and the large size of oat genome are major impediments to oat biotechnology, but despite these problems, substantial progress has been made in the application of molecular tools for oat improvement. Furthermore, there is a great deal of synteny among the genomes of the members of the Poaceae family to which oat belongs. Therefore, information gathered in other cereal crops, such as rice whose entire genome has been sequenced (IRGSP, 2005; <http://rgp.dna.affrc.go.jp/IRGSP/whatsnew.html>), may be used as a proxy species for the study of oat genome. However, heavy reliance on such a practice should be avoided because the loss of synteny, though slow, is reasonably steady due to genome rearrangements (Paterson *et al.*, 2000; Gaut, 2002), in particular in hexaploid oat, which has numerous chromosomal rearrangements (O'Donoughe *et al.*, 1995; Wight *et al.*, 2003). Despite the challenges mentioned above, the future of oat biotechnology could still be bright because of the increasing amount of health benefits associated with oats. These benefits include cholesterol-lowering effects and the blood glucose and insulin-reducing responses of oat bran, which is so crucial in controlling diabetes, a disease that is increasing becoming a global epidemic health issue.

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Sorghum

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The center of diversity and origin of sorghum is in sub-Saharan Africa, extending from the Horn of Africa in the east to the west coast in present day Senegal, and south to the Cape of Good Hope in South Africa. Evidence of sorghum cultivation extends back to approximately 5000 years ago, with archaeological findings suggesting sorghum cultivation in Ethiopia, Somalia, and around the Red Sea at least 4500 years ago. Doggett (1965) considered that sorghum was first cultivated in the Ethiopian highlands and southern Ethiopia and Sudan over 5000 years ago. Cultivated landraces are now found throughout Africa.

Sorghum spread to India and China over 2000 years ago, and there is evidence for the expansion by Arab traders both by boat to the coastal areas of India, and overland across the Silk Route. Molecular and other evidences seem to indicate that the Chinese sorghum types (Kaoliang) were introduced via India, and seem to form a group with distinct characteristics, very probably derived via introgression from wild sorghums such as *Sorghum propinquum* (Doggett, 1988).

Until the late 1800s, sorghum cultivation was confined almost entirely to the African and Asian continents, and was only of any major importance in India and China. The movement

to the Americas was initially concurrent with the human slave trade from Africa, and when introduced into the United States from West Africa was originally known as guinea corn. By the late 1880s, a number of separate introductions had been made with names such as milo, durra, hegari, and kafir corn. Forage and sweet sorghums were also introduced during this time, most notably as Sudangrass and sorgo (sweet sorghum) from Natal. Sorghum remains an important crop in the middle west states of Kansas, Texas, and to a lesser extent Nebraska and Oklahoma. It was not until the 20th century that sorghum became accepted as an important crop in parts of Latin America, most notably in Mexico, Argentina, and Brazil. Sorghum has become an important part of the farming system in the northern grain belt of Australia, and is now Australia's most important summer cereal crop.

1.2 Botanical Description

Sorghum is a diploid species ($2n = 2x = 20$), and a member of the family Andropogonae. Among the cultivated grasses, it is most closely related to the *Saccharums* including cultivated sugarcane. The species is quite variable, varying in height from 50 cm to almost 6 m. Sorghum has widely been referred to as guinea corn, chicken corn, milo, feterita, and locally as kafir corn in South Africa, great millet in West Africa, jowar in India, and kaoliang in China.

Morphologically, sorghum usually has an erect, often single stem, but can be profusely tillering. The inflorescence is usually a compact panicle and although generally self-pollinating, outcrossing can be as high as 5–15%. At maturity, the seeds vary from spherical, oval or in some cases more flattened with a lentil-like shape, and are smaller than maize grains. They can be covered by glumes, but in most modern sorghums the glumes are very small, and seed coat varies from white, pale yellow, gray, red, brown through to very dark purple, or even black.

Sorghum was originally classified as *Holcus* by Linnaeus in 1753, until Moench distinguished the genus *Sorghum* in 1794. The genus is found worldwide throughout the monsoonal and semi-arid tropics, yet distribution does extend into temperate regions of Australia, such as the case of *Sorghum leiocladum*.

Sorghum bicolor is of sub-Saharan origin, and the species includes all the annual taxa of the section *Sorghum* as recognized by Snowden (1936). This includes not only the cultivated taxa, but also the variable wild African types and weedy derivatives with three recognizable subspecies.

S. bicolor subsp. *bicolor*, which includes the domesticated and improved types, has been divided into five basic races, namely, *bicolor*, *kafir*, *caudatum*, *durra*, and *guinea* (Harlan and de Wet, 1972). It is believed that the race *bicolor* originated in Ethiopia, *durra* and *caudatum* types are most dominant in Ethiopia and Sudan and the East African highlands, the *guineas* are from West Africa and the *kafirs* are from Southern Africa, predominantly Zambia, Malawi, Mozambique, Zimbabwe, and South Africa. Subspecies *drummondii* is a weedy species found almost in all places where grain sorghum is cultivated and is easily interfertile with the cultivated species. The most variable wild sorghum is the subspecies *verticilliflorum*, which includes the former subspecies *arundinaceum*, and is found across the African savanna and has been inadvertently introduced into Australia, India, and the Americas.

In its original, tropically adapted form, sorghum is a tall, photoperiod sensitive long-duration grass. It is best adapted to summer dominant rainfall areas, and flowering is usually induced as days become shorter, which usually coincides with the cessation of the rainy season.

1.3 Economic Importance

Sorghum (*S. bicolor* L.) is an important cereal crop throughout the semi-arid tropical and subtropical regions of the world. Approximately 300 million people in sub-Saharan Africa and India rely on sorghum grain as a major staple food. In many developing countries, sorghum is a multipurpose crop, with the stover widely used as forage for cattle, goats, and sheep. The grain is used for breads and porridges, and in parts of Africa it is an important component of the local beer making process, whereas in China, a major use is for high alcohol beverage distillation. Elsewhere, sorghum is important as an animal feed grain and a forage crop. The major production areas are found in Africa, Asia, and the Americas, with the major producers being the United States, Mexico, India, Nigeria, and China (Figure 1).

In addition to grain types, sorghum has had historical importance as a sweet sorghum, with the stems crushed for sweet syrup or eaten as a sweet stalk. Special forage sorghum types are grown throughout the tropics and subtropics, and may be fed as a standing crop, or harvested and fed fresh, as silage or dried as hay. In many parts of the developing world, the stover may be just as economically important as the grain. Recent interest in the sweet sorghums has focused on their potential has a high biomass feedstock for bioethanol production.

Sorghum is commonly considered as a “poor man’s crop.” This perception stems predominantly from the fact that sorghum is mainly cultivated in the more agriculturally marginal areas, and the areas of least economic development. Even in developed countries, such as the United States and Australia, sorghum is mostly cultivated in areas too hot and/or dry for reliable yields of maize or other higher value crops. Indeed, it is the adaptation of sorghum that makes it a preferable proposition in more marginal areas of production. Sorghum crops can yield under low water conditions and produces viable pollen under higher temperatures than alternative crops, such as maize, which tends to suffer from tissue damage and poor pollination under high temperatures. Sorghum is remarkably tolerant of high temperatures, with the rate of metabolic processes unaffected up to 38 °C (Ludlow and Wilson, 1971), and reversible high temperature

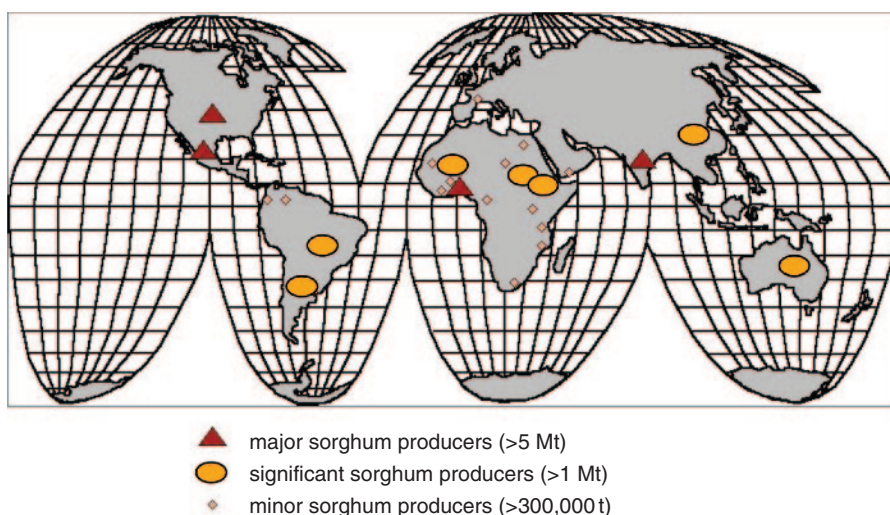


Figure 1 Important sorghum producing nations, based on annual grain production (tons) for 2005 (FAO, 2006)

damage does not occur until leaf temperature exceeds 46 °C (Ludlow, 1987).

Sorghum's lower value as a crop is a result of two factors:

1. The major food grains (wheat, rice, and maize) are preferred as food by most people, because of tradition, taste, and the variety of utilization.
2. Sorghum is less digestible to both humans and livestock than wheat, barley, maize, and oats.

In the semi-arid tropics, subsistence sorghums of Africa and South Asia generally yield 700 kg ha⁻¹ or less, with little change in yield over the last 50 years. Sorghums in these areas are commonly confined to the most marginal land with the millets, where land with higher rainfall or better soils is usually used for maize, cotton, or other higher value crops.

In the subtropical and temperate regions of the developed world, most notably in the United States, Argentina, Mexico, and Australia, average yields are substantially higher, often in excess of 3 t ha⁻¹. Better soils, rainfall distribution, and availability of hybrid seed as well as fertility and plant population management contribute to this. Average yields in the United States exceed 4 t ha⁻¹, and the major impact, as will be discussed in Section 1.4 has been of breeding programs, particularly the introduction of F₁ hybrid varieties.

1.4 Breeding Programs

As sorghum is predominantly grown in the tropical developing world, or the more marginal cropping areas of the developed nations, heat and drought remain the major abiotic stresses to production (Doggett, 1988). Much of the plant breeding focus is on maintaining production under the typical conditions of high temperature and low water availability at some stage of the summer production cycle. As with other cereals, nitrogen use efficiency is also a major issue in crop improvement and management. In tropical areas of Africa and South America, acid soils are a limitation to cereal and other grain crop cultivation, with associated problems of aluminium toxicity and phosphorus deficiency.

Worldwide, insect pests are the major biotic stress faced by sorghum producers, with an estimated grain loss well in excess of US\$1 billion annually (Nwanze *et al.*, 1995). In terms of economic damage, the most destructive pests are the lepidopterans, heliothis (*Helicoverpa armigera*), and stem borer (*Chilo partellus*, yellow stem borer) and the dipterans, midge (*Stenodiplosis sorghicola*), and shootfly (*Atherigona soccata*). Genetic sources of resistance have been combined to produce midge resistant hybrids in Australia (Henzell and Hare, 1996) and India (Sharma *et al.*, 1996), although this appears to have come at the expense of genetic variability (Jordan *et al.*, 1998).

There are no sources of true resistance to heliothis, stem borer, or shootfly.

Diseases are a constraint to both production and quality of grain and forage sorghums. Downy mildew is a major limitation to grain yield in the United States and other parts of the world (Frederikson, 1980). Other fungal leaf diseases usually do not impact greatly on grain yield, but some such as rust and leaf blight are important in reducing forage quality. Viral diseases such as Johnsongrass mosaic virus and maize dwarf mosaic virus can be particularly damaging to yield and forage quality, especially when infection occurs early in the crop cycle (Franzmann *et al.*, 1996).

Sorghum ergot, or sugary disease, is caused by two different fungi (*Claviceps sorghi*, *C. africana*). The disease has been important in Asia and Africa, and in the late 1990s, spread throughout the Americas and Australia. The disease can lead to total crop loss, and recent reports have demonstrated that the alkaloids produced by the fungus are toxic to livestock (Bailey *et al.*, 1999; Moss *et al.*, 1999). Ergot is particularly devastating to susceptible cytoplasmic male sterile parents in hybrid seed production (Meinke and Ryley, 1997; Isakeit *et al.*, 1998).

As already discussed, there has been little change in average yields in subsistence type sorghums in Africa and South Asia. Open-pollinated varieties and landraces still predominate among the cultivated sorghums in Africa. In both China and more recently India, hybrid sorghums have largely replaced the landraces or improved open-pollinated inbred varieties.

With the availability of the first commercial hybrids in the United States in 1957, sorghum productivity increased markedly. Within 4 years, most farmers had adopted the new hybrid cultivars, with the result that average yields more than doubled over this time. The original hybrids were predominantly made between East African *caudatum* and South African *kafir* types.

The United States Department of Agriculture (USDA) commenced the sorghum conversion program in the 1950s with the aim to “convert” unadapted tropical types with height of >3 m, photoperiod sensitivity and harvest indices of around 20. By the introduction of dwarfing and photoperiod insensitivity genes, the sorghums were converted to more temperate adaptation.

This involved shuttle breeding between the USDA stations in Puerto Rico and Texas, involving about five backcrosses to transfer at least eight major genes from a four-dwarf line BTx406 (Rosenow *et al.*, 1971). This particular program was instrumental in bringing into adapted breeding germplasm many traits for pest and disease resistance, as well as resistance to abiotic stresses, such as drought and soil toxicity.

2. SORGHUM TRANSFORMATION

2.1 Donor Genes

A successful transformation system typically requires the use of reporter genes, selectable marker genes (see Section 2.4), and appropriate promoters to regulate transgene expression. Promoters are regulatory sequences located upstream of a gene and determine patterns and levels of expression. Constitutive promoters direct expression in almost all tissues and function independently of developmental or environmental signals. Besides constitutive promoters, tissue-specific and inducible promoters can also be used. Several promoters have been evaluated in sorghum including those from the rice actin gene *Act1* (McElroy *et al.*, 1991), the maize *ubiquitin1* (*Ubi1*; Christensen *et al.*, 1992), and alcohol dehydrogenase-1 (*adh1*) genes, and the chimeric maize *Emu* promoter (Last *et al.*, 1991). These promoters drive strong, constitutive expression and result in significantly higher expression levels in monocotyledonous species compared with the 35S promoter from cauliflower mosaic virus (CaMV; Schledzewski and Mendel, 1994). Several reports have shown that the *Ubi1* promoter has the highest activity in sorghum compared with other promoters tested to date (Able *et al.*, 2001; Hill-Ambrose and Weeks, 2001; Jeoung *et al.*, 2002; Tadesse *et al.*, 2003). Able *et al.* (2001) ranked *Ubi1* promoter activity as highest in sorghum, followed by *Act1* then CaMV 35S. A similar trend of *Ubi1* > *Act1D* > *adh1* > CaMV 35S was also reported by Tadesse *et al.* (2003). Hill-Ambrose and Weeks (2001) examined transient β -glucuronidase (GUS) expression in embryogenic tissue and ranked the promoters as CaMV 35S \geq *Ubi1* \geq *adh1* \geq *Act1* \geq control. However, GUS activity was very low compared with expression in

similarly derived wheat callus. It was suggested that ineffective promoter strength could be one reason for low transformation rates in sorghum, and that future work should focus on the development of more effective promoters.

Several reporter genes have been used in transformation systems to monitor the efficiency of DNA transfer. These genes are also useful for monitoring the expression pattern of transformed genes in cells, tissues, or whole plants. In particular, they are useful for determining differential expression patterns of promoters in different tissues and at different developmental stages of growth. In addition, transient expression of reporter genes can be evaluated within 48h of DNA delivery. This rapid analysis can be used to assess the activity of genes or promoters without requiring stable integration of the DNA into the host genome. Ideally, reporter genes should have no detrimental effects on the plant's metabolism, there should be negligible background activity caused by the plant cells, and any assay should be sensitive and easy to carry out. The most commonly used reporter gene has been the β -glucuronidase gene (*GUS*; Jefferson *et al.*, 1987), encoded by the *uidA* locus of *Escherichia coli*. This gene meets most of the above criteria, but it does involve a destructive assay. Several groups investigating the sorghum transformation system have used this marker gene (Casas *et al.*, 1993; Emani *et al.*, 2002; Tadesse *et al.*, 2003; Girijashankar *et al.*, 2005; Howe *et al.*, 2006). Nondestructive marker systems have also been developed using the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994). The *GFP* gene has also been used as a reporter gene for visual selection of transgenic sorghum plants (Gao *et al.*, 2005a) and by Gao *et al.* (2005b) in a dual-marker system with mannose selection (see Section 2.4). Both the *GFP* and *GUS* genes were tested for suitability and effectiveness for the early detection of transgene expression when optimizing an *Agrobacterium*-mediated sorghum transformation with sorghum lines C401 and Tx430 (Jeoung *et al.*, 2002). *GFP* was found to be an effective reporter gene when optimizing conditions for transient expression. Of the two sorghum lines tested, Tx430 was identified as yielding the most instances of transient expression that could be correlated with stably transformed callus lobes.

2.2 Sorghum Transformation

Biolistic and *Agrobacterium*-mediated transformation methods currently underpin the genetic engineering of sorghum. While *Agrobacterium*-mediated transformation has been used extensively with dicotyledonous plants since first reported by Zambryski *et al.* (1983), monocotyledonous plants such as sorghum were considered outside the host range (DeCleene and DeLey, 1976). Significant effort has since been directed toward the development of *Agrobacterium*-mediated transformation systems for the economically important cereals, and there are an increasing number of systems being reported for sorghum. The proposed mechanisms by which transfer DNA (T-DNA) transfer and integration occur have been extensively reviewed elsewhere (Gelvin, 2000; Tzfira and Citovsky, 2002; Valentine, 2003; Tzfira *et al.*, 2004), as have many of the factors known to influence the success of *Agrobacterium*-mediated transformation of cereals (Cheng *et al.*, 2004).

The earliest sorghum transformation studies produced transgenic calli containing the neomycin phosphotransferase (*neo*) and β -glucuronidase (*uidA*) genes following electroporation of protoplast cultures (Battraw and Hall, 1991). However, the calli were nonmorphogenic and unable to be regenerated into plants. Regeneration from embryogenic suspension culture protoplasts had been reported by Wei and Xu (1990), but significant albinism, sterility, and morphological mutations were encountered. Stable transformation of cell cultures with the hygromycin phosphotransferase (*hpt*), *neo*, and *uidA* genes via microprojectile bombardment was also reported by Hagio *et al.* (1991). In this study, only eight cell colonies were recovered on hygromycin B and three were selected on kanamycin.

The first transgenic sorghum plants were created using a Biolistics PDS 1000/He system to bombard immature embryos (Casas *et al.*, 1993). While eight sorghum genotypes were used, P898012 was the only cultivar for which transgenic plants were regenerated (from 2 out of 600 bombarded embryos). Modified methods were also used by this group to produce sorghum carrying a range of reporter genes (Kononowicz *et al.*, 1995). A particle-inflow-gun was used by Rathus *et al.* (1996) to transform embryogenic calli derived from immature embryos. This allowed

several shoots to be selected on Basta[®]-containing medium, although only a single plant survived transfer to soil. These reports were followed by the production of sorghum carrying a rice chitinase gene and the *bar* gene that were introduced into immature zygotic embryos using a biolistic PDS/He system (Zhu *et al.*, 1998). A total of 1100 calli were bombarded to obtain just six fertile transgenic plants. Subsequent work with progeny of these plants has demonstrated that those constitutively expressing the chitinase gene have improved resistance to stalk rot (Krishnaveni *et al.*, 2001). Partial tolerance to insect damage has also been reported after transforming sorghum with a synthetic *cry1Ac* from *Bacillus thuringiensis* under the control of a wound-inducible promoter (Girijashankar *et al.*, 2005).

The optimization of microprojectile bombardment parameters has been reported in the years following these first reports of transgenic sorghum. The pressure, distance to the target tissue and the aperture of the helium inlet valve were optimized for a particle-inflow-gun, and used to produce fertile, transgenic sorghum plants carrying the *bar* gene by Able *et al.* (2001). Similar parameters were also optimized by Syamala and Devi (2004) in the development of their own transformation system. Additional optimization of transformation conditions was also conducted by Tadesse *et al.* (2003). Physical parameters such as acceleration pressure, target distance, gap width, and macroprojectile travel distance were tested, with gold and tungsten, using a PDS-1000/He biolistic device. The optimized bombardment conditions were applied to produce geneticin-resistant sorghum plants from immature embryos and shoot tips. This transformation system was used to generate transgenic sorghum plants with an altered biochemical pathway for increased lysine production (Dewaele *et al.*, 2002). The same type of device was again used to introduce the *bar* and *GUS* transgenes into immature sorghum embryos (Emani *et al.*, 2002). However, the *GUS* transgene was found to have been silenced. Subsequent examination of the transgenes indicated that methylation-based silencing was involved, and it was suggested that this mechanism could be responsible for many of the cases of transgene inactivation that have been reported.

Initial work with *Agrobacterium*-mediated transformation systems allowed Godwin and

Chikwamba (1994) to generate several chimeric plants, in which histochemical GUS assays provided evidence of transgene expression but Southern hybridization failed to confirm transgenicity. The first report of transgenic sorghum displaying stable Mendelian inheritance of the transgene introduced via *Agrobacterium* was from Zhao *et al.* (2000). This involved the introduction of the *bar* gene for herbicide resistance into immature embryos of a public inbred (P898012) and a commercial line (PHI391) of sorghum. Various transformation conditions and media were tested to produce 131 stable transformation events from 6175 inoculated embryos, an overall transformation frequency of 2.1%. Reporter genes were also transferred to sorghum lines Tx430, C401 (a Chinese inbred line), and Wheatland via *Agrobacterium* by Jeoung *et al.* (2002). Subsequent reports began to identify factors that had the greatest influence on the success of transformation. Carvalho *et al.* (2004) showed these to include the sensitivity of immature embryos to *Agrobacterium* infection, the growing conditions of the donor plant, the explant type, and the composition of the co-cultivation medium. A major problem encountered in the establishment of their protocols was a necrotic response displayed by explants after co-cultivation. Attempts to minimize the hypersensitivity reactions with antioxidants that have successfully been used in other species were unsuccessful. While a transformation efficiency of up to 3.5% was achieved, a high degree of variation in transient GUS expression and immature embryo survival after co-cultivation was found in many experiments, even under similar experimental conditions. This efficiency was similar to the 3.3% achieved for the inbred line C401 by Gao *et al.* (2005b). A rapid and reproducible *Agrobacterium*-mediated transformation system has recently been reported by Howe *et al.* (2006). A combination of changes to previously reported systems was used, including a hypervirulent strain of *Agrobacterium*, the *nptII* marker gene for selection on geneticin or paromomycin, and a medium salt composition to alleviate phenolic secretion and the associated problems in tissue culture. Their protocols resulted in transformation frequencies up to 4.5%, the highest reported to date.

The choice of genetic transformation method relies on both practical and biological requirements. Particle bombardment can offer

a number of advantages over *Agrobacterium*-mediated transformation as it could, in theory, be used with any plant species. However, the insertion and integration of DNA into the host genome in a biolistic system is essentially a random process. Transgenic plants produced from bombarded explants can have very high transgene copy numbers and complex integration patterns (Register *et al.*, 1994). Multiple copy inserts are undesirable in most transgenic plants, as clusters of the same gene can increase the risk of transgene silencing and DNA rearrangement (Kumpatla and Hall, 1998; Pawlowski and Somers, 1998). In contrast, *Agrobacterium*-mediated transformation often results in the integration of a single copy of the transgene. In addition, the transferred DNA can usually be better defined, as bombardment of plasmid constructs often leads to integration of vector fragments in addition to the transgene. However, in some comparisons of *Agrobacterium* and biolistic methods, no differences have been found in the occurrence of transgene rearrangements or undesirable integration patterns (Kohli *et al.*, 1999).

2.3 Tissue Culture

Tissue culture is used to describe a variety of techniques that exploit the totipotency and plasticity of plant cells, enabling the regeneration of whole plants from small tissue pieces or single cells. These methods include micropropagation and embryo rescue, and may involve the manipulation of callus, protoplasts, cell suspensions, or anther and microspore cultures. Such procedures are conducted *in vitro*, enabling the environment and growth medium to be manipulated to ensure a high frequency of regeneration. The ability to generate fertile plants from single cells has made this technology an integral step in the development of transgenic plants.

Tissue culture methodologies have been developed for sorghum over a number of decades. Early reports described systems using embryogenic callus (Masteller and Holden, 1970), immature embryo culture (Ma *et al.*, 1987), shoot portions of mature embryos (Cai *et al.*, 1987), and somatic embryos developed from embryogenic callus (MacKinnon *et al.*, 1987). Regeneration from sorghum protoplasts (Wei and Xu, 1990) has

been reported and immature inflorescences have been shown to have embryogenic potential (Cai and Butler, 1990). Callus initiation and growth has even been obtained from transverse thin cell layers of roots or epicotyls from sorghum seedlings (Gendy *et al.*, 1996). In addition, shoot apices have been used (Bhaskaran and Smith, 1989), as have nodes (Masteller and Holden, 1970) in organogenic systems. Plant regeneration has also been reported from immature and mature sorghum embryos (Gamborg *et al.*, 1977; MacKinnon *et al.*, 1986; Bhaskaran *et al.*, 1987; Ma *et al.*, 1987; Bhaskaran and Smith, 1988; Oldach *et al.*, 2001). However, many genotypes do not readily produce regenerable calli, as embryo culture appears to be media and genotype dependent. Genetic differences were also found to affect the regenerative potential of callus derived from immature inflorescences (Cai and Butler, 1990).

An ideal tissue culture system should not induce unwanted changes into the host genome, however, dedifferentiation of plant cells that occur during a callus phase is well known to induce such changes, i.e., somaclonal variation (Larkin and Scowcroft, 1981). To avoid this, tissue culture methodologies that involve little or no callus stage have been developed using shoot apices. These methods have been reported for sorghum (Zhong *et al.*, 1998) using similar procedures as those employed for maize, barley, and oats (Zhong *et al.*, 1992a, b; Zhang *et al.*, 1996, 1999). In these systems, multiple shoots are produced through differentiation of both axillary and adventitious buds when apical dominance is removed. An additional advantage of this system is that it is less genotype specific than many of the callus systems. A range of sorghum genotypes have been trialed in this culture system and all were shown to regenerate at a relatively high frequency (Zhong *et al.*, 1998; Williams *et al.*, 2004). An additional advantage is the ability to use mature seed-derived meristems, which removes any seasonal constraints to the production of immature embryos. Despite these advances, sorghum has remained one of the more difficult cereals to manipulate for tissue culture and transformation.

Improvements and variations to the sorghum tissue culture system have been reported more recently, including revised culture media for regenerating adventitious shoots from immature embryos, thereby enabling regeneration of shoots

from up to 100% of embryos (Hagio, 2002). Manipulation of media composition and supplementation with copper and aluminium has also been shown to improve callus induction and plant regeneration (Anas and Yoshida, 2002; Gupta *et al.*, 2002; Nirwan and Kothari, 2003). There have also been further reports of the regeneration of plants from shoot apices (Harshavardhan *et al.*, 2002; Syamala and Devi, 2004). Efficient protocols that can be used with any sorghum genotype are yet to be developed and this may have delayed the utilization of transformation technologies in sorghum breeding programs, however, Sato *et al.* (2004) have recently identified an elite sorghum genotype that exhibits efficient induction of embryogenic tissue and plant regeneration.

2.4 Selection Systems

Genetic transformation systems rely on an ability to differentiate transformed and nontransformed cells, as only a small proportion of cells will have the transgenic DNA integrated into the nucleus in a stable manner. To select the desired cells, a selectable marker gene is often used, such as one that confers resistance to an antibiotic or herbicide. In recent years, there has been a move away from the use of herbicide and antibiotic resistance genes, due to the public concern over perceived safety issues and the impact that such agents could have on the environment and human health. These perceptions include potential allergenicity and the transfer of antibiotic resistance to human pathogens. In addition, sorghum is able to outcross with Johnsongrass (*Sorghum halepense*), thus presenting a route by which bialaphos herbicide resistance could be passed to this weedy species (Arriola and Ellstrand, 1996, 1997). For these and other reasons, alternative selectable marker gene systems are being developed.

Sorghum transformation systems have commonly used the phosphinothricin acetyltransferase gene (De Block *et al.*, 1987; Thompson *et al.*, 1987) as a selectable marker. This gene confers resistance to the herbicidal compound bialaphos. Kanamycin and hygromycin have also been used by Hagio *et al.* (1991) as selectable markers with sorghum suspension cell cultures. However, it was found that sorghum cells had a high level of basal tolerance to kanamycin. The

bialaphos resistance gene (*bar*) was derived from *Streptomyces hygroscopicus*, which produces the tripeptide antibiotic bialaphos. The two L-alanine residues of bialaphos are removed from the compound by intracellular peptidases, leaving the phosphinothricin compound that is a strong inhibitor of glutamine synthetase (De Block *et al.*, 1987; Thompson *et al.*, 1987). This enzyme is essential for assimilating ammonia in plants and as such, plays a key role in nitrogen regulation. The inhibition of glutamine synthetase results in the accumulation of ammonia in the plant cell and ultimately cell death.

A more recent report has presented a selection system that uses galactose as the selective agent and a UDP-glucose:galactose-1-phosphate uridyltransferase gene as the selective gene (Joersbo *et al.*, 2003). This system avoids the use of herbicide and antibiotic resistance genes, and is based on conventional selection principles. Galactose is toxic to a broad range of plant species, and the system presented by Joersbo *et al.* (2003) sufficiently reduces the toxicity of galactose to allow transformed shoots to survive on selective media, while killing any nontransformed shoots.

Alternative selection systems have now been developed in which the growth and vigor of transformed cells is favored over nontransformed cells that are outgrown or starved, but not killed as they would be in lethal selection systems (Joersbo and Okkels, 1996). These systems include the use of phosphomannose isomerase (Joersbo *et al.*, 1998), xylose isomerase (Haldrup *et al.*, 1998b), and the novel use of GUS (Joersbo and Okkels, 1996). In addition to addressing some of the environmental and safety issues, positive selection systems offer a number of advantages over the use of herbicide and antibiotic selection systems. Primarily, cells transformed with the marker gene are able to utilize a compound that can be included into the tissue culture medium, resulting in enhanced growth of the transformed cells. However, nontransformed cells remain unaffected and less able to influence the growth of any transformed cells. This influence may be present in lethal selection systems, where dead or dying cells may release toxins or other substances detrimental to the growth of transformed cells, such as phenolic compounds. The dead cells may also form a partial or complete barrier between the culture medium and any transformed cells, thus inhibiting

or slowing the uptake of nutrients and hormones by the cells.

One positive selection system has been developed using phosphomannose isomerase (PMI; EC 5.3.1.8), which catalyses the conversion of mannose-6-phosphate to fructose-6-phosphate. In the PMI selection system, plants are transformed with the PMI gene (*manA*) from *E. coli* (Miles and Guest, 1984). They are then grown on media containing mannose, a hexose sugar that is readily absorbed and converted to mannose-6-phosphate by hexokinase, but which is not metabolized further by untransformed cells. In transformed cells, the PMI enzyme can convert it to fructose-6-phosphate, which is a metabolizable sugar. The use of the *PMI* gene to select transformed cells on media containing mannose has been reported for sorghum (Gao *et al.*, 2005b) and several other cereals, including maize (Evans *et al.*, 1996; Negrotto *et al.*, 2000; Wang *et al.*, 2000; Wright *et al.*, 2001), wheat (Reed *et al.*, 1999; Wright *et al.*, 2001), and rice (He *et al.*, 2004). Gao *et al.* (2005b) reported the successful dual-marker transformation of sorghum with *PMI* and *GFP* using an *Agrobacterium*-mediated transformation system. While the reported transformation frequencies were between 2.88% and 3.30%, further optimization of this system may offer the potential for reaching the high frequencies similar to those reported for maize (30%, Negrotto *et al.*, 2000).

2.5 Regeneration Systems

Cells that survive the transformation and selection processes are regenerated through the tissue culture system into plantlets that can be transferred to soil. Once in a glasshouse and growing vigorously, the presence of transgenes can be confirmed using a number of rigorous molecular techniques. While a number of studies have been conducted in sorghum to optimize the efficiency of genetic transformation (Able *et al.*, 2001; Tadesse *et al.*, 2003), little attention has been given to improving the survival rate of sorghum plants when transferred from *in vitro* culture to the open environment. Low efficiency during this stage of the transformation system can result in substantial losses of putatively transgenic plants.

It is well established that plantlets cultivated *in vitro* suffer from a range of morphological abnor-

malities. During the acclimatization period, several changes in leaf structure, water relations, and photosynthesis take place and these modifications can often be accelerated using antitranspirants and elevated CO₂ concentrations (reviewed by Pospíšilová *et al.*, 1999). A wide variety of methods have been used to acclimatize sorghum plants from all types of tissue culture before transfer to the glasshouse (Table 1). Many differences exist among the soil substrates, environmental conditions, and time frames used in each phase of the process. There is also great variation in the clarity with which these conditions are described. The efficiency of the systems has not always been recorded, however, Smith *et al.* (1983) reported that many of the rooted plants did not survive transfer, while Zhu *et al.* (1998) reported that only 60% of plants survived transplantation to soil.

We have previously reported on the generation of organogenic tissue cultures derived from the meristem of germinated sorghum seeds (Williams *et al.*, 2004). After observing considerable variation in the success of our hardening-off methods and those reported in the literature, we examined simple ways to improve the frequency of plantlet survival. Once plantlets had developed roots to between 15 and 30 mm in length, they were removed from the *in vitro* environment and at least 15 plantlets were transplanted into a variety of sterile substrates:

- A. seed raising mix in 50 mm pots with a clear plastic bag cover that was progressively opened and ultimately removed after 2 weeks;
- B. potting mix based on UC mix B;
- C. grade 2 vermiculite;
- D. 2:1 grade 3 vermiculite:sand;
- E. 4:1 perlite:grade 2 vermiculite, or;
- F. a filter-paper bridge in 10 mL of half-strength liquid culture medium in a glass test tube for 2 weeks before transfer into grade 2 vermiculite.

Liquid fertilizer (1 g l⁻¹ Aquasol™) was used to moisten the solid media once a week and sterile water was used as required between these times. One to two weeks after transplant, the lids of the culture vessels were progressively opened over the course of several days. Plants were retained in the tissue culture room until they were growing vigorously and their roots had reached 1–2 mm in diameter and new leaf

Table 1 Hardening-off conditions reported for sorghum plants derived from diverse tissue types

Tissue type (source)	Photo-period (h)	Light intensity ($\mu\text{Mm}^{-2} \text{s}^{-1}$)	Substrate	References
Callus (immature embryos)	20	27	Vermiculite:sand: peatmoss mixture	Gamborg <i>et al.</i> (1977)
	16	81	Potting compost	Dunstan <i>et al.</i> (1979)
	16	13.5–27	Soil	Casas <i>et al.</i> (1993)
	–	–	Soil	Zhu <i>et al.</i> (1998)
	16	30	Soil mix (nonsterile)	Able <i>et al.</i> (2001)
	16	70	Soil	Oldach <i>et al.</i> (2001)
	16	80	–	Hagio (2002)
Embryos and callus (immature embryos)	16	270	Universal mix	Zhao <i>et al.</i> (2000)
	–	50 or 120	Peatmoss mixture	Carvalho <i>et al.</i> (2004)
Callus (immature embryos and immature inflorescences)	16	13.5–27	–	Kononowicz <i>et al.</i> (1995)
Inflorescence (immature, unemerged)	16	4.7 or 40.5	Soil	Brettell <i>et al.</i> (1980)
Shoot tips, callus, and embryos (immature and mature)	16	43	1:1 soil:vermiculite (sterile)	Tadesse <i>et al.</i> (2003)
Callus (shoots of germinating seed)	16	24	Vermiculite	Smith <i>et al.</i> (1983)
	16	27	Vermiculite (sterile)	Bhaskaran <i>et al.</i> (1983)
Callus (mature embryos)	–	–	Soil-less potting mixture	Duncan <i>et al.</i> (1995)
	16	25	1:1 soil:compost	Nirwan and Kothari (2003)
Shoot apical meristems (excised 24 h after germination)	18	–	3:1 field soil and vermiculite (sterile)	Nahdi and de Wet (1995)
Shoot apices (excised 7 day after germination), somatic embryos (shoot apices)	16	–	1:1 soil:coconut peat (sterile)	Syamala and Devi (2003)
Shoot apices (excised 7 day after germination)	24	30	1:1 peat:perlite	Zhong <i>et al.</i> (1998)
	16	–	Vermiculite mixture (sterile)	Girijashankar <i>et al.</i> (2005)

blades had a width of more than 10 mm, approximately 4–5 weeks after transplant. The light intensity in the culture room was increased from $25 \pm 5 \mu\text{Mm}^{-2} \text{s}^{-1}$ during organogenic culture production to $80 \pm 10 \mu\text{Mm}^{-2} \text{s}^{-1}$ after rooting. Plants were transferred to the glasshouse for acclimatization for 1 week in their same vessel before being transplanted into 100 mm pots of UC mix. The survival of plants was assessed after 6 weeks in the glasshouse (Table 2). Analysis of the results, conducted with the λ^2 -test of independence (Minitab, R14; with a significance level corresponding to p -value < 0.05), demonstrated that there was a significant effect of the soil substrate on survival. The seed raising mix, vermiculite:sand and perlite:vermiculite substrates

Table 2 Survival of sorghum plants after transfer from tissue culture^(a) into various substrates

Substrate	Survival rate ^(a) (%)
(A) Seed raising mix	19
(B) Potting mix	87
(C) Vermiculite	76
(D) Vermiculite:sand	0
(E) Perlite:vermiculite	100
(F) Filter-paper bridge in liquid media	33

^(a)Plant survival was assessed 6 weeks after transfer to the glasshouse

were found to contribute the most to these differences (Table 2).

The use of these substrates, combined with a higher light intensity after rooting, produced

plants that were more robust and vigorously growing before transfer to the glasshouse, where they quickly adapted to the changed environment. The transfer of plants from tissue culture to soil media is an integral step in any plant transformation process. High survival rates during this critical transition to *ex vitro* conditions are particularly important if transformation frequencies are low. Significant improvements can be made to the survival rate of regenerated plants and it is hoped that this will contribute to improved transformation efficiency for sorghum.

3. THE FUTURE OF SORGHUM TRANSGENICS

3.1 Potential Products

There are currently three major targets for sorghum improvement via genetic modification, these being the improvement of nutritional quality, pest resistance, and disease resistance. Sorghum pests will continue to be targeted with a transgenic approach, such as that reported by Girijashankar *et al.* (2005) who used a synthetic *cryIAc* gene against stem borer. Sorghum yield and quality is also affected by many diseases (e.g., more than 50 have been identified in Australia; Ryley *et al.*, 2002), which can cause serious economic losses and endanger food security for a significant proportion of people in tropical and subtropical areas of the world who are reliant on sorghum. Many of the diseases of global importance are caused by fungi, including grain molds, stalk rots, ergot, smuts, anthracnose, and downy mildew (de Milliano, 2002). In Australia, ergot (caused by *C. africana*) is a major disease, which has a significant impact on all sectors of the industry, particularly hybrid seed production (Ryley *et al.*, 2002). Sorghum rust (caused by *Puccinia purpurea*) and leaf blight (caused by *Exserohilum turcicum*) are serious leaf diseases that result in direct yield losses. Sorghum rust is a pathogen found in most sorghum production areas of the world. Recurrent periods of wet and cool weather during the early vegetative growth stage can lead to a high incidence of the disease. The resulting high infection levels during flowering and early grain fill can have a serious impact on yield, and due to the decreased

photosynthetic leaf area, plants can be predisposed to lodging (Ryley *et al.*, 2002). Globally, the disease itself has little effect on sorghum yield, but infection by the fungus predisposes the plant to other more significant diseases such as grain molds, charcoal, and *Fusarium* stalk rots. Some reports have estimated grain yield losses up to 65% under conditions favorable for disease development (Bandyopadhyay, 1986). Host disease resistance genes typically provide defense against rust infection and a rust resistance gene has been identified in sorghum (Ramakrishna *et al.*, 2002; McIntyre *et al.*, 2004), which is a homolog of the extensively studied *Rp1-D* rust resistance gene from maize. The use of maize resistance genes in other cereal species has been investigated by Ayliffe *et al.* (2004) who generated transgenic wheat and barley with the *Rp1-D* gene. The ability to use resistance genes, particularly highly characterized ones such as these from maize, to control a range of pathogens in related species would have obvious benefits for increasing the production potential of these crops. The potential of the maize *Rp1-D* gene has been investigated as a source of novel resistance against rust pathogens in sorghum (Williams *et al.*, 2002).

Johnsongrass mosaic virus (JGMV) also occurs throughout the sorghum growing regions of Australia, particularly where the alternate host Johnsongrass (*S. halepense*) is common. JGMV is a single-stranded positive sense RNA virus of the genus *Potyvirus* (family Potyviridae), which infects sorghum, maize, and other species of Poaceae (Shukla and Teakle, 1989; Gough and Shukla, 1993). It causes a range of symptoms including mosaic, temperature-independent necrosis, stunting, delayed flowering, panicle necrosis, small seed, and reduced grain quality (Persley *et al.*, 1986). Resistance to JGMV has been a target of the sorghum breeding programs in Australia, and several lines have been developed that display mild symptoms and low levels of infection (Ryley *et al.*, 2002). However, as JGMV has previously overcome host resistance, preliminary work for the development of transgenic resistance has been undertaken. This included an assessment of the sequence diversity of the coat protein coding region of the virus (Laidlaw *et al.*, 2004), which suggested that a transgenic approach could be used to provide effective resistance against this pathogen.

The nutritional quality of sorghum, specifically the protein quality, and vitamin and mineral content, has become the focus of international research. Significant funding has been provided by the Bill and Melinda Gates Foundation to several institutions involved in the African Biofortified Sorghum (ABS) project. Their aim is to generate transgenic sorghum with increased levels of lysine, threonine and tryptophan, vitamins A and E, iron, and zinc (<http://www.supersorghum.org>). Various strategies have been developed to manipulate seed proteins, including modification of the protein sequence, metabolic engineering of free amino acids, generation of synthetic proteins, and expression of heterologous proteins. For example, amino acid synthesis pathways can be altered to preferentially accumulate a target amino acid (Sun and Liu, 2004). Much of the research reported to date has used cereal storage protein genes to improve the amino acid balance in noncereal crops. For example, maize γ -zein gene has been expressed in soybean to significantly increase cysteine and methionine content (Li *et al.*, 2005). As the maize and sorghum storage proteins (prolamins) are related, opportunities may exist to transfer some of this work to sorghum improvement. There is also potential to overexpress prolamins with high methionine and low cysteine content, such as the recently characterized δ -kafirin (Izquierdo and Godwin, 2005) to improve the methionine content of cereal and pulse crop seed storage proteins. Lysine is another essential amino acid for humans and monogastric livestock and it is the most limiting amino acid in cereal seeds. A number of approaches have been investigated to improve the lysine content in maize (Mazur *et al.*, 1999; Ferreira *et al.*, 2005). Since the isolation of the *opaque-2* mutant (*o2*; Mertz *et al.*, 1964) and the realization that the increase in lysine-rich proteins in the endosperm resulted from a decreased synthesis of 22-kD α -zeins, the trait has also been associated with low seed density and soft grain texture, leading to brittleness and increased susceptibility to insect damage. Maize germplasm that incorporates the *o2* protein quality without these detrimental problems has been developed (Quality Protein Maize; reviewed by Gibbon and Larkins, 2005). RNA interference (RNAi) technology has also been used to reduce the 22-kDa α -zeins in maize (Segal *et al.*, 2003). Instead of relying on the recessive *o2* mutation that only affects a subset of the α -zeins, an RNAi

construct was developed to produce a dominant mutation to suppress all α -zeins, without affecting the accumulation of other storage proteins. In addition, it led to increased lysine content in the grain, while reducing the levels of leucine, alanine, and glutamine. Huang *et al.* (2004) also used sense and antisense genes to reduce the expression of 19-kDa α -zeins, which led to increases in the level of lysine, tryptophan, and methionine in transgenic hybrids. The isolation and partial characterization of enzymes involved in lysine catabolism in sorghum seeds by Fornazier *et al.* (2005) is another important step toward a better understanding of lysine metabolism. The ability to alter the amino acid balance using these types of technology will be useful for manipulating the amino acid balance and further characterization of the storage protein genes in sorghum. Additional strategies for manipulating protein, mineral, and vitamin content in sorghum have been reviewed by O'Kennedy *et al.* (2006).

3.2 Improved Transformation Technologies

Significant efforts have been made to understand the mechanisms involved in *Agrobacterium*-mediated transformation in an effort to overcome some of the barriers that were preventing its routine use in the generation of transgenic cereals. For example, improvements in the efficiency of DNA transfer to rice were observed in the presence of a mutation in a virulence gene regulator that allowed constitutive expression of the *vir* genes (Ke *et al.*, 2001). Analysis of T-DNA transfer events in barley and wheat has also revealed that they occur preferentially in distinct anatomical regions of the cereal embryo that have a low capacity for regenerative growth (Ke *et al.*, 2002). Manipulation of *Agrobacterium* inoculum density and/or media composition has also been shown to alter the number and distribution of T-DNA transfer events, with the potential for a corresponding improvement of the transformation frequency. Efforts have also been made to understand and manipulate the host plant, rather than attempting to improve transformation efficiency by altering *Agrobacterium* or the inoculation conditions. For example, a population of *Brassica oleracea* segregating for transformation efficiency has been subjected to quantitative trait analysis, leading to the identification of genomic regions

that account for 26% of the observed variation (Cogan *et al.*, 2002). Several plant genes involved in *Agrobacterium*-mediated transformation have also been identified, and there is evidence that their overexpression in recalcitrant species may lead to marked improvements in transformation ability (recently reviewed by Gelvin, 2003).

The transformation of plants with large DNA fragments is also developing as a useful technology for genetic analysis and metabolic engineering in plants. One significant report by Song *et al.* (2004) demonstrated the introduction of a 90 kb bacterial artificial chromosome (BAC) clone from sorghum into maize via particle bombardment. The BAC clone consisted of ten tandem copies of the 22-kDa α -kafirin gene and neighboring genomic sequences. It was demonstrated that the entire kafirin gene cluster could be integrated into the maize genome as a single fragment and that tissue-specific expression and processing of the kafirins occurred in the maize endosperm. The ability to perform such transformations will enhance the analysis of promoters and transcription factors in their native chromosomal framework and provide another tool for functional genomics studies in the cereals. It should also be useful for transferring multigene transformation constructs for modifying complex traits and the manipulation of entire metabolic pathways. The release of the first draft of the sorghum genome sequence in 2007 has provided an important resource for the sorghum research community and anyone interested in cereal genetics (<http://www.phytozome.net/sorghum>).

3.3 Addressing Risks

It is essential that the potential impact of transgenic sorghum is assessed before release into the environment, whether in field trials or into the crop production system. Of particular concern is the ability of sorghum to hybridize with its weedy relative Johnsongrass (Arriola and Ellstrand, 1996, 1997). This provides an opportunity for transgenes to “escape” from sorghum into Johnsongrass. It is therefore important that the types of transgenes used will not confer a genetic advantage to related weeds if and when outcrossing occurs. The use of herbicide-resistant selectable markers is of particular concern, as these genes may have the potential to increase the weediness of

sorghum relatives and sorghum itself. A recent risk assessment of transgenic sorghum in Africa found that there is strong evidence that introgression of transgenes into sorghum crops and wild relatives will occur (Schmidt and Bothma, 2006). In effect, the question to pose may be not whether transgene “escape” will occur, but what the likely impact of the transgene will be. This is more effectively viewed on a gene-specific basis.

In preparation for the release of transgenic sorghum outside of the controlled laboratory and glasshouse environment, transformation systems with alternative selection strategies have been developed, such as the use of mannose in the PMI system (see Section 2.4). It is considered unlikely that the transfer of the *manA* gene to Johnsongrass would confer any adventitious traits to this weedy relative. In addition, a recent safety assessment of PMI has found no adverse effects with respect to mammalian toxicity and allergenicity, or any unintended effects on the agronomic or nutritional qualities of maize (Privalle *et al.*, 2000). There is also the possibility for other systems to be used in sorghum transformation system, such as those based on xylose isomerase and benzyladenine N-3-glucuronide. The *xylA* gene from *Thermoanaerobacterium thermosulfurogenes* encodes for xylose isomerase (EC 5.3.1.5), which catalyses the isomerization of D-xylose to D-xylulose, and has been used to successfully transform potato, tobacco, and tomato (Haldrup *et al.*, 1998b). It was found that this selection system gave up to eightfold higher transformation frequencies compared to kanamycin-based selection in potato. At the time of the publication, the selection system was not yet optimized for use with tobacco and tomato cultures. However, the frequency of transformation in tomato was increased by 20%, but was reduced by 30% in tobacco compared to a kanamycin system. In addition, Haldrup *et al.* (1998a) found that potato had endogenous xylose isomerase activity, which they used to support the harmlessness of this protein. The enzyme is also used in the food industry to produce high-fructose syrup from glucose (Quax, 1993). While, this system was not fully optimized for any of the plant species examined, it appeared to present significant advantages for use as a selectable marker system compared with existing systems. An additional selection system, based on benzyladenine N-3-glucuronide, relies on the requirement for cytokinin to obtain

favorable shoot regeneration from explants. In this system, the cytokinin is added as an inactive glucuronide derivative (benzyladenine N-3-glucuronide), which is converted to an active cytokinin (benzyladenine) through the hydrolyzing action of GUS (Joersbo and Okkels, 1996). This enzyme, encoded for by the *GUS* gene, is more commonly known for its visual transformation marker properties. Joersbo and Okkels (1996) reported up to threefold higher transformation frequencies compared to kanamycin selection systems. In addition to being a positive selection system, the GUS gene could be used as both a selectable and screenable marker. Alternatively, a number of cases of marker-free transgenic systems have also been reported which may have utility in sorghum (Sugita *et al.*, 2000; Jacob and Veluthambi, 2002; Zuo *et al.*, 2002; Permingeat *et al.*, 2003).

A number of concerns have also been raised in regard to the transformation systems used. There are reports that sequences from outside the T-DNA regions can be transferred to the host organism (Ramanathan and Veluthambi, 1995). De Buck *et al.* (2000) also showed that T-DNA vector backbone sequences were frequently integrated into the genome of transgenic plants following transformation with *Agrobacterium*. However, functional plant analogs of T-DNA border sequences have been identified in potato and rice, allowing genetically modified plants to be created using only native plant DNA (Rommens *et al.*, 2004). This advance addresses one significant objection that some consumers have toward genetically modified organisms, that is, the introduction of foreign or bacterial DNA into food crops.

The genetic modification of plants occurs within a strict framework provided by national regulatory bodies, such as the Australian Office of the Gene Technology Regulator (<http://www.ogtr.gov.au>). The regulatory powers of such bodies were demonstrated in 2006 when South Africa's biotechnology Executive Council rejected an application by the country's largest science research organization Council for Scientific and Industrial Research to conduct experiments with transgenic sorghum until more suitable containment measures were established (ABS Consortium, 2006). The development of transgenic sorghum is being undertaken within a highly regulated environment to ensure

the safety of the environment and end users. Improvements in transformation technology and our understanding of the sorghum genome are also contributing to the development of these improved sorghum lines and in so doing, helping to make transgenic sorghum a beneficial, acceptable and safe option for the plant breeder, grower, processor, and end user alike.

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Pearl Millet

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a staple food that supplies a major proportion of calories and protein to large segments of the populations living in the semi-arid tropical regions of Africa and Asia. Pearl millet was domesticated from wild grasses of the southern Sahara approximately 4000 years ago. Soon after its domestication, it spread from its homeland to East Africa and then to India. It has since become widely distributed across the semi-arid tropics of Africa and Asia. Pearl millet was first introduced into Europe in approximately 1566 where plants were grown in Belgium from seed received from India. This millet is sometimes referred to as *Pennisetum spicatum*, and is still grown in Spain and North Africa (National Research Council, 1996). Pearl millet was introduced into the United States as long ago as 1850, and is still extensively grown as a summer annual grazing crop in the south (Hanna, 2000).

1.2 Botanical Description

Pearl millet is an erect annual plant that ranges in height between 50 and 140 cm. Flowers are arranged around a cylindrical spike (8–140 cm in

length), and are yellow to green in color. The flowers are either self- or cross-pollinated. Cross-pollination occurs when the stigma emerges before anther maturation. Grain develops within 20–30 days after pollination, and seed color ranges from white to brown, and even purple. Grain is usually tear shaped, approximately 8 mg in weight, and usually smaller than wheat seed.

Pearl millet is generally a short-day plant, but breeding has produced some day-length neutral varieties. It tolerates a range of rainfall conditions depending on the variety, and is usually found growing in areas that receive between 250 and 700 mm rainfall per annum. Although pearl millet is considered to be drought tolerant, it requires an even distribution of rain over the growing season. Too much rain during flowering can lead to crop failure. The flowering and seedling stages are sensitive to low temperatures, but high day-time temperatures (typically 30 °C) are required for the grain to mature. Drought tolerance is attributed to rapid growth, a short life cycle, high temperature tolerance, and a deep root system that can reach to the soil layers untapped by other plants.

Pearl millet has an abundant natural diversity with approximately 140 species or subspecies belonging to the genus *Pennisetum* (Stapf and Hubbard, 1934; Brunken, 1977). The presence of many weedy relatives leads to cross-pollination between cultivated pearl millet and wild *Pennisetum* species, which often causes a drop in

production capacity. However, hybridization with and introgression from wild relatives often lead to new improved crop forms. Pearl millet breeding programs often take advantage of desirable traits in wild species to improve cultivated varieties. Such traits include disease and insect resistance, high yields under adverse conditions, apomixis, early maturity, and a range of morphological characteristics (National Research Council, 1996). Useful wild *Pennisetum* species for introgression of genes into pearl millet include *P. purpureum* (napier grass), *P. squamulatum*, *P. orientale*, *P. flaccidum*, *P. setaceum* (Dujardin and Hanna, 1989b), and the wild pearl millet species *P. glaucum* subsp. *monodii* and *P. glaucum* subsp. *stenostachyum*. Subspecies *monodii* has been a particularly useful source of germplasm for introgression of resistance to rust, *Pyricularia* leaf spot, and smut, as well as forage dry matter yield and seasonal distribution (Hanna, 2000).

In recent years, considerable advances have been made in the understanding of the genetics of the crop. *P. glaucum* is an annual, sexual diploid ($2n = 2x = 14$) with a haploid DNA content of 2.4 pg (picogram). However, many genetic states can be obtained, and different *Pennisetum* species have chromosome numbers in multiples of $x = 5, 7, 8$, and 9 . Furthermore, for each of these states, there are different ploidy levels. Detailed genetic maps of pearl millet of some 300 loci spread over 7 linkage groups are available (Liu *et al.*, 1994). An extended map from multiple crosses incorporates not only molecular markers but also significant phenotypic traits (Devos *et al.*, 2006). Despite these advances, pearl millet is poorly supported by science and politics. Over the past two decades, production in West Africa has increased only by 0.7% per year, the lowest growth rate of any food crop in the region (National Research Council, 1996). Over the decades, more and more farmers, especially in southern Africa, have abandoned pearl millet farming and switched over to maize. This is due to a number of reasons. First, research efforts have made maize more productive than pearl millet; second, government incentives have given maize an added financial advantage; and finally, easier processing has made maize more convenient to use. However, with water steadily becoming a limiting resource to numerous economies, pearl millet could resurge as a vital multipurpose crop.

1.3 Economic Importance

In 2003, pearl millet was the world's sixth largest cereal crop with 29 million metric tons produced on 36 million hectares of cultivated land (<http://apps.fao.org/>). Approximately half of the world's pearl millet is grown in Africa, with the continent producing 14 million metric tons in 2003 (<http://apps.fao.org/>). In southern Africa, the commercialization of agriculture has resulted in maize partially or completely displacing pearl millet as a traditional food crop. In South Africa, only 12 000 t of pearl millet was produced in 2003, and this was mainly for subsistence purposes. Almost all millet is produced by small-scale farmers for household consumption and localized trade.

Pearl millet is a crop of vital importance to millions of African families living in semi-arid regions of the continent. Millet is one of the world's most resilient crops. In many areas where millet is the staple food, nothing else will grow. Pearl millet is supremely adapted to heat and aridity and production is likely to increase as the world gets hotter and drier. Of all the major cereals, it is the one most able to tolerate extremes of heat and drought. It yields reliably in regions too hot and too dry to consistently support good yields of maize or even sorghum. Pearl millet is easy to grow and suffers less from disease and insect pests than sorghum, maize, or other grains (National Research Council, 1996).

Pearl millet grain is nutritious, and has higher protein and energy levels than maize or sorghum (National Research Council, 1996). Carbohydrates usually make up about 70% of the dry grain, and they consist almost exclusively of starch. The grain contains at least 9% protein and a good balance of amino acids. It has roughly twice the fat content (5–7%) that of most standard cereals, and is particularly high in calcium and iron. The vitamin values of pearl millet grain are generally somewhat lower than those of maize, although the levels of vitamin A and carotene are good, particularly for a cereal. Importantly, it has neither the tannins nor other compounds that reduce digestibility in sorghum. Pearl millet is a versatile foodstuff, and is mainly used as whole seed, cracked seed, ground flour, dough, or a grain like rice. These are made into unfermented breads (*roti*), fermented foods (*kisra* and *gallettes*), thin

and thick porridges (*toh*), steam cooked dishes (*couscous*), nonalcoholic beverages and traditional beers. Grain from certain cultivars is roasted whole and consumed directly (<http://africancrops.net>). Although grain is the most important consumable from pearl millet in developing countries, the crop also provides forage/silage, building materials, and fuel (Devos *et al.*, 2006). Its primary use in industrialized countries is as forage, but interest in grain production for animal feed is increasing (Andrews *et al.*, 1996).

1.4 Traditional Breeding

The semi-arid tropics are characterized by unpredictable weather, limited and erratic rainfall and nutrient-poor soils, and suffer from a host of agricultural constraints (Sharma and Ortiz, 2000; Maqbool *et al.*, 2001). There is an urgent need to focus on improving crops relevant to the small farm holders and poor consumers in the developing countries of the humid and semi-arid tropics (Sharma *et al.*, 2002).

Traditional plant breeding methods have made, and will continue to make, important contributions toward meeting the need for more food. Breeding objectives for pearl millet include tolerance to drought, enhanced nutritional quality, adaptation to saline conditions, stover feeding value, high tillering, and resistance to major pearl millet diseases such as downy mildew (*Sclerospora graminicola*), ergot (*Claviceps fusiformis*), smut (*Moesziomyces penicillariae*), rust (*Puccinia substriata*), and head mold of pearl millet. The latter diseases are more serious on commercial F₁ hybrids than on open-pollinated varieties.

Although pearl millet is regarded as an “orphan” crop, the development of molecular marker systems and molecular marker-based genetic maps was initiated early, in 1990, within a Department for International Development (DfID)-funded program (Bertin *et al.*, 2005). Pearl millet genome mapping and molecular breeding were reviewed by Devos *et al.* (2006). Specific achievements are highlighted below.

In many areas of the world, staple foods lack certain essential nutrients even though enough energy is available from these food crops. Micronutrient-rich pearl millet cultivars are being bred by the International Crops Research Institute for

the Semi-Arid Tropics (ICRISAT) to address micronutrient deficiency in the semi-arid tropics with funding support from HarvestPlus (Rao *et al.*, 2006; www.harvestplus.org). Plant breeders are aware of the need to improve not just the grain component but also the quantity and nutritive value of the vegetative part of the crop, especially where it is an important animal feed (Zerbinia and Thomas, 2003). An initiative to improve pearl millet grain carotenoid content with marker-assisted selection is underway with team members from Cornell University, University of Georgia, ILRI and Waite Institute in Australia (www.icrisat.org). A panel of genotypes (including the best-known sources of elevated grain β -carotene levels) having diverse grain characteristics are used to assess single nucleotide polymorphism (SNP) diversity in genes for enzymes controlling the carotenoid biosynthesis pathway and to establish a near-infrared spectroscopy (NIRS) calibration curve for grain β -carotene content. The development of mapping populations based on crosses of best-known trait sources and elite inbred lines will follow.

The general complexity of drought stress is aggravated in the semi-arid tropics and subtropics by highly unpredictable rainfall, and by high temperatures, high levels of solar radiation, and low soil fertility (Serraj *et al.*, 2005). The resulting large variability in the nature and occurrence of drought stress and our insufficient understanding of its complexity have made it generally difficult to identify the physiological traits required for improved crop performance under drought stress conditions. Improving the adaptation or enhancing tolerance of pearl millet to drought stress, one of the most common and serious environmental constraints, is an important objective in most pearl millet breeding programs (Yadav *et al.*, 2004; Devos *et al.*, 2006) as it reduces mean yields, increases the magnitude of the annual variation in harvests, and the incidence of crop failure. In addition, tremendous variation in the timing and in the severity and duration of drought stress, and the high degree of interaction between the timing and intensity of the stress, and the crop growth stage and genotype are prevalent in drought-prone environments. Quantitative trait loci (QTL) mapping dissects complex phenotypic characters, such as drought tolerance, into their component traits (Yadav *et al.*, 2004) and subsequently allows

the identification of molecular markers linked to desirable QTLs, so that these can be directly used in marker-assisted selection. A genetic linkage map to detect genomic regions associated with grain and stover yield, and aspects of drought tolerance, was constructed (Yadav *et al.*, 2004) to aid breeding programs. Serraj *et al.* (2005) showed that superior grain yield performance of the introgression line hybrids was accompanied by increased biomass yields and reduced grain harvest indices instead of the reduced biomass yield and increased grain harvest index that appears to contribute to the higher grain yield potential and superior terminal drought tolerance of hybrids of donor parent PRLT 2/89-33 and the QTL-based topcross pollinator.

S. graminicola, an obligate biotrophic oomycetous fungal phytopathogen, is a widespread and destructive disease of pearl millet in India and in African countries (Singh and Talukdar, 1998) and losses up to 30% amounting to 260 million US dollars are reported (Singh *et al.*, 1993; Shetty and Kumar, 2000). Although *S. graminicola*, the causal agent of downy mildew in pearl millet can be chemically controlled, host-plant resistance provides a more attractive and cost-efficient method of disease management (Devos *et al.*, 2006). Mapping QTLs for downy mildew resistance in pearl millet has been done (Jones *et al.*, 1995, 2002). Recently, Hash *et al.* (2006) have shown the effectiveness of pyramiding/stacking of disease resistance genes. The pyramided resistance genes from ICML 22 and 843B that were present in ICMB 99022 proved effective against eight of the nine pathogen isolates used in their study. Overexpression of a pearl millet cysteine protease inhibitor that exhibits potent antifungal activity against important phytopathogenic fungi (Joshi *et al.*, 1998) might be another option.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Traditional breeding has been for many years the main avenue for crop improvement in pearl millet, but is limited in that it only allows the exploitation of variation present in these species or in wild relatives with which they can be crossed. Recombinant DNA technology permits a more precise and predictable introduction of a broader array of traits than does traditional plant breeding.

Modern agricultural biotechnology, which involves the application of cellular and molecular techniques to transfer DNA that encodes a desired trait to food and feed crops, is proving to be a powerful complement to traditional methods to meet global food requirements. An important aspect of biotechnology is that it provides access to a broad array of traits that can help to meet this need for nutritionally improved cultivars (CRFSFS, 2004) and other desirable traits.

2. TRANSGENIC PEARL MILLET

2.1 Tissue Culture Amenability

The establishment of a reliable transformation protocol for pearl millet would form the basis for future genetic enhancement of this crop by complementing classical breeding programs for the benefit of India and sub-Saharan Africa. As a first step toward a routine transformation system, it is essential to establish a reliable and highly efficient regeneration system for selected pearl millet lines. An improved regeneration capacity for selected pearl millet lines would underpin the development of a reliable transformation system. *In vitro* culture of cereals shows strong genotype dependence, and production of the appropriate culture is generally limited to selected genotypes (Lambé *et al.*, 1999).

Procedures for the regeneration of pearl millet plants via somatic embryogenesis have been described for immature zygotic embryos (IZEs) (Vasil and Vasil, 1981; Lambé *et al.*, 1995; Oldach *et al.*, 2001; Goldman *et al.*, 2003; O'Kennedy *et al.*, 2004b), mature embryos (Botti and Vasil, 1983), immature inflorescences (Vasil and Vasil, 1981; Pinard and Chandrapalaiah, 1991; Pius *et al.*, 1993; Goldman *et al.*, 2003), shoot apices (Lambé *et al.*, 1999, 2000; Devi and Sticklen, 2002), and apical meristems (Goldman *et al.*, 2003). The addition of L-proline to the tissue culture induction medium resulted in a highly efficient embryogenic regeneration system for pearl millet, obtaining on average 80 regenerants per immature zygotic embryo explant (O'Kennedy *et al.*, 2004b). Shaved immature inflorescences provided a greatly increased surface area in contact with the culture medium for the generation of embryogenic tissues in comparison with immature inflorescence segments and even immature embryos (Goldman *et al.*, 2003).

2.2 Transformation and Selection Methodology

A transformation protocol was established using the herbicide resistance selectable marker gene, *bar*, and the particle inflow gun (PIG) (Girgi *et al.*, 2002). However, the transformation efficiency obtained was very low (0.02%) (Table 1). Using the same selectable marker gene, much higher transformation efficiency and far fewer escapes were achieved with shaved immature inflorescence cultures and the biolistic device from Bio-Rad (Goldman *et al.*, 2003). Subsequently, the *manA* (POSITECH, Syngenta) as positive selectable marker gene was investigated (O’Kennedy *et al.*, 2004a). The system employs the phosphomannose isomerase (PMI) expressing gene (*manA*) as a selectable marker and mannose, converted to mannose-6-phosphate by endogenous hexokinase, as selective agent (Joersbo *et al.*, 1999). The mannose positive selection system favors the regeneration and growth of the transgenic cells while the nontransgenic cells are starved but not killed. Therefore, untransformed tissue is separated from transgenic tissue by carbohydrate starvation of the untransformed cells. The use of *manA* selection limited the number of escapes

to less than 10%, whereas using the *bar* gene and selecting with 3–5 mg l⁻¹ bialaphos (the active ingredient of the herbicide) resulted in more than 90% nontransformed escapes (Girgi *et al.*, 2002). The *manA* selection system not only improved the transformation efficiency but also avoided the use of antibiotic or herbicide resistance genes as selectable markers in pearl millet transformation (O’Kennedy *et al.*, 2004a). To date, no *Agrobacterium*-mediated transformation of pearl millet has been reported.

Current transformation efficiencies are sufficient for testing of candidate genes, but a more efficient optimized system will be required for pearl millet transformation in high-throughput functional genomics projects.

2.3 Decreased Disease Susceptibility in Transgenic Pearl Millet

The most important applications of biotechnology for plant protection amongst the ICRISAT mandate crops, especially in Africa, include downy mildew resistance in pearl millet (Sharma and Ortiz, 2000).

Table 1 A summary of published work reporting on the production of transgenic pearl millet plants

Explant source	Genotypes	Transformation methodology and efficiency	Gene of interest	References
Embryogenic calli	N.E.	Biolistics	35S ^(a) - <i>uidA</i> ^(b) and 35S- <i>hph</i> ^(c)	Lambé <i>et al.</i> (1995, 2000)
IZEs	7042, 842B Manga Nara Bongo Nara	Biolistics 0.02–0.28%	Ubi ^(d) - <i>bar</i> ^(e) and Ubi- <i>uidA</i> 35S- <i>bar</i>	Girgi <i>et al.</i> (2002)
Apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues	HGM100	Biolistics	Ubi- <i>bar</i> and Ubi- <i>uidA</i> 35S- <i>gfp</i>	Goldman <i>et al.</i> (2003)
IZEs	842B	Biolistics 0.72%	Ubi- <i>manA</i> ^(f)	O’Kennedy <i>et al.</i> (2004a)
IZEs	7042 Manga Nara	Biolistics 0.14%	35S- <i>bar</i> and Ubi- <i>afp</i> ^(g)	Girgi <i>et al.</i> (2006)
Shoot-tip-derived embryogenic calli	ICMP 451	Biolistics	35S- <i>bar</i> and 35S- <i>pin</i> ^(h)	Latha <i>et al.</i> (2006)

^(a)CaMV 35S cauliflower mosaic virus promoter

^(b) β -glucuronidase coding sequence

^(c)Hygromycin phosphotransferase selectable marker gene

^(d)Maize ubiquitin promoter

^(e)Phosphinothricin acetyl transferase herbicide selectable marker gene

^(f)Phosphomannose isomerase positive selectable marker gene

^(g)Antifungal protein AFP from the ascomycete *Aspergillus giganteus*

^(h)Synthetic prawn antifungal protein encoding gene

The antimicrobial protein gene *afp* from the mold *Aspergillus giganteus* was introduced into two pearl millet genotypes by particle bombardment (Girgi *et al.*, 2006). Stable integration and expression of the *afp* gene was confirmed in two independent transgenic T₀ plants and their progeny using Southern blot and reverse transcription polymerase chain reaction analysis. *In vitro* infection of detached leaves and *in vivo* inoculation of whole plants with the basidiomycete *P. substriata*, the causal agent of rust disease, and the oomycete *S. graminicola*, causal agent of downy mildew, resulted in a significant reduction of disease symptoms in comparison to wild-type control plants. The disease resistance of pearl millet was increased by up to 90% when infected with two diverse, economically important pathogens. Disease resistance against *S. graminicola* was also obtained by expressing the puroindoline protein encoded by the synthetic prawn antifungal gene in the downy mildew susceptible genotype ICMP451 (Latha *et al.*, 2006). These are the first published reports of genetic engineering for resistance of pearl millet against infections by plant microbes.

Trichoderma atroviride is a well-known biological control agent, which can be used in combination with *Bacillus* spp. to combat *S. graminicola*, causal agent of downy mildew in pearl millet (Shetty and Kumar, 2000). Previous studies showed that a 78-kDa β -1,3-glucanase enzyme from *T. atroviride* exhibited potent antimicrobial activity to the oomycetous pathogen *Phytophthora* (Fogliano *et al.*, 2002). The *gluc78* gene (Donzelli *et al.*, 2001) from *T. atroviride*, which degrades glucan in the microbe's cell walls, was placed downstream of the potato proteinase inhibitor IIK wound inducible promoter followed by the rice *Act1* intron, and also downstream of the maize ubiquitin constitutive promoter and introduced into the genome of pearl millet (O'Kennedy *et al.*, unpublished results). Pathogenecity trials are currently underway.

2.4 Donor Gene: Isolation and Functional Analysis of Genes from Pearl Millet

All transgenic pearl millet studies undertaken to date have introduced genes from nonplant sources in order to confer tolerance to microbial pathogens

(see Section 2.3). While these genes have been shown to effectively confer tolerance to downy mildew and/or rust in transgenic pearl millet, they are of microbial origin. This may slow down the release of transgenic pearl millet lines due to the necessity of rigorous toxicity testing procedures, and also have implications for subsequent public acceptance of transgenic varieties. It would, therefore, be more suitable to isolate genes of interest (e.g., conferring abiotic and biotic stress tolerance) from existing pearl millet landraces and cultivars, and introduce these into stress susceptible pearl millet lines. Although very few examples of pearl millet gene isolation experiments exist in the literature, searches on National Centre for Biotechnology Information's (NCBI) Web site indicate that 3309 pearl millet sequences have been deposited in their database (www.ncbi.nlm.nih.gov). Although transformation with individual genes confers some degree of stress tolerance in transgenic plants, it is felt that regulated expression of more genes via overexpression of transcription factors or components of signaling pathways can lead to expression of a large number of relevant downstream genes, and sustained tolerance. This discussion will, therefore, focus on such examples of isolated pearl millet genes/gene fragments, and their potential application in future transgenic pearl millet projects.

One of the major objectives of pearl millet research is to introduce broad-spectrum disease tolerance into pearl millet lines. A study was undertaken to identify pearl millet genes that are differentially expressed in response to treatment with the fungal and bacterial pathogen elicitors, chitin and flagellin, respectively, and wounding (van den Berg *et al.*, 2004). Complementary DNA (cDNA) libraries were constructed from treated plants, and used in subsequent experiments to identify genes that are up- or down-regulated in response to treatment with the defense signaling molecules nitric oxide, salicylic acid, and methyl jasmonate, and also in response to rust treatment (Crampton and Berger, unpublished results). Substantial overlap was found to exist between jasmonate and salicylate signaling pathways, and genes involved in defense, signaling, oxidative burst, stress, basic/secondary metabolism, protein synthesis, and photosynthesis were identified. Identified candidate genes for pearl millet transgenic

studies that could confer broad-spectrum disease tolerance include a calcium binding EF hand protein and an ethylene responsive element binding protein (EREBP1). Calcium binding EF hand proteins are involved in detecting perturbations in cellular calcium levels following pathogen infection, and subsequent changes in metabolism and gene expression (Harmon *et al.*, 2000). Transcription factor EREBP1 mediates gene expression changes in response to various pathogens and defense elicitors (Euglem, 2005).

Mitogen-activated protein kinase (MAPK) cascades are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses, and are found in all eukaryotes analyzed to date (Innes, 2001). The basic assembly of a MAPK cascade is a three kinase module conserved in all eukaryotes. MAPK, the last kinase in the cascade, is activated by a kinase relay consisting of a MAPK kinase (MAPKK or MEK), which in turn is activated by a MAPKK kinase (MAPKKK or MEKK). MAPKs are activated by a variety of stress stimuli including wounding, temperature, drought, salinity osmolarity, ultraviolet (UV) irradiation, ozone, and reactive oxygen species. GenBank searches indicate that partial sequences exist for pearl millet MAP kinase kinase 1 mRNA (messenger RNA) (accession number AY147852), and a MEK1-like MAP kinase kinase mRNA (accession number AY147853), which are induced under salinity stress. Introduction of full-length sequences of these genes into pearl millet under the control of a saline-inducible promoter will potentially activate the MAPK cascade, which will in turn induce downstream genes involved in salinity tolerance.

Postflowering drought stress is one of the most common and serious environmental constraints of pearl millet production in sub-Saharan Africa and the Indian subcontinent (van Oosterom *et al.*, 1996). A number of drought responsive gene sequences exist in the GenBank (www.ncbi.nlm.nih), but of particular interest are a partial drought responsive element binding factor (DREB) sequence (accession number AY823566) and a complete pearl millet *DREB2A* gene sequence (accession number DQ227697). DREBs are transcription factors that induce a set of abiotic stress related genes, and impart stress endurance to plants. The DREB transcription factors are independent of

abscisic acid signaling, and can be dichotomized as DREB1 and DREB2, which are involved in two separate signal transduction pathways under low temperature and dehydration, respectively (Agarwal *et al.*, 2006). A number of transgenic plants have been made expressing DREB genes (as summarized in Agarwal *et al.*, 2006), and overexpression of a stress-inducible DREB transcription factor was found to activate the expression of many target genes having DRE elements in their promoters, and the resulting transgenic plants showed improved stress tolerance.

Many of the pearl millet sequences submitted to the GenBank represent partial mRNA sequences. It will, therefore, be necessary to isolate the coding sequence in its entirety before introducing the gene into pearl millet. Once isolated, functional analyses of pearl millet genes can be achieved by direct introduction into pearl millet and assessing the effect of their overexpression. Alternatively, RNA silencing of the gene of interest (Miki *et al.*, 2005) would enable researchers to study the effect of drastically down-regulating the gene, and determine its role in a particular stress response; namely, if the plant is highly susceptible to a stress following knockdown, then the expression of the gene plays an important role in the stress response. Stress-related pearl millet gene sequences will also provide a novel source of material for promoter mining studies. Genome walking (Rishi *et al.*, 2004) experiments upstream of the transcription start site will reveal regulatory sequences that could be a useful source of inducible promoters for transgenic studies.

2.5 Testing of the Activity and Stability of Inheritance of Transgenes, and Their Adverse Effects on Growth, Yield, and Quality

The concerns of consumers and regulatory authorities mean that transgenic crops are currently being subjected to detailed scrutiny to assess their safety for use in food. Random insertion of transgenes by the biolistic and *Agrobacterium*-mediated approaches has led to the suggestion that transgenes may have unpredicted effects on the expression of other endogenous genes, depending on their sites of insertion. Even an infrequent event could have serious consequences if it resulted in

the production of deleterious components or the loss of components that currently contribute to nutritional or processing quality. A key element of this comparative safety assessment is that a food or feed derived from a genetically modified (GM) crop is shown to be as safe as its conventionally bred counterpart. Application of the principle of substantial equivalence involves identifying the similarities and any differences between a product and its closest traditional counterpart and subjecting the differences to a rigorous safety assessment.

“Substantial equivalence” has recently been shown for transgenic wheat lines expressing the high molecular weight subunit transgenes assessing field performance (Shewry *et al.*, 2006), transcriptome of wheat grain in comparison with conventional breeding (Baudo *et al.*, 2006), and metabolomic studies of substantial equivalence of field-grown GM wheat (Baker *et al.*, 2006), which can only promote public acceptance of genetically produced food crops.

3. FUTURE ROAD MAP

3.1 Future Research Areas: Enhancement of Nutritional Quality

Bioavailable iron, vitamin A, and zinc are mainly provided in the human diet by animal food sources. In the developing world, where poorer individuals consume predominantly plant-based diets, deficiencies of these micronutrients are common and can occur in the same individual (Hess *et al.*, 2005). In the past decade, micronutrient malnutrition has been identified as a major underlying cause of numerous human health problems in developing countries (Toenneissen, 2002) with nutritional deficiency most acute amongst the poor. The magnitude of persistent deficiencies of micronutrient, iron and zinc, iodine and vitamins are particularly alarming among children, women at reproductive age, and pregnant and lactating women. Current efforts to combat micronutrient malnutrition in the developing world focus on providing vitamin and mineral supplements on fortifying foods during processing. With molecular markers, it is now possible to tag the genes responsible for the trait of interest, which then facilitate incorporating the genes into new breeding lines. Mutant cereal lines with

high β -carotene (provitamin A) content in the nonphotosynthetic edible tissues can be used in breeding; if such mutants do not exist, the carotenoid biosynthetic pathway can be introduced by genetic engineering. Paine *et al.* (2005) successfully introduced sufficiently high ($37 \mu\text{g g}^{-1}$) levels of β -carotene into the genome of rice. Based on a retinol equivalency ratio for β -carotene of 12:1, 50% of the children's recommended dietary allowance is delivered by 72 g of dry Golden Rice 2. This technology development can effectively be applied to other cereals, such as pearl millet.

Studies on the interaction between vitamin A and iron have produced quite compelling evidence that vitamin A deficiency decreases iron utilization, as well as clear evidence of the public health benefits of dual supplementation of iron and vitamin A to populations deficient in both nutrients so as to improve the impact of the iron on hemoglobin levels (Hess *et al.*, 2005). An interaction between vitamin A and zinc also appears to occur, but its public health importance remains unclear and will likely remain that way until a better measure of zinc status is developed (Hess *et al.*, 2005).

3.2 Addressing Risks and Concerns: Damage to Human Health and Environment

It is essential to reduce direct pollen-mediated gene flow from GM pearl millet to non-GM plants, as pearl millet is indigenous to Africa. In order to adhere to biosafety regulations and to minimize inadvertent introgression of transgenes into non-GM pearl millet varieties, the following strategies have potential to be applied to transgenic pearl millet. Firstly, the removal of antibiotic or herbicidal selectable marker genes that confer resistance to antibiotics or herbicides (Scutt *et al.*, 2002) and secondly the use of a positive selectable marker gene, such as *manA* (O'Kennedy *et al.*, 2004a), will be desirable. The latter should not confer any selective advantage to weedy subspecies if outcrossing was to occur. Thirdly, cytoplasmic-nuclear male sterility (CMS) can be used to severely restrict, but not eliminate, pollen-mediated gene flow. CMS has been well characterized in pearl millet and is routinely used for the production of hybrid seed (Andrews *et al.*,

1996). Gene flow through pollen could be restricted by CMS in fields where maintainer lines are sown for cross-pollination. However, it is likely that pollen from wild subspecies that transmits restorer genes would be present under some circumstances, and a male-fertile transgenic plant eventually would arise. Gene flow between wild subspecies and pearl millet has been documented (Renno *et al.*, 1997). Other strategies for transgene containment in addition to male sterility have been proposed (Daniell, 2002; Stewart *et al.*, 2003). These include maternal inheritance, linkage disequilibrium, gene use restriction technology, transgene mitigation, and apomixis. Gene use restriction technology includes the “terminator technology” for seed sterility and has been an unpopular approach. More empirical data for effectiveness of transgene containment are available for maternal inheritance and transgene mitigation. Maternal inheritance typically is achieved through chloroplast transformation. Chloroplast transformation has been repeatedly accomplished in tobacco (Daniell *et al.*, 2005) but has not yet been attempted in pearl millet. Chloroplast transformation most easily is carried out with chlorophyll-containing tissues. Therefore, chloroplast transformation may be more feasible using a shoot regeneration system for pearl millet such as that developed by Devi and Sticklen (2002) instead of the more commonly used embryogenic culture system, although recent progress indicates that plastids in embryogenic tissues can be transformed (Daniell *et al.*, 2005). Transgene mitigation and linkage disequilibrium (LD) are related in that transgene mitigation takes advantage of two closely linked genes that have a low probability of recombination (LD), one gene of which would reduce the competitiveness of weeds. Domestication traits, such as dwarf and nonshattering seed heads, are examples of traits selected for agricultural crops that would reduce the competitiveness of weeds (Gressel, 1999). Even though apomixis (asexual reproduction through seeds) occurs in wild relatives of pearl millet and has been introgressed into the crop species (Dujardin and Hanna, 1989c; Goel *et al.*, 2003), pollination of graminaceous apomicts is required for endosperm development. Therefore, male sterility accompanying apomixis still would require the interplanting of a pollinator as for CMS lines. In contrast to CMS, production

of recombinant embryos would be less likely. Furthermore, the existing apomictic pearl millet introgression lines have been developed at the tetraploid level (Dujardin and Hanna, 1989a, 1989c; Ozias-Akins *et al.*, 2003) and crosses with wild diploid relatives would yield rare and unfit triploids. Lastly, the necessity for transgene containment may vary depending upon the trait. The introduction of transgenes for nutritional quality improvement that confer no agronomical or competitive advantage to weedy species and non-GM varieties should not only be a safe technology but also would help to meet the acute need for nutritionally enhanced staple food crops in Africa.

3.3 Expected Technologies

Although no *Agrobacterium*-mediated transformation system yet exists for pearl millet, it is still worth exploring whether *Agrobacterium* transformation can be more efficient than particle bombardment in this species. *Agrobacterium* has become the standard for rice transformation (Hiei *et al.*, 1997). It also is now preferred for maize transformation (Zhao *et al.*, 2001; Frame *et al.*, 2002; Sidorov *et al.*, 2006) but remains relatively inefficient for sorghum transformation (Howe *et al.*, 2006). A distinct advantage of *Agrobacterium* transformation is the lower number of gene copies introduced into the transformed host genome compared with biolistic methods. Nevertheless, transfer DNA (T-DNA) insertions are not precise as once thought and plasmid backbone sequences beyond the T-DNA borders frequently are found in the transformed genome (Shou *et al.*, 2004).

The biolistic approach in combination with clean gene technologies or minimal transgene expression cassettes is a useful tool to produce a product that is free of antibiotic resistance genes in order to adhere to regulatory food safety requirements especially for a staple food crop indigenous to Africa. Minimal transgene cassettes (promoter-coding sequence-terminator sequence) were successfully introduced into the genome of pearl millet (O’Kennedy, unpublished results) in order to (1) eliminate backbone sequences containing repetitive sequences, which could lead to gene silencing and overload of non-sense DNA into the genome of a food crop, and (2) eliminate

antibiotic resistance marker genes (e.g., *Amp^R*), which are necessary for mass production of the genes of interest in *Escherichia coli* cells, but redundant for pearl millet transformation. The approach of introducing minimal transgene cassettes by biolistic transformation potentially enhances low copy number integration of transgenes, and minimizes transgene rearrangements and gene silencing as previously reported (Kohli *et al.*, 1998; Fu *et al.*, 2000). Vector backbones have the tendency to promote transgene rearrangements, since the vector backbone has been shown to provide a number of recombination hotspots, and the removal of these sequences would limit the influence of recombinogenic elements on the process of integration (Fu *et al.*, 2000).

3.4 IPR, Public Perceptions, Industrial Perspectives, and Political and Economic Consequences

In spite of substantial introduction of new pearl millet varieties in semi-arid sub-Saharan Africa during the recent decades (Monyo, 2002), inorganic fertilizers and improved water management are essential for large yield increases (Ahmed *et al.*, 2000). Government policies, transportation infrastructure, and market development of the target African countries and crops also need to be addressed for conventional and/or transformation-based crop cultivar improvement to significantly contribute to food supply.

New lines of millets containing transgenes will need to be tested at least as stringently as any other introduced or improved cultivars of these crops. A thorough assessment of their allergenic potential and the monitoring of any unintended effects on food composition will provide a solid basis for food safety assessment. The FAO/WHO have provided decision trees for a rigorous assessment and testing for GM foods (Halsberger, 2003), which would be applicable to transgenic pearl millet expressing the genes of interest. Furthermore, the phenotypes of transgenic pearl millet plants produced and the milling and processing qualities of the transgenic seed need to be assessed.

Finally, the gains in food production provided by the green revolution have reached their ceiling while the world population continues to rise

(Wisniewski *et al.*, 2002). A new green revolution will necessitate the application of recent advances in plant breeding, including new tissue culture techniques, marker-aided selection, mutagenesis, and genetic modification (Wisniewski *et al.*, 2002) to meet our increasing requirement for food, feed, fodder, and fuel, with cereal grains playing a pivotal role (Hoisington *et al.*, 1999). Whereas the affluent nations can afford to adopt elitist positions and pay more for food produced by the so called natural methods, the one billion chronically poor and hungry people of this world cannot (Wisniewski *et al.*, 2002). Therefore, despite the diverse and widespread potential for beneficial applications of transgenic products in agriculture, there remains a critical need to present these benefits to the general public in a real and understandable way that stimulates an unbiased and responsible public debate (Sharma *et al.*, 2002) and pro-GM government policies.

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Finger Millet

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1. INTRODUCTION

Millets are one of the world's important food crops among cereals, primarily grown in arid and semi-arid regions of Asia and Africa. The various millets, designated as coarse cereals, are known to thrive well in the harsh climates such as high temperatures and low rainfall areas, where other crops fail to grow (Goldman *et al.*, 2003). Millets are nutritionally at par with other cereals and are the principal sources of energy, proteins, vitamins, and minerals. The projected food demand for 2025 warrants yields of millets to be increased from 2.5 to 4.5 t ha⁻¹ (Borlaug, 2002). These increases are expected to come from genetically improved varieties and transgenics exhibiting durable resistance to abiotic and biotic stresses using tertiary gene pools.

1.1 History, Origin, and Distribution

Finger millet, *Eleusine coracana* (L.) Gaertn., also known as African millet or *ragi*, has outstanding attributes as a subsistence food crop. *E. coracana* (L.) Gaertn. ssp. *coracana* belongs to a relatively small genus with 9 of its 11 species predominantly African in distribution (Phillips, 1972; Hilu and de Wet, 1976a). The crop was believed to have been domesticated in India from its weedy relative *E. indica* (L.) Gaertn. (Cobley, 1963; Vishnu, 1968). Others supported an origin in Africa from

E. coracana ssp. *africana* (Kennedy-O'Bryne, 1956; Mehra, 1963; Porteres, 1970; Hilu and de Wet, 1976b). However, Kennedy-O'Bryne (1956) and Jameson (1970) proposed independent origins for the crop in both Africa and India from subspecies *africana* and *E. indica*, respectively. Hilu and de Wet (1976a, b) and Hilu *et al.* (1978), based on biosystematic, ethnobotanic, and linguistic evidences, proposed the East African origin of *E. coracana* and its differentiation and domestication from the subspecies *africana*. Most probably the crop was later introduced into India via sea routes as early as the third millennium BC (Hilu *et al.*, 1979).

1.2 Botanical Description

The cultivated *E. coracana* is an erect, annual grass, growing to about 2–4 feet height with tillering-tufted stems. When mature, the stems are somewhat laterally flattened, bearing a whorl of 4–6 digitate, straight or slightly incurved spikes. The spikes are about 1/2 in. broad and 5–6 in. long; the numerous spikelets (about 70) are arranged alternately on the rachis. Each spikelet contains 4–7 seeds varying in diameter from 1 to 2 mm. The seeds are globose or somewhat flattened, smooth or rugose, and differ in color from dark reddish brown to nearly white.

Eleusine (L.) Gaertn. (Poaceae) is a small genus comprising nine species closely allied to *Acrachne*

and *Dactyloctenium* belonging to subtribe Eleusinae of the subfamily Chloridoideae (Phillips, 1972; Clayton and Renvoze, 1986). This genus is characterized by spikes of overlapping spikelets and an ornate grain enclosed by a free pericarp. The center of diversity for *Eleusine* is located in Ethiopia (East Africa) where eight of its species, namely, diploid annuals *E. indica* (L.) ($n = 9$), *E. multiflora* ($n = 8$), and diploid perennials *E. floccifolia* ($n = 9$), *E. intermedia* ($n = 9$), *E. jaegeri* ($n = 10$), *E. semisterilis*, as well as tetraploid perennial *E. kigeziensis* ($n = 19$), tetraploid annual *E. coracana* (L.) subsp. *africana* ($n = 18$), have been found. The remaining species, diploid annual *E. tristachya* ($n = 8$ or 9) is indigenous to the New World extending from South America to southwestern North America (Hilu, 1980); it also occurs in Africa as an occasional weed of limited distribution. The various species of *Eleusine* occupy diverse habitats ranging from open, dry places to understories of forests, and from sea level to high elevations. Some of the species, the cosmopolitan *E. indica* and its close relative *E. tristachya* grow as wild weeds beyond their indigenous ranges. Moreover, the genus includes the economically important cereal crop finger millet, *E. coracana* subsp. *coracana*, which is an annual tetraploid ($n = 18$) grown extensively in the semi-arid regions of Africa, India, and other countries. However, the diploid parents, genome composition, and evolutionary history of finger millet are incompletely understood (Hilu *et al.*, 1979).

Results from cytogenetic studies amply suggested that finger millet is an allotetraploid, derived directly from the wild tetraploid *E. coracana* subsp. *africana*, an annual weed occurring across much of Africa (Chennaveeraiah and Hiremath, 1974; Hiremath and Chennaveeraiah, 1982). Conspecificity of the *E. coracana* crop with, and its derivation from, its weedy relative subsp. *africana* is further supported by evidences from morphology, flavonoid chemistry, as well as analysis of chloroplast and ribosomal DNA (Hilu and De Wet, 1976a b; Hilu *et al.*, 1978; Hilu, 1988; Hilu and Johnson, 1991). That the *E. indica* ($n = 9$)—long suspected of being ancestral to *E. coracana* (Greenway, 1945)—is indeed the source of one of its genomes is now well established (Hilu, 1988; Hilu and Johnson, 1991; Hiremath and Salimath, 1992). However, the identity of the other parental diploid ($n = 9$) species remains unknown.

Among the polyploids are the cultivated *E. coracana* subsp. *coracana* and its putative wild ancestor, *E. coracana* subsp. *africana*. *E. coracana* was, thus, believed to be an allotetraploid derived from hybridization between *E. indica* and an unknown diploid. To evaluate this hypothesis, 16 isozyme loci coding for 9 enzymes were compared among 7 of the 9 *Eleusine* species. Substantial genetic variability was observed among the diploid species, ranging from $P = 0.563$, $A = 1.6$, $H = 0.208$ in *E. indica* to $P = 0.188$, $A = 1.2$, $H = 0.042$ in *E. jaegeri*. The diploids tended to be genetically distinct with values of Rogers' Similarity ranging from $S = 0.294$ (*E. jaegeri/floccifolia*) to $S = 0.794$ (*E. indica/tristachya*). Both the tetraploid subspecies *coracana* and *africana* disclosed fixed heterozygosity at several loci, confirming their allotetraploid status. These tetraploids invariably possessed *E. indica* marker alleles at all the loci, corroborating ancestry by this taxon. Genotypes of the non-*indica* ancestor, inferred separately for each tetraploid, differed substantially from all candidate diploids and also from each other. These data suggested that (1) none of the diploids investigated is likely to have been the non-*indica* ancestor of *E. coracana*, and (2) the non-*indica* diploid ancestor of the wild tetraploid may differ from that of the cultivated subsp. *coracana*. The latter conclusion, however, is inconsistent with the complete chromosomal homology observed between the two tetraploid subspecies, indicating the need for additional evidence bearing on their relationships (Charles *et al.*, 1994).

Deshpande and Ranjekar (1980) analyzed the genome of finger millet using DNA reassociation kinetics. The genome size has been estimated as 3.0×10^8 bp. The T_m values of cot 25 and cot 1 fractions were reported to be lowered by 10.8 and 12.8 °C, respectively, as compared to sonicated DNA indicating a base pair mismatch.

1.3 Economic Importance

Finger millet is grown in more than 4 million hectare worldwide and is the primary food source for millions of people in dryland regions of India and Central Africa. It occupies about 1.8 million hectares yielding about 2.34 million tons of grain with an average productivity of 1300 kg ha⁻¹ ([http://www.icrisat.org/Pearl Millet.htm](http://www.icrisat.org/Pearl%20Millet.htm)).

Finger millet is a typical dryland crop grown in semi-arid tropics of Africa and India. In East Africa, Ethiopia, and Somalia, it serves as the principal staple food of several tribes besides its use for malting and brewing. In India, grains are usually converted into flour and a variety of preparations, namely, cakes, puddings, porridge, etc., are made. A fermented drink is prepared from the grain in some parts of Maharashtra and in the Himalayan tracts in India. The grain is also malted and the flour of the malted grain is used as a nourishing food for infants and invalids, besides using it as a popular food supplement for diabetic patients. Its slow digestion leads to lesser increase in blood sugar levels, thereby acting as a safer food for diabetics.

Finger millet possesses excellent storage properties and is said to improve in quality as a result of storage; seeds of finger millet can be stored safely for several years without insect damage, and is a traditional component of farmers' risk avoidance strategies in the drought-prone areas (Sastri, 1989). The nutrient-rich grains are mainly used for making unleavened bread, while straw serves as a nutritious feed for the bovines. It is one of the richest sources of vitamins, proteins, and minerals such as calcium, while the iodine content is reported to be the highest among cereal grains. Finger millets have nutritional qualities superior to that of rice and is on a par with that of wheat. It is also a good source of essential amino acids like lysine and methionine. The malted grains of finger millet have a significantly higher saccharifying enzyme activity useful in brewing (Sastri, 1989).

1.4 *In Vitro* Plant Regeneration in Finger Millet

Development of a genotype-independent high-frequency regeneration system is an essential prerequisite for successful production of transgenic plants. Earlier reports on the morphogenetic response of different explants of finger millet are listed in Table 1.

Callus formation was induced from the cut end of the shoot tips of finger millet seedlings within 4–7 days. Among the 20 finger millet genotypes tested for their efficiency of callus induction, PGEC-2, showed the highest frequency (99%) of callus initiation on Murashige and Skoog (MS) medium supplemented with 2,4-D.

The calli were differentiated into embryogenic and nonembryogenic sectors within 10–12 days, and the frequency of embryogenesis, in PGEC-2, approximated to 90%. Embryogenic sectors of the calli were compact, nodular, and creamy-yellow, while nonembryogenic regions were white and friable. Embryogenic regions were subcultured on the callusing medium for 2 weeks for proliferation of the embryoids. These embryoids were later regenerated into plants with well-developed roots on the MS basal medium supplemented with 6-benzylaminopurine (BAP) (1.0 mg l^{-1}) and kinetin (0.5 mg l^{-1}). From each embryogenic callus (1–2 mm) more than 100 plantlets were regenerated. Thus, from a single shoot-tip explant, a maximum of >4000 plantlets could be established within 75 days in the glasshouse (Madhavi *et al.*, 2005).

1.5 Limitations of Traditional Breeding

That cultivated *E. coaracana* subsp. *coaracana* is a polyploidy is also evidenced by duplicate loci for various morphological characters. The inheritance of numerous qualitative traits, such as purple pigmentation, grain color, sterility, pericarp color, elongation of earhead (spike), glume length, earhead (spike) shape, etc., were investigated; whereas, for characters important in breeding, very limited genetic studies have been made (Richaria, 1957).

In the past, mass and pure line selections were used to isolate improved strains from introductions and mixed local varieties. Hybridization followed by pedigree, bulk, and backcross methods have been used to improve grain yield, maturity, lodging resistance, disease resistance, grain quality, etc. (Chavan and Shendge, 1957; Poehlman and Borthakur, 1969).

Sixteen fungal, three viral, and at least one bacterial pathogen have been reported to infect finger millet. Leaf blast disease caused by the fungus *Pyricularia grisea* has been identified as the most devastating disease in finger millet affecting its production in South Asia and Africa (Ramakrishnan, 1963). *P. grisea* is pathogenic to more than 50 graminaceous hosts including common crops such as rice, finger millet, foxtail millet, and wheat. It causes complete harvest loss when it occurs prior to grain formation (Ekwanu, 1991). Various finger millet landraces were found

Table 1 Morphogenic response in finger millet

Explant	Callusing medium	Regeneration medium	References
Apical domes	MS + 2,4-D (0, 1, 1.5, 2.0, or 3.0 mg l ⁻¹) or MS + 2,4-D (1.0 or 2.0 mg l ⁻¹) + kinetin (0.5 mg l ⁻¹)	MS + GA ₃ (1.0 mg l ⁻¹) or MS + NAA (1.0 mg l ⁻¹)	Kumar <i>et al.</i> , 2001
Apical domes from germinating caryopses	MS + 2,4-D (3.0 mg l ⁻¹) + thiamine (1.0 mg l ⁻¹) + kinetin (0.1 mg l ⁻¹) + casamino acids (500 mg l ⁻¹) + coconut milk (5%)	MS + thiamine (1.0 mg l ⁻¹) + kinetin (0.1 mg l ⁻¹)	Wakizuka and Yamaguchi, 1987
Shoot apices or mesocotyls	MS + picloram (4.0 mg l ⁻¹) + kinetin (0.5 mg l ⁻¹)	MS basal	Eapen and George, 1990
Germinating embryos	MS + 2,4-D (1.0 or 3.0 mg l ⁻¹)	MS + kinetin + 2,4-D	Sivadas <i>et al.</i> , 1990
Immature embryo	MS + 2,4-D (1.0 or 2.0 mg l ⁻¹) or MS + 2,4-D (1.0 or 2.0 mg l ⁻¹) + kinetin (0.5 mg l ⁻¹)	MS basal	Vishnoi and Kothari, 1995
Seeds/caryopses	MS + 2,4-D (9.05 μM) + kinetin (2.32 μM) + CuSO ₄ (0, 0.1, 0.2, 0.5, 0.6, 1.0, or 2.0 μM) or ZnSO ₄ (0, 29.91, 59.82, or 149.55 μM)	MS + NAA (5.37 μM) + CuSO ₄ (0, 0.1, 0.2, 0.5, 0.6, 1.0, or 2.0 μM) or ZnSO ₄ (0, 29.91, 59.82, 89.73, 119.64, 149.55, or 179.46 μM)	Kothari <i>et al.</i> , 2004
Seeds/caryopses	MS + 2,4-D (1.0–5.0 mg l ⁻¹)	MS basal	Gupta <i>et al.</i> , 2001
Seeds/caryopses	MS + 2,4-D (2.0 mg l ⁻¹) + kinetin (0.5 mg l ⁻¹)	MS + NH ₄ NO ₃ (0, 20, 40, 80, 100, or 120 mM) with or without NAA (1.0 mg l ⁻¹)	Poddar <i>et al.</i> , 1997
Seeds/caryopses	MS + 2,4-D (2.0 mg l ⁻¹) + kinetin (0.5 mg l ⁻¹)	MS + BAP (0.5, 1.0, 2.0, or 4.0 mg l ⁻¹)	Kavi Kishore <i>et al.</i> , 1992
Caryopses and excised shoot tips	MS + picloram (4.0 mg l ⁻¹) + kinetin (0.5 mg l ⁻¹)	MS basal	Eapen and George, 1989

to be highly susceptible to blast disease resulting in heavy yield losses. In India, blast disease caused annual yield losses ranging from 28% to 50% in the endemic areas (Sastri, 1989). Adoption of conventional methods of breeding met with limited success for developing durable resistance to leaf blast disease (Coca *et al.*, 2004).

1.6 Rationale for Production of Transgenics

Genetic transformation is extensively used as a method of choice for transferring desired genes into commercial crop cultivars for enhancing various agronomic attributes. Developments in gene transfer methods have made it feasible to rapidly introduce exotic stress tolerance genes into diverse crop plants. There are several successful examples

of transgenics showing enhanced tolerance to various biotic and abiotic stress factors. Because of the nonavailability of desired resistance genes in the primary and secondary gene pools of *Elusine* for developing durable resistance to leaf blast disease and salt tolerance, genetic engineering approaches deploying novel resistance genes from tertiary gene pools have been adopted.

2. TRANSGENICS DEVELOPED IN FINGER MILLET

Development of reproducible gene transfer systems is essential for the introduction of candidate exotic genes into the genomes of different crop plants for enhancing various agronomic traits. *Agrobacterium*-mediated and

microprojectile bombardment methods are widely employed to produce various transgenic plants in diverse crops. Most of the major cereal crops such as rice, wheat, maize, etc., have been successfully transformed using these methods.

Transformation of different millets has so far lagged behind in comparison to other cereals. However, limited attempts have been made in finger millet for the establishment of gene transfer system. Gupta *et al.* (2001) used different plasmid constructs containing *gusA* gene driven by five promoters, namely, Actin1, ubiquitin 1, RbcS, cauliflower mosaic virus (CaMV) 35S, and ppcA-L-Ft, to assess the efficacy of these promoters based on the transient β -glucuronidase activity. These constructs were bombarded into calli derived from mature embryo, leaf lamina, and leaf sheath of finger millet using biolistic TMPDS-1000/He particle delivery system. They reported the highest transient expression of *gusA* gene driven by the maize ubiquitin promoter when compared to other promoters, and concluded that ubiquitin 1 is the most efficient promoter. Shiva Kumar *et al.* (2005) reported standardization of transformation protocols in finger millet employing particle bombardment and *Agrobacterium* methods using *gusA* as a reporter gene.

2.1 Plasmids Used for Production of Transgenics by Particle-Inflow-Gun Method

Plasmid vectors, pPur, pBar, pPin, and pPcSrp carrying *gusA*, *bar*, *pin*, and *PcSrp* genes, driven by CaMV 35S, rice actin, and *Porteresia coarctata* (*Pccp*) promoters along with *nos* terminator, were used for production of finger millet transformants. For optimization of transformation conditions using particle-inflow-gun method, plasmid constructs containing *gusA* and *bar* genes were employed. Embryogenic calli, derived from shoot-tip cultures, were used as target tissue for production of transgenic plants. The calli were bombarded with 1.1 μ m size tungsten particles using the optimized parameters, namely, 15 kg cm⁻² helium gas pressure, 7 cm target distance, 4 h of pre- and postbombardment osmotic treatments with 0.2 M equimolar concentration of sorbital and mannitol (Madhavi *et al.*, 2005).

2.2 Development of Blast-Resistant and Herbicide-Tolerant Transgenic Plants

For production of leaf blast-resistant and herbicide-tolerant finger millet, plasmids carrying *pin* gene and *bar* gene were introduced into the genome of finger millet employing particle-inflow-gun mediated transformation. The *pin* gene was chemically synthesized based on prawn antimicrobial peptide using two oligos with *Bam*HI and *Sac*I restriction enzyme sites (Madhavi *et al.*, 2005). The synthetic gene encoded a 38 amino acid antifungal peptide that was designated as PIN. The *pin* coding sequence was cloned between CaMV 35S promoter and *nos* terminator at *Bam*HI and *Sac*I sites of pPur after excising the *gusA* sequence, and was used for the production of blast-resistant finger millet plants.

Embryogenic calli, co-bombarded with pPin 35S and pBar 35S constructs, were subjected to phosphinothricin (PPT) selection, ranging from 5 to 12 mg l⁻¹ in a sequential manner, using a four-step-selection strategy for selecting stable transformants. About 1.9% of the bombarded calli could survive four stages of selection with PPT. The PPT-resistant calli were then transferred to the regeneration medium containing 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ kinetin for plant regeneration. Regenerants with well-developed root system were transferred to the glass house and were grown to maturity. Most of the primary transformants were healthy and fertile with normal seed set.

The primary transformants (T₀) obtained with *bar* and *pin* genes were identified based on herbicide Basta test, polymerase chain reaction (PCR), Southern blot and Northern blot analyses. The various putative transformants when tested with Basta showed different levels of tolerance to the herbicide. The primary transgenic plants, subjected to PCR, revealed expected amplification products corresponding to the transgenes. Southern analysis, using enzymes with unique sites in the plasmid, showed 1–3 hybridizing bands of higher molecular weight than the plasmid size; it also disclosed 1–10 copies of transgenes with single to multiple integrations in the genomes of transgenic plants. An approximate 30% of the transformants showed the presence of single copies of the introduced transgenes. About 50% of transformants, in general, showed silencing of transgenes as evidenced by Northern blot analysis. Most of these



Figure 1 Transgenic finger millet plants expressing *pin* gene showing blast resistance

transformants were found positive for both *bar* and *pin* genes, confirming stable integration of transgenes into the genome of finger millet.

Three T_1 progenies of finger millet, expressing *pin* and *bar*, were subjected to fungal bioassays and herbicide Basta test to analyze the inheritance mode of introduced transgenes. When T_1 transgenics were scored on a scale of 0–9 for resistance/susceptibility reaction against *P. grisea*, the transgenic plants expressing *pin* displayed high-level resistance to the leaf blast disease (Figure ??). The fungus caused 0.1–0.5-mm lesions on resistant transgenics compared to 5–20-mm lesions observed on susceptible plants. Different T_1 progenies segregated into distinct resistant and susceptible phenotypes conforming to the monogenic ratio. The herbicide Basta test on transgenics also disclosed a clear segregation of 3 tolerant:1 susceptible plants (Madhavi *et al.*, 2005).

2.3 Production of Marker-Free and Salinity-Tolerant Finger Millet

Transgenic finger millet lines, tolerant to NaCl stress were produced by transferring a complementary DNA (cDNA) clone, designated as *PcSrp*, isolated from the cDNA library of salt stressed roots of *P. coarctata*, a salt-tolerant wild grass (Mahalakshmi *et al.*, 2006a). The *PcSrp* gene was cloned downstream to the actin promoter and the expression unit was introduced into calli of finger millet through the particle-inflow-gun method. For selection of transformed calli, 250 mM NaCl was used as a selection agent; selected NaCl-tolerant calli were then regenerated on the

medium containing BAP (1.0 mg l^{-1}), kinetin (0.5 mg l^{-1}), and NaCl (250 mM). Regenerated plants were subjected to NaCl stress and grown to maturity in the glass house. Southern blot analysis of transgenic plants revealed the presence of *PcSrp* gene in the genome of finger millet, and its expression was authenticated by Northern analysis. When T_1 transgenic plants were subjected to 250 mM NaCl salt stress, they segregated into 3 resistant:1 susceptible plants conforming to monogenic ratio. Tolerance of T_1 transformants to salt stress was assessed by subjecting 3-week-old $1T_1$ plants to 250 mM NaCl salt stress up to maturity. Transgenic plants under salt stress showed marked increases in root length, shoot length, and seedling fresh weight as compared to untransformed control plants, which failed to survive. The seed yield of $1T_1$ plants, subjected to 250 mM NaCl stress, ranged between 1.11 and 5.57 g/plant with a mean seed yield of 3.10 g/plant. On the other hand, under unstressed conditions, transgenic plants produced seed yield in the range of 7.73–14.4 g/plant, while in the untransformed plants the seed yield ranged between 15.44 and 29.48 g/plant (Mahalakshmi *et al.*, 2006a). These results amply suggest that use of a salt-inducible, root-specific promoter might obviate the energy drain caused by the constitutive expression of *PcSrp* in transgenic plants.

Under 250 mM NaCl stress, the transgenic plants accumulated higher amount of Na^+ ions in the roots compared to the roots of untransformed plants. On the contrary, the Na^+ ion content was higher in the shoots of untransformed plants compared to the shoots of transgenic plants. However, the untransformed control plants grown

under unstressed conditions accumulated lower Na^+ ions in the roots and shoots when compared to the stressed transgenic and control plants (Mahalakshmi *et al.*, 2006a). The K^+ ion content was higher in the roots of transgenic plants when compared to the roots of untransformed plants. Whereas, shoots of untransformed plants accumulated higher K^+ ion content than the shoots of transgenic plants. On the other hand, the roots of untransformed plants, grown under unstressed conditions, showed lesser K^+ ion content compared to the shoots. An overview of ion profiles amply demonstrates the unequivocal role of *Pcsrp* in bestowing salinity tolerance, by maintaining ion homeostasis, at the whole plant level (Mahalakshmi *et al.*, 2006a).

2.4 Evaluation of *Porteresia coarctata* Promoter in Finger Millet

Employing the *Prosearch* tool developed by us for identification of plant promoters, a promoter sequence, *Pccp*, containing 633 bp has been identified from the genomic sequences of *P. coarctata*, and the same was cloned upstream to *gusA* having *nos* terminator downstream. Using the particle-inflow-gun method, the fusion construct was introduced into callus and various other tissues of finger millet. The transformed tissues/plants showed transient and stable expression of *gusA* gene, confirming the predicted sequence (*Pccp*) of *P. coarctata* as a constitutive promoter (Mahalakshmi *et al.*, 2006b).

3. FUTURE ROAD MAP

Reproducible protocols have been optimized in finger millet for high-frequency *in vitro* plant regeneration as well as genotype-independent genetic transformation system using the particle-inflow-gun method. Employing these protocols, a synthetic antifungal *pin* gene was successfully introduced into finger millet. Stable transgenics, expressing puroindoline (PIN) protein, exhibited high-level resistance to the leaf blast disease. These transgenic lines, endowed with the exotic *pin* gene, appear promising as a novel genetic resource in conventional crossbreeding, besides their use for direct commercial cultivation in areas endemic to blast disease.

The novel *PcSrp* gene cloned from *P. coarctata*, upon introduction into the finger millet, imparted marked tolerance to salt stress at the whole plant level. The marker-free, prototype transgenic finger millet, fortified with *PcSrp*, might serve as a key parent in improving the salinity tolerance of elite finger millet cultivars.

Furthermore, the standardized plant regeneration and transformation protocols offer enormous scope for enhancing various agronomic attributes, such as disease and insect resistance, early maturity, lodging resistance, high harvest index, improved seed quality, etc., of finger millet by deploying desired genes isolated from diverse gene pools. Development of efficient gene transfer protocols using *Agrobacterium* should be given high priority, as this method facilitates single copy transgene integrations, which minimize gene silencing effects in subsequent generations as opposed to the particle-inflow-gun method.

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Cool-Season Forage Grasses

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Although the grass family is very large, relatively few species have been developed as forage crops, and even fewer species have been genetically transformed. Cool-season grasses make most of their growth under cool environment and serve as the primary forage for ruminant animals in temperate regions of the world. Cool-season grass species in which transgenic plants have been developed include: tall fescue (*Festuca arundinacea* Schreb.), perennial ryegrass (*Lolium perenne* L.), Italian ryegrass (*Lolium multiflorum* Lam.), orchardgrass (*Dactylis glomerata* L.), Kentucky bluegrass (*Poa pratensis* L.), and Russian wildrye (*Psathyrostachys juncea* (Fisch.) Nevski) (Wang and Ge, 2006). This chapter focuses on these forage-type grass species.

Tall fescue is indigenous to Europe, also naturally occurring on the Baltic coasts throughout the Caucasus, in western Siberia and extending into China (Jauhar, 1993). Tall fescue introductions have been made into North and South America, Australia, New Zealand, Japan, and South and East Asia (Barnes, 1990). It was introduced into the United States from Europe in the early 1800s. It is widely grown in southern Europe and is the predominant cool-season perennial grass species in the United States (Sleper and West, 1996; Stadelmann *et al.*, 1999).

Perennial and Italian ryegrasses are indigenous to parts of Europe, Asia, and North Africa (Jauhar, 1993; Jung *et al.*, 1996). The first records of cultivated use of perennial ryegrass came from England in about 1677, and Italian ryegrass was grown in Italy under irrigation as a cut forage crop in the 13th century (Jung *et al.*, 1996). The spread of ryegrasses is closely associated with the development of livestock farming. Ryegrasses are widely distributed throughout the temperate zones and are extensively used in Europe, Australia, New Zealand, North America, and Japan (Jung *et al.*, 1996).

Orchardgrass is native to Europe, North Africa, and parts of Asia. This species was introduced into the United States probably in the 1750s, and in 1763 seed was shipped to the United Kingdom from Virginia (Christie and McElroy, 1995). Orchardgrass was first described in North America from plants found growing in an orchard in Virginia. Its British common name, “cocksfoot” refers to the characteristic shape of the panicle (Van Santen and Sleper, 1996). Orchardgrass is perennial forage widely adapted on all continents including the Kerguelen and Crozet Islands of Antarctica (Van Santen and Sleper, 1996).

Kentucky bluegrass is native to Europe, northern Asia, and the mountains of Algeria and Morocco. It is now ubiquitous in permanent pastures in humid temperate regions of the United States, occurring in every state and extending to Canada (Balasko *et al.*, 1995).

Russian wildrye is indigenous to the Eurasian interior. The first recorded introduction into the United States was made in 1927 by United States Department of Agriculture (USDA) (Asay and Jensen, 1996). In North America, it has been seeded most often on arid and semi-arid rangelands of the Northern Great Plains and intermountain regions. Russian wildrye occurs naturally from Iran northward to the lower Volga River and lower Don River regions of Russia in the north, eastward into western Siberia and across Asia to Mongolia and northern China (Asay and Jensen, 1996).

1.2 Taxonomy, Genome Size, and Cytological Features

Festuca is a diverse and widely adapted genus which contains over 80 species, of which approximately 20 exist in the United States (Barnes, 1990). Tall fescue (*F. arundinacea* Schreb.) is the most important forage species worldwide of the *Festuca* genus (Sleper and West, 1996). Tall fescue is a polyploid ($2n = 6x = 42$), wind-pollinated species with a high degree of self-incompatibility. Its genome size is approximately 6×10^3 Mbp (mega base pair) (Seal, 1983). Tall fescue is an outcrossing hexaploid that contains three genomes (P, G1, and G2). The P ($2x$) genome is from a diploid species, meadow fescue (*F. pratensis*), while the G1 and G2 ($4x$) genomes are from a tetraploid species, *F. arundinacea* var. "glauescens" (Sleper, 1985).

Lolium L. is a small genus which includes only eight diploid ($2n = 2x = 14$) species (Terrell, 1966). The commercially most important ryegrasses in cool temperate climates throughout the world are perennial ryegrass (*L. perenne* L.) and Italian or annual ryegrass (*L. multiflorum* Lam.). Both species are outcrossing with genome sizes of about 1.7×10^3 Mbp for perennial ryegrass (Bert *et al.*, 1999) and 2.0×10^3 Mbp for Italian ryegrass (Hutchinson *et al.*, 1979; Inoue *et al.*, 2004).

The genus *Dactylis* is monospecific genus, *D. glomerata* L., divides into many subspecies with ploidy level ranging from diploid ($2n = 2x = 14$) to hexaploid ($2n = 6x = 42$). Tetraploids arose from diploids by chromosome doubling. Cultivated orchardgrass belongs to the subspecies *glomerata*, which is a tetrasomic polyploid with $4x$ chromosomes (Van Santen and Sleper, 1996). The genome size of the tetraploid populations varies

from 3.2×10^3 to 4.3×10^3 Mbp (Creber *et al.*, 1994).

Kentucky bluegrass (*P. pratensis* L.) is a facultative apomictic species. The form of apomixis in Kentucky bluegrass is pseudogamous apospory, although it is also capable of reproducing sexually to varying degrees. A complex series of polyploidy and aneuploidy exists in Kentucky bluegrass. Within the species, chromosome numbers range between 24 and 124 with skewed distributional modes in the 56–59, 63–70, and 84–91 chromosome-number classes (Wedin and Huff, 1996).

Psathyrostachys contains about 10 diploid species including diploids, autotetraploids, and autohexaploids, and they all have the Ns genome (Vogel *et al.*, 1999). Russian wildrye is the only species of *Psathyrostachys* that has proven to be an important forage grass. In its native habitat, it is widely distributed on open slopes and steppes from the Middle East and Russia across central Asia to northern China. The genome size of Russian wildrye is estimated to be 7.5×10^3 Mbp (Vogel *et al.*, 1999). It is typically a cross-pollinated diploid ($2n = 2x = 14$); however, autotetraploid ($2n = 4x = 28$) forms have been artificially induced, and such types may occur in nature (Asay, 1995).

1.3 Economic Importance

Cool-season grasses have played an important role in providing pasture and hay for the livestock industry and are important in soil conservation and environmental protection. Besides being used as forage, some of the grasses, such as turf-type tall fescue, ryegrasses, and Kentucky bluegrass have been widely used for amenity purpose on lawns, parks, sports fields, golf courses, and roadsides.

Tall fescue is a leafy, course-textured, vigorous perennial bunchgrass that reproduces vegetatively through tillering and by seed. It forms the basis for beef cow-calf production in the east-central and southeast United States, supporting over 8.5 million beef cows on 12–14 million hectares of planted areas, and is used for sheep and horse production (Buckner *et al.*, 1979; Hoveland, 1993; Sleper and West, 1996). Its widespread use in the United States can be attributed to a long list of desirable agronomic characteristics, including high yields of herbage, excellent persistence, adaptation

to a wide range of soil conditions, compatibility with various management practices, long grazing season, and low incidence of pest problems (Hanson, 1979; Sleper and West, 1996).

Perennial ryegrass is a palatable, persistent grass of high tillering density that shows resistance to treading and good response to high nitrogen application. In New Zealand, perennial ryegrass is grown on 7 million hectares providing high quality forage to support 60 million sheep and cattle. Perennial ryegrass is—with an estimated 55 million kilograms of seed consumed annually—the main grass species in the European Union (EU) (Spangenberg *et al.*, 1998a).

Italian ryegrass is an annual or biennial highly palatable nutritious grass that shows a rapid establishment from seed, good production in the seeding year, and rapid recovery after defoliation (Jauhar, 1993). Italian ryegrass represents—with an estimated 36 million kilograms of seed—27% of the yearly grass seed consumption in the EU (Spangenberg *et al.*, 1998a). Perennial and Italian ryegrasses form the basis of most pastures for grazing and silage in New Zealand, Australia, and Europe. They are also widely grown in grasslands in the United States and Japan.

Orchardgrass is one of the most important forage grasses in Japan, it is especially adapted in the cooler climates such as the island of Hokkaido (Van Santen and Sleper, 1996). Orchardgrass is the second most commonly sown grass species contributing to a maximum of 10% to pasture production in New Zealand (Van Santen and Sleper, 1996). In the northeast and north-central United States, it is one of the major grass species for pasture (Christie and McElroy, 1995).

Kentucky bluegrass spreads by rhizomes to form sod. Its rhizomatous growth habit makes heavy grazing or frequent cutting are not as detrimental to its sod formation as they are for more upright species (Wedin and Huff, 1996). Kentucky bluegrass is used for grazing on >15 million hectares of pastures throughout the northeastern and north-central United States and substantial areas of Europe and Canada (Eaton *et al.*, 2004).

Although Russian wildrye was introduced into North America in 1926, its potential in range improvement was not fully recognized until the 1950s. It since became an important forage grass on semi-arid rangelands of the intermountain West and Northern Great Plains. Russian wildrye is

exceptionally cold and drought tolerant (Asay and Jensen, 1996). The species provides valuable pasture that is often used to complement native rangeland during the late summer and fall when nutritive quality of most forages is relatively low (Asay, 1991).

1.4 Traditional Breeding: Objectives, Strategies, and Achievements

Breeding for improved forage grasses in North America began in earnest in the 1930s and 1940s. Compared to centuries of human involvement in domestication and selection in maize and other cereal crops, forage grass breeding is a relatively recent development. Many of the first grass cultivars widely used in the United States, such as “Kentucky-31” tall fescue, “Linn” perennial ryegrass, and “Merion” Kentucky bluegrass, were ecotypes subjected to little or no selective breeding for specific traits (Wang *et al.*, 2001a).

The release of new cultivars has tremendous impact on the adoption and expansion of grasses. For example, tall fescue grew on approximately 16 000 ha in 1940 in the United States, with the release of the “Kentucky-31” and “Alta” cultivars during the early 1940s by the Kentucky and Oregon Agricultural Experiment Stations, by 1973 it had become the predominant cool-season perennial grass occupying 12–14 million hectares (Buckner *et al.*, 1979; Sleper and Buckner, 1995).

Cultivars have been developed with documented improvement in traits such as forage quality (Vogel *et al.*, 1991) and seedling vigor (Asay *et al.*, 1985). For some traits, improvement has been inconsistent or slow, particularly with regard to forage yield. It was estimated that the average rate of genetic gain for yield was 13.5% per decade for several grain crops compared to 4% per decade for forage yield of several grasses and legumes (Humphreys, 1997). Several reasons have been suggested for such disparity, including the genomic complexity (e.g., polyploidy, outcrossing) typical of forage grasses, and insufficient breeding efforts devoted to improving forage yield of a given species (Casler *et al.*, 2000).

Persistence, a combination of several traits, such as drought, heat, and cold tolerance, is an important target for grass cultivar improvement. Similarly, improving forage quality, often via

selection for increased digestibility, is a critical objective in many forage grass breeding programs. It has been shown that increased digestibility can lead to substantial improvements in animal gains per hectare (Casler, 2001).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Genetic improvement of cool-season grasses by conventional plant breeding is slow since many of these species are predominantly, if not completely, allogamous wind-pollinated grasses and generally self-sterile (Kasperbauer, 1990; Jauhar, 1993; Spangenberg *et al.*, 1998b). These features represent a great challenge to the breeder because these plants are usually heterozygous, and self-incompatibility limits inbreeding to concentrate desired genes for use in rapid development of new cultivars (Kaul, 1990). Although many improved forage varieties have been developed through conventional breeding efforts, progress in improving particular traits has slowed (Ha *et al.*, 1992). Transgenic approaches are expected to accelerate or complement conventional breeding approaches, since they offer the opportunity to generate novel genetic variations that is either absent or has very low heritability.

2. DEVELOPMENT OF TRANSGENIC COOL-SEASON FORAGE GRASSES

2.1 Donor Genes, Promoters, and Selectable Markers

Much of the reported transformation work on cool-season forage grasses has been on method development using selectable marker genes and reporter genes (Table 1). The selectable marker genes used are: hygromycin phosphotransferase gene (*hph*) from *Escherichia coli*, phosphinothricin acetyltransferase gene (*bar*) from *Streptomyces hygroscopicus*, and neomycin phosphotransferase II gene (*nptII*) of transposon Tn5 (Table 1). The reporter genes are β -glucuronidase gene (*gusA*) from *E. coli* and green fluorescent protein gene (*mgfp*) from *Aequorea victoria*. For *Agrobacterium*-mediated transformation, the *gusA* reporter gene needs to have an intron (e.g., a

catalase intron) inside the coding sequence to ensure that expression of glucuronidase activity is derived from eukaryotic cells, not from expression by residual *A. tumefaciens* cells (Wang and Ge, 2005a, b).

The promoters used to construct the chimeric genes are: cauliflower mosaic virus (CaMV) 35S promoter, rice actin promoter, maize ubiquitin promoter, or tissue-specific promoters. The terminators used are the CaMV 35S terminator or the *nos* terminator from *Agrobacterium*.

Overexpression of heterologous genes is a straight forward approach for trait improvement. The target genes are often from unrelated species (Table 1). For example, to improve protein quality of forage grass for ruminant nutrition, the sunflower seed albumin 8 (*SFA8*) transgenes were constructed under the control of constitutive (CaMV 35S) and light-regulated (wheat cab) promoters (Wang *et al.*, 2001b). The transgenes were introduced into tall fescue and led to the accumulation of sulfur-rich SFA8 protein rich in sulfur-containing amino acids (methionine and cysteine) (Wang *et al.*, 2001b). To improve cold tolerance of perennial ryegrass, two fructan biosynthetic genes from wheat, *wft1* and *wft2*, which encode sucrose-fructan 6-fructosyltransferase (6-SFT) and sucrose-sucrose 1-fructosyltransferase (1-SST), respectively, were introduced into perennial ryegrass under the control of CaMV 35S promoter (Hisano *et al.*, 2004).

For down-regulation of endogenous genes, the target gene is normally isolated from a species and transferred back to the same species or closely related species. To improve forage digestibility by down-regulation of lignin biosynthesis, genes encoding two key enzymes involved in lignin biosynthesis, cinnamyl alcohol dehydrogenase (CAD), and caffeic acid *O*-methyltransferase (COMT), were cloned from tall fescue (Chen *et al.*, 2002). Transgenic tall fescue plants were produced using antisense and sense CAD and COMT gene constructs under the control of maize ubiquitin promoter. Severely reduced mRNA levels and significantly decreased enzymatic activities were found in some transgenic lines. These transgenic tall fescue plants had reduced lignin content, altered lignin composition, and increased *in vitro* dry matter digestibility (Chen *et al.*, 2003, 2004). In order to develop hypo-allergic grasses to reduce

Table 1 Transgenic plants obtained in major cool-season forage grasses

Plant species	Transgene	Method	References
<i>Festuca arundinacea</i> (tall fescue)	<i>hph</i> ^(a) , <i>bar</i> ^(b)	Protoplasts	Wang <i>et al.</i> (1992)
	<i>hph</i> , <i>gusA</i> ^(c)	Protoplasts	Ha <i>et al.</i> (1992)
	<i>hph</i>	Biolistics	Spangenberg <i>et al.</i> (1995a)
	<i>hph</i>	Protoplasts	Dalton <i>et al.</i> (1995)
	<i>hph</i>	Whiskers	Dalton <i>et al.</i> (1998)
	<i>hph</i> , <i>gusA</i>	Protoplasts	Kuai <i>et al.</i> (1999)
	<i>hph</i> , <i>bar</i> , <i>gusA</i>	Biolistics	Cho <i>et al.</i> (2000)
	<i>sfa8</i> ^(d) , <i>hph</i>	Biolistics	Wang <i>et al.</i> (2001b)
	<i>hph</i> , <i>gusA</i>	Biolistics	Wang and Ge (2003)
	<i>CAD</i> ^(e) , <i>hph</i>	Biolistics	Chen <i>et al.</i> (2003)
	<i>hph</i> , <i>gusA</i>	<i>Agrobacterium</i>	Bettany <i>et al.</i> (2003)
	<i>COMT</i> ^(f) , <i>hph</i>	Biolistics	Chen <i>et al.</i> (2004)
	<i>Ipt</i> ^(g) , <i>bar</i>	Biolistics	Hu <i>et al.</i> (2005)
	<i>hph</i> , <i>gusA</i>	<i>Agrobacterium</i>	Dong and Qu (2005)
	<i>hph</i> , <i>gusA</i> , <i>gfp</i> ^(h)	<i>Agrobacterium</i>	Wang and Ge (2005a)
<i>Lolium perenne</i> (perennial ryegrass)	<i>hph</i> , <i>gusA</i>	Biolistics	Spangenberg <i>et al.</i> (1995b)
	<i>npt2</i> ⁽ⁱ⁾ , <i>gusA</i>	Protoplasts	Wang <i>et al.</i> (1997)
	<i>hph</i>	Whiskers	Dalton <i>et al.</i> (1998)
	<i>hph</i> , <i>gusA</i>	Biolistics	Dalton <i>et al.</i> (1999)
	<i>Npt 2</i>	Biolistics	Alpeter <i>et al.</i> (2000)
	<i>RgMV-CP</i> ^(j) , <i>npt2</i>	Biolistics	Xu <i>et al.</i> (2001)
	<i>Lol p1</i> ^(k) , <i>Lol p2</i>	Biolistics	Petrovska <i>et al.</i> (2004)
	<i>Wft1</i> ^(l) , <i>wft2</i> ^(m) , <i>bar</i>	Biolistics	Hisano <i>et al.</i> (2004)
	<i>arg E</i> ⁽ⁿ⁾ , <i>hph</i>	Biolistics	Chen <i>et al.</i> (2005)
	<i>OsNHX1</i> ^(o) , <i>bar</i>	<i>Agrobacterium</i>	Wu <i>et al.</i> (2005)
	<i>hph</i> , <i>gusA</i>	<i>Agrobacterium</i>	Bajaj <i>et al.</i> (2006)
	<i>hph</i> , <i>gusA</i>	<i>Agrobacterium</i>	Sato and Takamizo (2006)
<i>Lolium multiflorum</i> (Italian ryegrass)	<i>hph</i> , <i>gusA</i>	Biolistics	Ye <i>et al.</i> (1997)
	<i>npt2</i> , <i>gusA</i>	Protoplasts	Wang <i>et al.</i> (1997)
	<i>hph</i> , <i>gusA</i>	Whiskers	Dalton <i>et al.</i> (1998)
	<i>hph</i> , <i>gusA</i>	Biolistics	Dalton <i>et al.</i> (1999)
	<i>sacB</i> ^(p) , <i>hph</i>	Biolistics	Ye <i>et al.</i> (2001)
	<i>hph</i> , <i>gusA</i>	<i>Agrobacterium</i>	Bettany <i>et al.</i> (2003)
	<i>Lol p1</i> , <i>Lol p2</i>	Biolistics	Petrovska <i>et al.</i> (2004)
	<i>Ipt</i> , <i>gusA</i> , <i>hph</i>	Biolistics	Li <i>et al.</i> (2004)
<i>Dactylis glomerata</i> (orchardgrass)	<i>RCC2</i> ^(q) , <i>hph</i>	Biolistics	Takahashi <i>et al.</i> (2005)
	<i>Hph</i>	Protoplasts	Horn <i>et al.</i> (1988)
	<i>bar</i> , <i>gusA</i>	Biolistics	Denchev <i>et al.</i> (1997)
	<i>hph</i> , <i>bar</i> , <i>gusA</i>	Biolistics	Cho <i>et al.</i> (2001)
	<i>hph</i> , <i>gusA</i>	<i>Agrobacterium</i>	Lee <i>et al.</i> (2006)
<i>Poa pratensis</i> (Kentucky bluegrass)	<i>hph</i> , <i>gusA</i> , <i>gfp</i>	Biolistics	Ha <i>et al.</i> (2001)
	<i>hph</i> , <i>bar</i> , <i>gusA</i>	Biolistics	Gao <i>et al.</i> (2006)
<i>Psathyrostachys juncea</i> (Russian wildrye)	<i>hph</i> , <i>gusA</i>	Biolistics	Wang <i>et al.</i> (2004a)

^(a)Hygromycin phosphotransferase from *E. coli*^(b)Phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus*^(c) β -glucuronidase from *E. coli*^(d)Sunflower albumin8^(e)Cinnamyl alcohol dehydrogenase gene from tall fescue^(f)Caffeic acid *O*-methyltransferase gene from tall fescue^(g)Isopentenyl transferase gene from *Agrobacterium tumefaciens*^(h)Green fluorescent protein gene from *Aequorea victoria*⁽ⁱ⁾Neomycin phosphotransferase II of transposon Tn5^(j)Ryegrass mosaic virus coat protein^(k)ryegrass pollen allergen gene from perennial ryegrass^(l)sucrose-fructan 6-fructosyltransferase (6-SFT) from wheat^(m)sucrose-sucrose 1-fructosyltransferase (1-SST) from wheat⁽ⁿ⁾N-acetylornithinase gene from *E. coli*^(o)rice vacuolar Na⁺/H⁺ antiporter^(p)levansucrase gene from *Bacillus subtilis*^(q)rice chitinase

hayfever and seasonal allergic asthma caused by grass pollen, genes encoding major pollen allergenic proteins, Lol p1 and Lol p2, were cloned from perennial ryegrass. The antisense *Lol p1* and *Lol p2* transgene under the control of a pollen-specific promoter were introduced into perennial and Italian ryegrasses (Petrovska *et al.*, 2004). The accumulation levels of Lol p1 and Lol p2 allergens in pollen were reduced in the transgenic ryegrass plants (Petrovska *et al.*, 2004).

2.2 Methods Employed for Transformation

Transgenic cool-season grass plants were first obtained by direct gene transfer to protoplasts (Horn *et al.*, 1988; Wang *et al.*, 1992). Because protoplast culture and transformation are very delicate systems to work with, in recent years biolistic transformation and *Agrobacterium*-mediated transformation have become the main methods for producing transgenic grasses.

As shown in Table 1, most of the protoplast works were done in the 1990s. Recovery of transgenic plants from protoplasts was reported for orchardgrass (Horn *et al.*, 1988), tall fescue (Ha *et al.*, 1992; Wang *et al.*, 1992; Dalton *et al.*, 1995; Kuai *et al.*, 1999), Italian ryegrass (Wang *et al.*, 1997), and perennial ryegrass (Wang *et al.*, 1997).

Biolistics or microprojectile bombardment employs high-velocity gold or tungsten particles to deliver DNA into living cells for stable transformation (Sanford, 1988; Christou, 1992). Because biolistic transformation is a physical process that involves only one biological system, it is a fairly reproducible procedure that can be easily adapted from one laboratory to another laboratory. Transgenic forage plants have been obtained by microprojectile bombardment of embryogenic cells in tall fescue (Spangenberg *et al.*, 1995a; Cho *et al.*, 2000; Wang *et al.*, 2001b, 2003a; Chen *et al.*, 2003, 2004), perennial ryegrass (Spangenberg *et al.*, 1995b; Dalton *et al.*, 1999; Altpeter *et al.*, 2000; Xu *et al.*, 2001; Petrovska *et al.*, 2004; Chen *et al.*, 2005), Italian ryegrass (Ye *et al.*, 1997, 2001; Dalton *et al.*, 1999; Li *et al.*, 2004; Petrovska *et al.*, 2004), orchardgrass (Denchev *et al.*, 1997; Cho *et al.*, 2001), Kentucky bluegrass (Ha *et al.*, 2001; Gao *et al.*, 2006), and Russian wildrye (Wang *et al.*, 2004a) (Table 1).

Agrobacterium-mediated transformation has the advantage of allowing for low copy number integration of the transgenes into the plant genome. In recent years, significant progress has been made in developing transformation protocols using *Agrobacterium tumefaciens* as a vector. Transgenics have been obtained by *Agrobacterium*-mediated transformation in tall fescue (Dong and Qu, 2005; Wang and Ge, 2005a), perennial ryegrass (Altpeter *et al.*, 2004; Wu *et al.*, 2005; Bajaj *et al.*, 2006; Sato and Takamizo, 2006), Italian ryegrass (Bettany *et al.*, 2003), and orchardgrass (Lee *et al.*, 2006).

2.3 Selection of Transformed Tissue

The establishment of efficient selection schemes by applying suitable selection pressure for an appropriate length of time is one of the critical aspects of successfully generating transgenic grasses. Because many grasses have high endogenous tolerance to antibiotics, particularly kanamycin, most of the reports in forage grass transformation used *hph* or *bar* as selectable marker gene and hygromycin or phosphinothricin (PPT) as selection agent (Table 1).

The stable integration and expression of a chimeric *hph* gene result in the ability to phosphorylate hygromycin, thus rendering the transformed plant cells resistant to concentrations of this antibiotic, which are lethal to untransformed cells. The expression of chimeric *bar* gene in transfected cells results in the acetylation of PPT and thus renders plant cells resistant to this herbicide. The concentrations of hygromycin and PPT used for selection varied with the species and the selection system used. For example, hygromycin concentrations of 50–250 mg l⁻¹ were used for selection in tall fescue. In some cases, a stepwise increase in hygromycin concentration (e.g., from 50 to 200 mg l⁻¹ for perennial ryegrass and from 25 to 100 mg l⁻¹ for Italian ryegrass) and a doubling of the selection pressure in every 2-week subculture step were used to recover resistant calluses (Spangenberg *et al.*, 1995b; Ye *et al.*, 1997). It should be noted that different cultivars and genotypes could have different levels of endogenous tolerance to antibiotics, which might lead to the recovery of escapes if not enough selection pressure is applied.

2.4 Regeneration of Whole Plant

For both biolistic transformation and *Agrobacterium*-mediated transformation, the identification and propagation of compact, yellowish/whitish, embryogenic calluses have been considered as key factors affecting transformation frequency (Xiao and Ha, 1997; Spangenberg *et al.*, 1998b; Zhang *et al.*, 2003; Li and Qu, 2004; Luo *et al.*, 2004; Dong and Qu, 2005; Wang and Ge, 2005a). The use of nonmorphogenic cultures only allowed the generation of resistant callus lines but not transgenic plants (Hensgens *et al.*, 1993; van der Maas *et al.*, 1994).

The establishment of an efficient plant regeneration system from embryogenic cultures is a prerequisite for stable transformation. In most of the reports, embryogenic calli were first induced and then used for particle bombardment or for incubation with *Agrobacterium*; resistant calluses were obtained after several weeks of antibiotic selection. In general, if highly embryogenic cultures were used for transformation, transgenic plants could be regenerated from the resistant calluses.

2.5 Testing of Transgenics

Detailed molecular analyses should be carried out to demonstrate the stable integration and expression of the transgenes in grasses. Insufficient molecular characterization may have contributed to the lack of reproducibility of some reports. Southern hybridization analysis, Northern hybridization analysis, enzyme assays, or other data confirming the functionality of the transgene should be provided (Wang *et al.*, 2001).

Transmission of foreign genes to progenies is critical for any potential use of transgenic material for producing novel germplasm. Because of the outcrossing nature and vernalization requirement of most of the cool-season grasses, only limited information is available on transmission of foreign genes in plants obtained by biolistic and *Agrobacterium*-mediated transformation (Spangenberg *et al.*, 1998b; Wang *et al.*, 2003a; Wang and Ge, 2005a).

The meiotic transmission of transgenes was studied in selected transgenic tall fescue and Italian ryegrass plants after reciprocal crossings

with wild-type untransformed control plants (Spangenberg *et al.*, 1998b; Wang *et al.*, 2003a; Wang and Ge, 2005a). T₁ seedlings of transgenic tall fescue and Italian ryegrass obtained by particle bombardment and transgenic tall fescue obtained by *Agrobacterium*-mediated transformation were subjected to polymerase chain reaction (PCR) and southern hybridization analyses. Expected 1:1 segregation ratios of the transgenes were observed in screened T₁ seedlings from independent T₀ transgenic plants. The results demonstrated stable meiotic transmission of the transgenes following Mendelian rules in transgenic grass plants obtained by biolistic or *Agrobacterium* transformation.

Transgenic forage-type tall fescue plants were transferred to the field and evaluated regarding their agronomic performance and pollen viability (Wang *et al.*, 2003b, 2004b, c). Although agronomic performance of the primary transgenics was generally inferior to the seed-derived plants, no major differences were found between the progenies of transgenics and the progenies of seed-derived plants (Wang *et al.*, 2003b). Progenies of primary transgenics had similar pollen viability when compared with that of seed-derived plants (Wang *et al.*, 2004b). The results indicate that once seeds are obtained from the primary transgenic plants, normal pollen viability and agronomic performance of the progenies can be expected. No indication of weediness of the transgenic tall fescue plants was observed. The field study provided evidence that outcrossing grass plants generated through transgenic approaches can be incorporated into forage breeding programs.

2.6 Specific Regulatory Measures Adopted

Prior to 2005, field trials of transgenic forage grasses required only a notification to APHIS (Animal and Plant Health Inspection Service) of USDA. Since 2005, field release of outcrossing transgenic grasses has been much tightened, requiring permit application, environmental assessment, and public comments. The major concern is pollen-mediated transgene flow in outcrossing grasses. Because human consumption is indirect, biosafety evaluation of transgenic grasses will likely to focus on their environmental or ecological impacts. Two questions need to be

answered regarding biosafety of transgenic grasses. First, how far can grass pollen disperse and still remain viable, and second, what is the probability of transgene escape by crossing with related grass species under natural conditions? Rigorous scientific studies using convincing molecular techniques are urgently needed to answer these questions.

3. FUTURE ROAD MAP

3.1 Expected Products

With the development of transgenic technologies in forage grasses, efforts have been made to produce transgenic plants with improved agronomic characteristics or evaluate novel strategies for molecular breeding. One of the most promising aspects is to genetically modify lignin biosynthesis to improve digestibility of forage grasses.

Lignification of cell walls during plant development has been identified as the major factor limiting digestibility of forages (Buxton and Russell, 1988; Buxton and Redfearn, 1997; Vogel and Jung, 2001). Feeding and grazing studies have shown that small changes in forage digestibility can have a significant impact on animal performance (Vogel and Sleper, 1994; Casler and Vogel, 1999). Lignin in forage grasses comprises guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, and *p*-hydroxyphenyl (H) units derived from *p*-coumaryl alcohol. Transgenic modification of lignin biosynthesis has been well studied in dicot species, such as tobacco, *Arabidopsis*, alfalfa, and poplar (Baucher *et al.*, 1998; Dixon *et al.*, 2001; Humphreys and Chapple, 2002; Reddy *et al.*, 2005). In grasses, it has been shown that down-regulation of CAD and COMT led to altered lignin and significantly increased *in vitro* dry matter digestibility of transgenic tall fescue (Chen *et al.*, 2003, 2004). Genetic modification of lignin has been considered to be of low risk because it is unlikely that lignin reduction will increase plant fitness (Bradford *et al.*, 2005). Engineering of such a low-risk trait is likely to reduce biosafety concerns of transgenic grasses.

The development of hypo-allergic grasses is another interesting approach. Grass pollen is a

widespread source of airborne allergens and is a major cause of hayfever and seasonal allergic asthma. The introduction of antisense *Lol p1* and *Lol p2* transgenes into ryegrass plants led to the reduction in accumulation levels of *Lol p1* and *Lol p2* allergens in pollen, as demonstrated by immunoblots using polyclonal antibodies raised against the recombinant *Lol p1* and *Lol p2* allergens as well as with sera from grass pollen sensitized patients (Petrovska *et al.*, 2004). The development of hypo-allergenic grass cultivars will have tremendous benefits for public health.

It is feasible to improve many other traits in grasses by transgenic technologies, such as tolerance to drought, cold, salt, diseases, and insects. Risk assessments for such traits will probably be very demanding.

3.2 Addressing Risks and Concerns

Pollen-mediated gene flow in outcrossing grasses is a natural process that has happened in the past and will continue to happen in the future. The introduction of transgenic techniques has brought new attention to the process and raised both ecological and economic issues for scientists and policymakers to consider. As novel transgenic grasses are tested and grown in the field, preventing unwanted gene flow will present technical and regulatory challenges as well as possible economic conflict (Wang *et al.*, 2004d). In recent years, strategies have been developed to allow desired traits to be obtained through precise genetic modification within the plant's own genome rather than through introduction of genetic variation from unrelated species (Nielsen, 2003; Rommens *et al.*, 2004). By cloning genes and promoters from a species and reinserting the sequences into the same species, no new genetic information is introduced into the transgenics from other species. Such strategy can be easily utilized for the effective improvement of some traits, such as digestibility by lignin modification or down-regulation of pollen allergens.

To limit pollen-mediated gene flow in transgenic grasses, development of nonflowering plants or male-sterile materials are obvious strategies. However, such strategies need to overcome the challenges of seed production and economics of commercialization.

3.3 Future Prospects

With the availability of more and more gene sequencing information, such as expressed sequence tags (ESTs), gene isolation has become easier than ever before. Functional characterization of genes and their gene regulatory elements has become the major bottleneck. Although much progress has been made in developing transformation methods in the last decade, the production of transgenic grasses is still time consuming, laborious, and inefficient. There is a great need to improve transformation efficiency in the important forage grasses and thus allow the production of large numbers of transgenic plants in a relatively short time. In view of the rapid development and improvement of transformation methods for different grasses in the last decade, it is foreseeable that further optimization of the *Agrobacterium* or biolistics-based methods will lead to more efficient and refined transformation systems in the near future.

Transgenic technologies developed for forage grasses have opened up new opportunities for molecular breeding of these species. The major challenge now is how to apply the technology to produce new germplasm and cultivars that satisfy regulatory requirements. By efficient incorporation of novel materials into applied forage grass breeding programs, it is expected that transgenic technology will accelerate the development of landmark cultivars in the 21st century.

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Bahiagrass

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1. INTRODUCTION

Bahiagrass (*Paspalum notatum* Flüggé) is a perennial, warm-season turf and forage grass, with wide distribution in the tropical and subtropical regions of the world. Bahiagrass is one of the most drought-tolerant grasses due to an extensive root system. It performs well in infertile, sandy soils and does not require high inputs. Unlike many other warm season grasses, bahiagrass produces large amounts of seeds. Bahiagrass resists most pests and diseases, and tolerates overgrazing. Main targets for genetic improvement of bahiagrass include forage quality, turf quality parameters including seedhead suppression, leaf texture, turf density, further enhancement of its abiotic stress tolerance and low input characteristics, mole cricket, and herbicide resistance.

1.1 Origin, Distribution, and History

Species of the genus *Paspalum* are abundant in the tropical and warm temperate regions, especially Brazil, eastern Bolivia, Paraguay, and northeastern Argentina. This South American center of diversity is believed to be the center of origin of the species (Gates *et al.*, 2004). “Pensacola”, the predominantly used diploid cultivar of bahiagrass belongs to *P. notatum* var. *saurae*. The var. *saurae* types are native to Corrientes, Entre Rios and the eastern edge of the Santa Fe Provinces in Argentina, and

large natural populations with genetic diversity were found in these areas (Burton, 1967). Common autotetraploid bahiagrass races like the popular cultivar “Argentine” share homologous chromosomes with races of var. *saurae*.

“Pensacola” bahiagrass is found in most countries of the western hemisphere. Several South American countries use the cultivars “Pensacola” and the derived cultivar “Tifton 9” for forage as well as turf purposes. Bahiagrass plantings are found in southern Brazil where it is an important forage crop. In the United States, “Pensacola” bahiagrass escaped from cultivation and has become naturalized in the southeastern States. The tetraploid races of bahiagrass are found in a wide variety of habitats such as open areas, savannas and cultivated pastures in central Mexico, Argentina, and throughout the West Indies. Common bahiagrass is found as the primary constituent in many native pastures in southern Brazil, Paraguay, Uruguay, and northeastern Argentina. Australia has naturalized populations of both “Pensacola” and tetraploid races. Bahiagrass is used in pastures in southwestern Japan. In addition, the use of bahiagrass is being explored in Taiwan and Zimbabwe. Thus bahiagrass is widely distributed in tropical and subtropical regions of the world.

The Bureau of Plant Industry (SPI No. 35067, Office of Foreign Seed and Plant Introductions) first introduced bahiagrass in the United States in 1913 (Scott, 1920). Seeds from Cuba were used to establish the first experimental plantings in Georgia in 1930. Seeds of “Argentine” bahiagrass

and “Paraguay 22” bahiagrass arrived in 1944 and 1947, respectively and were distributed for testing by United States Department of Agriculture (USDA) and the Florida Experimental Agriculture Research Station. “Pensacola” bahiagrass seeds probably arrived on a ship from Argentina before 1926 and were first found growing near the docks in “Pensacola”, FL (Burton, 1967). The introduction and use of “Pensacola” bahiagrass for pastures in the southeastern United States was a major achievement (Hoveland, 2000). “Pensacola” bahiagrass is more cold tolerant than “Argentine” and grows throughout the southeastern United States, from Texas to North Carolina, and extends into Arkansas and Tennessee. “Pensacola” bahiagrass was even reported in southeastern Oklahoma and Virginia (Gates *et al.*, 2004) but it shows a low productivity and persistence in colder environments.

1.2 Botanical Description

1.2.1 Taxonomy

Bahiagrass was first described using a plant collected by Schrader and Ventenat from St. Thomas Island in 1810. It is classified as *P. notatum* Flüggé. There are several synonyms including *Paspalum saltense* Arechavaleta, *Paspalum uruguayense* Arechavaleta, and *P. notatum* var. *latifolium* Doell. The common names used in different countries are bahiagrass (United States), pasto horqueta (Argentina), capii cabayu (Paraguay), and grama batatis (Brazil). The sexual diploid form is classified as *P. notatum* var. *saurae* Parodi. It is also known as *Paspalum sauræ* (Parodi) Parodi (Gates *et al.*, 2004).

1.2.2 Habit

Bahiagrass is perennial, with strong, shallow, horizontal rhizomes that have short, stout internodes. The internodes are usually covered with old, dry leaf sheaths. Culms are simple, ascending, geniculate at the node between the first and second elongated internodes, otherwise erect, 10–60 cm tall. Leaves are mostly crowded at the base with overlapped keeled sheaths, glabrous, or with ciliate margins mainly toward the summit. Blades are usually flat or somewhat folded toward

the base, linear lanceolate 3–30 cm long, 3–12 mm wide, usually glabrous or ciliate toward the base and rarely pubescent throughout. Subconjugate inflorescences emerge as a 60–90 cm long stalk with a short almost imperceptible common axis of typically two 3–14 cm long racemes (rarely three). Racemes are ascending or recurved-divergent in some races. The rachis is glabrous, flexuous, and green or purplish. Spikelets are solitary in two rows on one side of the rachis, obovate or ovate, shining, glabrous, 2.5–4 mm long and 2–2.8 mm wide. Anthers and stigmas are usually purple, the fruit is oval about 1.2–1.8 mm wide (summarized from Gates *et al.*, 2004).

On the basis of botanical characteristics, common, tetraploid bahiagrass is the typical form of *P. notatum*, characterized by broad leaves, strong roots, and stout rhizomes with short internodes. When compared to common, tetraploid bahiagrass, the diploid “Pensacola” type is taller, with longer and narrower leaves, smaller spikelets, and more racemes per inflorescence. It belongs to *P. notatum* var. *sauræ*.

1.2.3 Cytological features

The base chromosome of bahiagrass is $x = 10$. Ploidy levels in the wild range from diploid ($2n = 2x = 20$) to pentaploid ($2n = 5x = 50$). The botanical variety *P. notatum* var. *sauræ* comprises all races with $2n = 2x = 20$, commonly known as “Pensacola” bahiagrass. Studies of chromosome numbers of naturally occurring bahiagrass accessions from South America revealed that most of them were tetraploids ($2n = 4x = 40$), with a few diploids ($2n = 2x = 20$), one triploid ($2n = 3x = 30$), and one pentaploid ($2n = 5x = 50$) (Gates *et al.*, 2004). Diploids found in “Argentine” were considered indigenous, while those found in Brazil were escapes from cultivated pastures. Pentaploids ($2n = 5x = 50$) and hexaploids ($2n = 6x = 60$) have been created artificially by pollinating apomictic tetraploids ($2n = 4x = 40$) with pollen from diploids and tetraploids, respectively, by the fertilization of an unreduced egg ($2n + n$) (Burton 1948; Martínez *et al.*, 1994). Tetraploid plants have been produced by doubling the chromosomes of diploid plants by colchicine (Forbes and Burton, 1961a). Octaploid plants with 80 chromosomes have also been produced from tetraploid plants using the same approach (Quarin, 1999).

The tetraploid races are believed to have originated by autopolyploidy. Evidence for this was provided by chromosome pairing studies carried out in induced sexual autotetraploids, natural apomictic tetraploid strains, and sexual \times apomictic tetraploid hybrids (Forbes and Burton, 1961b). It has been observed that in the pollen mother cells (PMC) of tetraploid plants, the chromosomes pair primarily as multivalents during meiosis I, forming 2–10 quadrivalents (Forbes and Burton, 1961b; Fernandes *et al.*, 1973). Also, triploid hybrids from crosses between diploid and tetraploid plants have as many as ten trivalents per PMC during meiosis I. This indicates that there is complete homology between the genomes of the diploid parent and both genomes of the tetraploid male parent (Forbes and Burton, 1961b). These data provide evidence for the assumption that *P. notatum* is an agamic complex containing many cytotypes with different ploidy levels, mostly autotetraploids.

1.3 Economic Importance

Bahiagrass is widely used in tropical and subtropical regions of the world including southwestern Japan, South America, and the southeastern United States, covering an estimated 5 million acres in Florida alone. The primary use of Bahiagrass is for forage, in Florida alone, bahiagrass supports about 1.2 million head of beef cattle. In addition it is also popular as utility or low-input turf. Bahiagrass is well adapted to sandy soils and remains productive with low fertility and low pH. It also tolerates periodic flooding. Bahiagrass is one of the most drought tolerant grasses due to its extensive root system. Bahiagrass has very few disease and pest problems. Mole crickets and fall armyworms may cause some damage. Ergot may reduce seed yields, even though damage is usually minimal (Blount *et al.*, 2002). The rhizomes of bahiagrass store large amounts of organic and inorganic nutrients and contribute to the persistence of the species.

1.3.1 Production

Bahiagrass, in contrast to many warm season grasses, is an abundant seed producer. Due to dor-

mancy, seed germination typically occurs slowly over a period of time (West and Marousky, 1989). In addition, seedlings are not very competitive against weeds (Williams and Webb, 1958). Hence the scarification of seeds to break the dormancy, preparation of a good seedbed and careful seed placement is important for quick establishment of a new bahiagrass stand (Burton, 1939, 1940). In Florida, the six warmest months of the year (April–September) comprise the major growing season and account for more than 85% of the annual production (Beatty *et al.*, 1980; Mislevy and Everett, 1981). Day length and temperature play important roles in the productivity of bahiagrass in subtropical and more temperate regions (Gates *et al.*, 2004). Following shorter days and the first freeze events bahiagrass becomes dormant, and alternative forage like cool season grasses becomes necessary to support the cattle industry. The timing of nitrogen application has an impact on the herbage accumulation pattern (Beatty *et al.*, 1980). Bahiagrass pastures are easy to manage as they tolerate overgrazing. However, the forage quality is comparatively low due to relatively low protein content and antinutritional factors. The antinutritional factors include secondary metabolites such as tannins, alkaloids, and phenolic compounds (e.g., lignins) or structural components of cells and tissues (Gates *et al.*, 2004). Bahiagrass can tolerate intense grazing and clipping (Sampaio and Beatty, 1976).

The tolerance of bahiagrass to drought conditions, periodic flooding, and light sandy soils with low fertility makes it desirable as forage and low-input turf. However, bahiagrass does not tolerate severe cold temperatures. This limits production and use in transition zones to temperate climate.

1.3.2 Crop rotation

Bahiagrass is also valuable in crop rotations. The use of bahiagrass has been reported to effectively control the population of root-knot nematodes of peanut (Rodriguez-Kabana *et al.*, 1988). Reduction in the incidence of stem rot and limb rot in peanut was reported following rotation with bahiagrass (Johnson *et al.*, 1999). Similarly, there was a reduction in root diseases from *Rhizotonia solani* or *Pythium* spp. in snap bean and cucumber (Sumner *et al.*, 1999).

1.3.3 Seed production

This is a supplementary source of income on some ranches. An acre of bahiagrass can yield from 23 to 68 kg of clean seed (Chambliss and Adjei, 2002).

1.3.4 Hay

If bahiagrass is harvested prior to flowering, it can provide good quality hay. However, harvesting at this stage may be difficult because the grass is low growing and dense. Usually surplus growth on pastures is cut and sold as hay (Chambliss and Adjei, 2002).

1.3.5 Sod production

Sod provides a popular alternative to seeds for establishment of new lawns. Lawns are established quicker by using sod, and establishment success is not dependent on seed quality and less dependent on environmental factors. Typically well-established pastures are utilized for sod production. Bahiagrass is the predominant utility turf grass, planted along highway right-of-ways throughout the southeastern United States and is used as low-input lawn grass in large turf areas. It has an excellent persistence and low or no irrigation requirement. The major disadvantage as turf is the prolific production of 60–90 cm tall seedheads throughout the fall and summer months, requiring frequent mowing. “Argentine” is the preferred cultivar for use as turf because it forms a denser turf and has a shorter period of seedhead production compared to “Pensacola.”

1.4 Traditional Breeding

Breeding efforts for bahiagrass have been focused on the sexual diploid germplasm, “Pensacola.” The naturally occurring tetraploid cultivars are apomictic and hence options for their genetic improvement are very limited. The generation of sexual tetraploids by doubling the chromosomes of diploid plants using colchicine allows for unidirectional gene transfer from such apomictic cultivars to sexual tetraploids (Forbes and Burton,

1961a). The tetraploid cultivars used at present are superior ecotypes selected from the introduced germplasm (Gates *et al.*, 2004). Seed for the following cultivars is available commercially: “Argentine”, “Paraguay”, and “Paraguay 22.” The most important breeding objective for bahiagrass has been above ground biomass yield. Two approaches have been used to improve bahiagrass. In the first approach, inbred lines of “Pensacola” were established in alternate rows in a field and the F₁ seeds were collected. “Pensacola” is partially self-incompatible. Hence most of the seeds resulted from out crossing (Burton, 1974). This approach was not very efficient since establishing the seed production fields from vegetatively propagated clones is very labor intensive.

The second approach was called “restricted recurrent phenotypic selection” (RRPS), a modification of the mass selection procedure (Burton, 1974). Selection was started from an initial population collected from 16 farms in Georgia. Twenty-three cycles of selection for increased forage production were completed by this approach. In this procedure, 1000 space-planted individuals were used for selection and 20% of these were selected based on scoring for above ground herbage accumulation. They were intermated in a polycross to produce seeds for the subsequent selection cycle. Seeds from the polycross were germinated in the greenhouse during winter and transferred to the field during spring. “Tifton 9” was released as a cultivar from the ninth selection cycle. Bahiagrass is characterized by a seasonal reduction in forage quality with an increase in fiber concentration. This results in low forage quality, i.e., *in vitro* dry matter digestability (IVDMD). Procedures similar to RRPS were used to select for increased yield and IVDMD. During seed germination in the winter in the advanced selection cycles, it was found that high yielding selections also showed early germination and reduced dormancy (Gates and Burton, 1998). Low forage production in the fall and winter months poses severe problems for dairy and beef cattle production in the southeastern United States. Selections for higher yield using RRPS also had a changed day-length response and more advanced cycles showed less response to supplemental light. Gates *et al.* (2001) suggested that this might result in higher growth rates of the advanced cycles during the cool season when day length is short.

A 2-year study in Quincy, FL indicated distinct cycle differences in sensitivity to day length (Blount *et al.*, 2001). It also identified day-neutral plants and also plants that could tolerate temperatures of -4°C . This was a significant achievement since improving cold tolerance is also one of the main breeding objectives for bahiagrass. Breeding effort was also directed to reducing the time taken for stand establishment and the improvement of forage quality and seedling vigor (Blount *et al.*, 2001).

“AU Sand Mountain” bahiagrass (*P. notatum* var. *saurae*) is a recent release of Auburn University. This is a natural selection of an introduction planted at the Sand Mountain Substation (Crossville, AL) about 30 years ago. It is characterized by narrow leaves, fine tillers, and shorter inflorescences. This variety has been shown to yield more than “Argentine”, “Pensacola”, Tifton 9, and even bermudagrass.

Evaluation of related germplasm is another activity being carried out at the University of Florida (Blount *et al.*, 2001). This includes new *P. notatum* accessions as well as *Paspalum* species. A genetic linkage map for diploid *P. notatum* has recently been constructed (Ortiz *et al.*, 2001). This map was developed as a framework for genetic studies as well as breeding purposes. The map was created using heterologous restriction fragment length polymorphism (RFLP) clones from maize, rice, and oat. The creation of this map will make it possible to study complex and simple traits and should prove to be a useful tool for plant breeders.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

In the case of diploid cultivars, introgression of desired traits by traditional hybridization or identification of superior ecotypes has been successful. However, genetic variability for the desired trait is necessary for the success of this approach. Transgenic technology may help to expand bahiagrass improvement to include traits that are difficult or impossible to incorporate by traditional breeding like herbicide resistance or drastic improvement of forage and turf quality as well as cold tolerance.

Some of the most popular cultivars of bahiagrass like cv. “Argentine” are apomictic

tetraploids. This prevents their improvement using conventional hybridizations. The use of transgenic technology may help to overcome these problems. The apomictic nature of these tetraploid cultivars may prove to be an advantage for the generation of transgenic lines. In obligate apomictic types, there is no segregation resulting in a uniform seed progeny. Also the likelihood of unintended transgene dispersal by pollen is very low.

2. DEVELOPMENT OF TRANSGENIC BAHAGRASS

The genetic engineering of plants is largely dependent on the ability of cultured cells and tissues to regenerate whole plants, an efficient gene delivery system, and a selection protocol that helps to distinguish transgenic tissues and plantlets from nontransgenic tissue and plantlets at an early stage.

2.1 Traits of Interest

Most desirable traits for bahiagrass improvement include improvement of forage and turf quality, further enhancement of its abiotic and biotic stress tolerance and herbicide resistance. The containment of transgenes is also an important target for stress-tolerant perennial grasses.

2.2 Methods of Transformation

The first transgenic bahiagrass plants were produced by Smith *et al.* (2002) using biolistic gene transfer into a highly embryogenic genotype (Tifton 7) with no commercial relevance. Efficient selection and regeneration systems following biolistic gene transfer have been established for the commercially important cultivars “Argentine” (Altpeter and James, 2005), “Pensacola” (Gondo *et al.*, 2005), and “Tifton 9” (Luciani *et al.*, 2007). Two of the main advantages of biolistic gene transfer are that it efficiently supports co-integration and co-expression of multiple transgenes without vector backbone sequences, and that it is applicable to a wide range of genotypes (reviewed by Altpeter *et al.*, 2005). Sandhu and Altpeter (2008) reported a comparatively high co-integration and co-expression frequency

following biolistic gene transfer of two unlinked, linear, minimal expression cassettes lacking vector backbone sequences into apomictic bahiagrass.

Alternative gene transfer systems like *Agrobacterium*-mediated gene transfer (Hiei *et al.*, 1994) or protoplast-mediated gene transfer (Ha *et al.*, 1992) are applicable to a more limited number of genotypes and although these protocols were successful for the genetic transformation of a number of cereal and grasses, they have not yet been established for bahiagrass.

2.3 Selection of Transformed Tissue

In the first bahiagrass transformation report using the *bar* gene as selectable marker and 3 mg l^{-1} glufosinate as selective agent more than 90% of nontransgenic plants escaping the selection process were recorded (Smith *et al.*, 2002). The subsequent study by Gondo *et al.* (2005) replaced glufosinate with 3 mg l^{-1} bialaphos for selection of *bar*-transgenic bahiagrass plants and reported an escape rate of more than 50%. In a direct comparison of alternative selectable marker genes for bahiagrass transformation, James *et al.* (2008b) identified the *nptII* selectable marker in combination with the selection on paromomycin-containing medium during callus subculture and regeneration as superior to *bar* and bialaphos or *EPSPS* and glyphosate in transformation efficiency and suppression of nontransgenic plants escaping selection.

Following this *nptII*/paromomycin selection protocol several hundred transgenic bahiagrass plants have been produced so far. The *nptII*/paromomycin selection strategy has been reported to be successful in a range of monocotyledonous plants including ryegrasses (Altpeter *et al.*, 2000), fescue (Altpeter and Xu, 2000), and rye (Popelka and Altpeter, 2003). A number of alternative selectable markers have been reported for the transformation of other monocotyledonous plants and are reviewed by Miki and McHugh (2004). The strategy supporting the highest transformation efficiency with the lowest number of nontransgenic plants escaping the selection process can be combined with excision of selectable marker genes from transgenic plants following the selection process as reviewed by Hare and Chua (2002).

2.4 Regeneration of Plants

The genetic engineering of plants is largely dependent on the ability of cultured cells and tissues to regenerate whole plants following gene transfer and selection. Vimla Vasil and Indra Vasil described the first detailed accounts of embryogenic cultures from monocotyledonous plants (Vasil and Vasil, 1980) following the culture of meristematic tissues like immature embryos of *Pennisetum americanum* at a precisely defined stage of development on auxin-containing culture media. Modifications of these protocols made them applicable to a wide range of monocotyledonous species including cereals, turf, and forage grasses. Embryogenic bahiagrass callus was initiated from immature inflorescences (Bovo and Mroginski, 1986), young leaf tissue (Bienick, 1989), mature and immature embryos (Bovo and Mroginski, 1989), mature or germinating seeds (Marousky and West, 1990; Akashi *et al.*, 1993; Grando *et al.*, 2002; Altpeter and Positano, 2005; Gondo *et al.*, 2005). The donor plant or seed quality, culture media composition, amount of microparticles used for biolistic gene transfer, the developmental stage of the embryogenic target tissue at the time of bombardment and the concentration, type and timing of the selective agent, environmental conditions as well as the overall time in tissue culture determine plant regeneration efficiency, which highly correlates with the transformation efficiency.

2.5 Testing Transgenic Plants

Typically a few weeks after establishing the putative transgenic plants in soil, they are subjected to testing to confirm their transgenic nature. Amplification of a part of or the whole transgene sequence using polymerase chain reaction (PCR) provides a quick method for screening a large number of plants. Following PCR, expression analysis is carried out. This is done using reverse-transcriptase PCR (RT-PCR) and RNA as a template. Alternative screening procedures involve commercially available kits for expression analysis of common selectable marker genes like *bar* or *nptII* (Sandhu and Altpeter, 2008). In contrast to PCR-based methods, there are typically no false positives detected with such

Enzyme-linked immunosorbent assay (ELISA) or immunochromatography kits. The latter, however, has a relatively high detection limit and is not a quantitative assay. Further molecular characterization is carried out using Southern and Northern blots or real-time RT-PCR to estimate the number of copies of the transgene and level of expression, respectively. Plants that have been tested positive by molecular methods are then further evaluated for the expected phenotype under controlled environment or field conditions following approval by USDA-APHIS (Animal and Plant Health Inspection Services). The progeny of the transgenic plants is again subjected to molecular characterization to confirm stability of transgene expression.

2.6 Specific Regulatory Measures Adopted

Generation and evaluation of transgenic plants involve a regulatory procedure. The first step in this process is the Institutional Biosafety Committee (IBC), which oversees all applications for laboratory, controlled environment, and field testing of transgenic plants. The applications for work with genetically modified plants are first sent to the IBC for review. The IBC then inspects the facilities to check for compliance with safety guidelines. When the IBC preapproves an application for field evaluation of transgenic plants, they are forwarded to USDA-APHIS for final approval.

APHIS is a part of USDA that deals with transgenic organisms. Permit applications for field testing are sent to this agency for approval. The application should contain all the details of each transgenic line to be evaluated in the field including the source of the gene of interest, the expected trait, any molecular characterization data, field design, and the GPS co-ordinates of the proposed field site. USDA-AHPIS permit number 05-364-01r is currently supporting the field evaluation of genetically engineered dwarf bahiagrass to evaluate its turf quality and water use efficiency. USDA-APHIS permit number 05-294-01r is currently supporting the field evaluation of transgenic bahiagrass overexpressing stress-inducible transcription factors to evaluate its cold and drought tolerance. USDA-APHIS permit number 05-294-02r is currently supporting a risk assessment study, which evaluates short distance gene

transfer from transgenic, apomictic bahiagrass to different nontransgenic bahiagrass cytotypes. An environmental assessment for this study is available at <http://www.isb.vt.edu/biomon/releapdf/0529402r.ea.pdf>.

3. FUTURE ROAD MAP

3.1 Expected Products

In the last years, a large number of transgenic bahiagrass plants have been produced and several transgenic lines displayed desirable phenotypes. The introduction of a number of different transgenes in bahiagrass has resulted in plants with improved turf quality (Agharkar *et al.*, 2007; Zhang *et al.*, 2007), abiotic (Xiong *et al.*, 2006; James *et al.*, 2008a), and biotic (Luciani *et al.*, 2007) stress tolerance and herbicide resistance (Sandhu *et al.*, 2007).

3.1.1 Abiotic stress tolerance

Plant adaptation to abiotic stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Engineering genes that protect and maintain the function and structure of cellular components as reviewed by Vinocur and Altman (2005) can enhance tolerance to stress.

Abiotic stress severely limits the productivity and persistence of forage and turf grasses like bahiagrass (*P. notatum* Flugge). Overexpression of a single stress-inducible transcription activator of the C/DRE stress response pathway CBF1(=DREB1B) or DREB1A(=CBF3) in transgenic *Arabidopsis*-enhanced freezing, drought, and salt tolerance with a concomitant expression of target genes (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999). The C/DRE stress response pathway is conserved in grasses (Jaglo *et al.*, 2001; Choi *et al.*, 2002; Dubouzet *et al.*, 2003). A *DREB1A* transcription factor ortholog was isolated from xeric, wild barley (*Hordeum spontaneum* L.) and introduced into the apomictic bahiagrass cultivar "Argentine" under control of the stress-inducible barley *HVA1s* promoter by James

et al., (2008a). The *HsDREB1A* gene was able to transactivate both the stress-inducible *HVA1s* and *Dhn8* promoters as shown with reporter gene assays in bahiagrass. *HsDREB1A* expression under control of the *HVA1s* promoter was detected in primary transgenic bahiagrass plants and their apomictic seed progeny by RT-PCR, real-time RT-PCR, and Northern blot analysis. Transgenic bahiagrass plants with stress-inducible expression of *DREB1A* survived severe salt stress and repeated cycles of severe dehydration stress under controlled environment conditions in contrast to nontransgenic plants (James *et al.*, 2008a).

The *WRKY* gene family represents a major group of plant-specific transcription factors implicated in many different processes (Eulgem *et al.*, 2000). The barley *Hv-WRKY38* transcription factor has been shown to be expressed during low temperature and freezing stress in an ABA-independent manner (Marè *et al.*, 2004). Transgenic bahiagrass lines with constitutive overexpression of the *Hv-WRKY38* transcription factor produced significantly more biomass than wild type following severe drought stress under controlled environment conditions (Xiong *et al.*, 2006).

The rice OsMYB4 transcription factor is strongly induced by low temperatures and has been shown to transactivate cold-inducible promoters (Vannini *et al.*, 2004). Overexpression of this gene in *Arabidopsis* significantly increased cold and freezing tolerance (Vannini *et al.*, 2004). Transgenic bahiagrass lines containing the above transcription factors have been generated and they are being evaluated under field conditions (USDA-APHIS permit number 05-294-01r) for enhanced cold and drought tolerance compared to wild-type 'Argentine' bahiagrass plants.

3.1.2 Biotic stress tolerance

Fall armyworm, *Spodoptera frugiperda* (J. E. Smith) is a destructive migratory pest of many tropical and subtropical grasses including bahiagrass. Engineering resistance to this pest in bahiagrass may further improve the potential of bahiagrass both as turf and forage. Bt toxins have been expressed in commercially important crops, such as cotton, soybean, and corn, to provide resistance to several lepidopteran pests.

To generate transgenic bahiagrass plants resistant to fall armyworm, a gene encoding an optimized, synthetic *Bacillus thuringiensis* endotoxin (Bt toxin) *cry1Fa* was subcloned under the control of the constitutive ubiquitin promoter. Co-transfer of constitutive *nptII* and Bt-toxin expression cassettes into seed-derived callus cultures from forage type bahiagrass cultivar Tifton, was carried out by biolistic gene transfer. Transgenic nature of the regenerated plants has been confirmed by PCR, Southern blot analysis, RT-PCR, and immunoassays. Neonate larvae fed with transgenic bahiagrass leaves showed a significant higher mortality than larvae fed with wild-type bahiagrass leaves (Luciani *et al.*, 2007).

3.1.3 Improvement of turf quality

Bahiagrass has several characteristics that make it desirable as low-input turf such as tolerance to drought, low fertility requirements, and few disease and pest problems. However, the turf quality of bahiagrass is reduced due to its open growth habit and prolific seedhead production during the fall and summer months.

In order to improve the turf quality of bahiagrass, a gibberellin-catabolizing enzyme, gibberellin 2-oxidase (*GA2ox1*) from *Arabidopsis* was introduced into bahiagrass under the control of the enhanced constitutive 35S promoter from cauliflower mosaic virus or the maize ubiquitin promoter with its first intron. Gibberellin 2-oxidases (*GA2ox*) are a family of enzymes involved in the degradation of bioactive gibberellins. These are 2-oxoglutarate-dependent dioxygenases (2ODDs) that inactivate bioactive gibberellins by hydroxylation of their C-2 (Martin *et al.*, 1999; Thomas *et al.*, 1999; Sakamoto *et al.*, 2001). Transgenic bahiagrass lines expressing the *GA-2* oxidase and displaying a semi-dwarf phenotype, while producing a larger number of tillers and showing delayed seedhead formation and reduced seedhead length, were identified under controlled environment conditions (Agharkar *et al.*, 2007). A field experiment has been established with these lines under USDA-AHPIS permit number 05-364-01r to evaluate turf density, seedhead production, mowing, and irrigation requirements.

Transcription factors involved in the control of flowering and cell expansion also have the

potential to contribute to improved turf quality. To evaluate this, the *Arabidopsis* repressor of cell expansion *ATHB16* has been overexpressed in bahiagrass under the control of the enhanced, constitutive 35S promoter. *ATHB16* (*Arabidopsis thaliana* Homeobox16) is a member of the HDZip family of plant transcription factors (Wang *et al.*, 2003). Transgenic expression of *ATHB16* in *Arabidopsis* resulted in alteration of flowering time, leaf expansion, and shoot elongation (Wang *et al.*, 2003). The *Arabidopsis* plants appeared dwarf with proportional reduction of leaf length and leaf width. Overexpression of the *Arabidopsis* *ATHB16* transcription factor in bahiagrass significantly changed plant architecture. Transgenic plants produced significant more vegetative and less reproductive tillers, shorter leaves, and shorter tillers. Overexpression of *ATHB16* resulted in proportional reduction of leaf width and leaf length. Formation of seedheads under natural photoperiod was delayed in some transgenic bahiagrass lines for approximately 4 weeks. Total root or shoot biomass and seed set were not compromised in semi-dwarf bahiagrass plants overexpressing *ATHB16* (Zhang *et al.*, 2007).

3.1.4 Herbicide resistance

Bahiagrass displays an open growth habit and slow establishment from seeds due to seed dormancy. Weed encroachment and low tolerance to commercially available herbicides make weed management a difficult task in bahiagrass. Fertile, herbicide-resistant transgenic bahiagrass lines from the apomictic cultivar “Argentine” were recently described by Sandhu *et al.* (2007). Integration of the constitutive *bar* gene expression cassette into genomic DNA of bahiagrass was confirmed by Southern blot analysis. Stable *bar* gene expression was detected by an immunochromatographic assay in primary transgenic lines and the apomictic progeny plants. Several independent transgenic lines showed no growth inhibition, chlorosis, or necrosis even after spray application of a solution with 1.0% glufosinate ammonium under greenhouse conditions. The selected transgenic plants did not differ morphologically from wild-type plants and produced viable seeds in the greenhouse and field (USDA-APHIS permit no.

05-365-01r). Transgenic plants resisted the field application of 0.6% glufosinate ammonium, which is twice the recommended rate for weed control, without any injury symptoms.

3.2 Addressing Risks and Concerns

Concerns about potential environmental risks involved with the commercialization of genetically modified turf or forage grasses currently slow their deregulation. Concerns associated with genetically modified (GM) grasses are putative invasiveness, vertical or horizontal gene flow, and other ecological impacts, like effects on biodiversity (Conner *et al.*, 2003). To address these concerns, risk assessment as well as development of containment strategies needs to be integrated into any grass biotechnology program.

3.2.1 Risk Assessment in transgenic bahiagrass

Perennial grasses containing genes that may contribute to enhanced fitness, such as stress protective genes, may display increased weediness or outcompete native species in natural areas. Hence, assessment of the relative fitness of transgenic plants under different environmental conditions can alleviate these concerns. Another important aspect of risk assessment studies is to monitor gene flow to predict the spread of transgenic plants in the environment. “Argentine” is a preferred bahiagrass cultivar target for genetic engineering, due to its apomictic reproduction mode, which contributes to uniform asexual seed progeny. However, “Argentine” bahiagrass produces viable pollen and there is a possibility of gene flow from the transgenic apomictic tetraploids to the sexual diploids. To address this, Sandhu *et al.* (2005b) are currently determining hybridization frequencies and gene flow between apomictic tetraploid bahiagrass and diploids under controlled environment and field conditions (USDA-APHIS permit number 05-294-02r) using Glufosinate resistance as a marker. The results of this study will provide an insight if apomixes in bahiagrass is a suitable natural transgene containment system.

3.2.2 Transgene containment strategies

The spread of transgenes through pollen, seeds as well as vegetative propagules can be reduced by transgene containment strategies. Although the apomictic nature of “Argentine” bahiagrass may provide an effective, natural containment system, concerns regarding the degree of sexuality of this species have prompted the consideration of alternative approaches to further enhance transgene containment. For perennial species, it would be desirable to have a system in place that would allow the selective elimination of transgenic plants, leaving the native species unharmed. The *ArgE* gene system (Kriete *et al.*, 1996) is an inducible cell death system, which may have potential for this purpose. The *argE* gene product of *Escherichia coli*, is an N-acetylornithin deacetylase, which converts nonphytotoxic N-acetyl-L.phosphinothricin (N-acetyl PPT) to phytotoxic phosphinothricin (glufosinate). Transgenic perennial ryegrass plants expressing the *ArgE* gene were shown to be selectively controlled by the application of proherbicide, N-acetyl-L.phosphinothricin (N-acetyl PPT) (Chen *et al.*, 2005). Transgenic bahiagrass plants expressing the *ArgE* gene have been generated and will be used to evaluate its potential as an environmentally neutral marker or inducible cell death system for selective elimination of transgenic plants (Sandhu *et al.*, 2005b).

3.3 Expected Technologies

Transformation technologies have made impressive progress since the first transgenic plants were produced. The development of an *Agrobacterium*-mediated genetic transformation protocol appears to be desirable for bahiagrass since it will, compared to biolistic gene transfer, likely enhance the frequency of single copy events, associated with a higher potential for transgene expression stability. Factors that need to be considered and optimized for monocots in order to establish an efficient protocol for *Agrobacterium*-mediated transformation have been reviewed by Cheng *et al.*, (2004).

While chloroplast transformation systems have been established for several dicotyledonous crops including cotton and soybean, homotransplas-

omic monocotyledonous plants have not been developed yet. Chloroplast transformation has made it possible to create plants with high levels of gene expression, while reducing line-to-line variation and also providing a natural transgene containment system, since chloroplasts are maternally inherited. The establishment of chloroplast transformation technology for bahiagrass can be based on the existing biolistic transformation protocol. In addition, the construction of vectors that support the integration of the transgene into the bahiagrass chloroplast genome by homologous recombination and high-level expression of selectable marker genes in nongreen plastids will be necessary.

The replacement of antibiotic resistance selectable markers by markers that provide cells a growth advantage in culture media containing mannose, xylose, or cytokinin glucuronides (reviewed by Joersbo, 2001) can alleviate some of the public concerns. Alternatively selectable marker derived from the host plant DNA like the mutant form of acetolactate synthase genomic DNA from rice could become available for genetic transformation of grasses. However, the use of antibiotic resistance genes will and should be continued if they support the highest transformation efficiency with the lowest escape rate. If elimination of antibiotic resistance genes is desirable, this strategy can be combined with excision of selectable marker genes from transgenic plants following the selection process as reviewed by Hare and Chua (2002).

The progress made in functional genomics of plants allows to predict and generate synthetic regulatory elements and transcription factors which will support the fine tuning of transgene and gene expression in bahiagrass.

RNA interference is a powerful technology for targeted gene silencing and will be useful to enhance forage quality in bahiagrass by down-regulation of genes in the lignin biosynthetic pathway and to engineer virus resistance.

3.4 Intellectual Property Rights (IPRs)

The University of Florida has filed a provisional patent application on the use of the *ATHB16* repressor of cell elongation for improvement of turf quality.

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Soybean

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1. INTRODUCTION

Soybean, *Glycine max* (L.) Merr., is the most valuable grain legume crop, representing the primary source of protein and vegetable oil throughout the world. Currently global seed production is estimated at 217 million metric tons, with the major producers being the United States, Brazil, Argentina, and China. In the past decades, soybean crops have been improved significantly in agronomic traits such as yield, disease and pest resistance, and seed composition by both conventional and transgenic breeding methods. Global demand for soybean and soybean products is growing faster than ever, which in turn increases the demand for genetic improvements. Scientists have used biotechnology to meet this challenge by providing soybean with higher yield and seed quality. In this chapter, we describe the history of soybean development and distribution, trait improvement by conventional breeding, the advancement of transformation and transgenic traits, current and future soybean products, and new technologies in modern agricultural practices.

1.1 History, Origin, and Distribution

The soybean is often called the “miracle crop” because it is the world’s foremost provider of

protein and oil. Soybeans were domesticated between the 17th and 11th centuries BC in the eastern half of China where they were cultivated as a food crop. Soybean probably reached central and southern China and Korea by the 1st century AD. The movement of soybean germplasm within the primary gene center of China is largely associated with the evolvement of the China territories and Chinese dynasties (Hymowitz, 1970). Soybeans were subsequently introduced into several other countries including Japan, Indonesia, the Philippines, Vietnam, Thailand, Malaysia, Burma, Nepal, and India, aided by the establishment of sea and land trade routes (Hymowitz and Newell, 1980). These regions comprise a secondary gene center. The earliest Japanese reference to the soybean is in the classic *Kojiki* (Records of Ancient Matters), which was completed in 712 AD.

Soybeans were introduced to Europe in 1712 by Englebert Kaempfer, a German botanist who had studied in Japan. Swedish botanist Carl von Linne made the first scientific study of the soybean in the West. He named it *G. max* because of the unusually large nitrogen-producing nodules on its roots. Unfortunately, poor climate and soil conditions in Europe limited attempts to produce soybeans as a crop there. In 1765, Samuel Bowen introduced soybean from China into the Colony of Georgia and Henry Yonge planted soybean on

his farm at the request of Bowen (Hymowitz and Harlan, 1983). In 1767, Bowen received a royal patent for making soy sauce (Woodcraft, 1854) and James Mease was the first person in American literature to use the word “soybean” (Hymowitz and Shurtleff, 2005). Another early introduction of soybean into North America was by Benjamin Franklin in 1770 (Hymowitz and Harlan, 1983). In 1929, Morse spent 2 years doing research on soybeans in China, where he gathered more than 10 000 soybean varieties. It was not until the 1940s that farming of soybeans became widespread in America.

Today, more soybeans are grown in the United States than in any other country in the world. Farmers in more than 30 states grow soybeans, with the major producing states being Illinois, Iowa, Missouri, Minnesota, Indiana, and Ohio. Soybeans are the United States’ second largest crop in cash sales and the number one value crop for export. In 2004, US soybean farmers harvested 3.141 billion bushels (86 million metric tons) of soybeans. Nearly half of the total value of the US soybean crop was exported as whole soybeans, soybean meal, or soybean oil.

1.2 Soybean Biology

The soybean is a papilionoid legume (family Fabaceae, subfamily Faboideae), and a member of the tribe Phaseoleae, subtribe Glycininae. The genus *Glycine* is unique within the subtribe on several morphological and chromosomal characteristics, and does not seem to bear an especially intimate relationship with any other genus in the subtribe (Lackey, 1977). The genus *Glycine* is divided into two distinct subgenera: *Glycine* and *Soja*. The first consists of perennial species primarily from Australia. There are 22 recognized perennial species, of which *Glycine tabacina* and *Glycine tomentella* are neopolyploid ($2n = 78, 80$) (Hymowitz, 2004). The second consists of two annual species from Asia: *G. max* and *Glycine soja* Sieb. and Zucc. The first species is the cultivated soybean; the second species is the wild form of the soybean (Hymowitz, 2004). There are few cytogenetic barriers to gene flow between *G. max* and *G. soja* (Hymowitz, 1970; Hymowitz and Newell, 1981). Natural hybrids are easily produced at a relative low frequency by

Table 1 Soybean growth stages

Vegetative stages	Reproductive stages
VE (emergence)	R1 (beginning bloom, first flower)
VC (cotyledon stage)	R2 (full bloom, flower in top 2 nodes)
V1 (first trifoliolate)	R3 (beginning pod, 3/16" pod in top 4 nodes)
V2 (second trifoliolate)	R4 (full pod, 3/4" pod in top 4 nodes)
V3 (third trifoliolate)	R5 (1/8" seed in top 4 nodes)
V(n) (nth trifoliolate)	R6 (full size seed in top 4 nodes)
V6 (flowering will soon start)	R7 (beginning maturity, one mature pod)
	R8 (full maturity, 95% of pods on the plant are mature)

pollen flow from the cultivated soybean to the wild soybean during their simultaneous flowering with adequate pollinators (Nakayama and Yamaguchi, 2002). The cultivated soybean (*G. max*) is a predominantly self-pollinated species with an outcrossing rate of less than 1% (Chiang and Kiang, 1987). Soybean plants are thus virtually pure breeding homozygous lines, although manual cross-pollination is practiced routinely in breeding programs (Fehr, 1987). Soybean plants can be described in various growing stages (Table 1) during their development (McWilliams *et al.*, 1999).

Based on the timing of the termination of apical stem growth, soybean can be divided into two stem growth habits: determinate and indeterminate (Bernard, 1972). A determinate stem terminates apical growth abruptly and generally produces a thick stem tip. An indeterminate stem continues terminal growth as long as lateral growth continues, and produces a stem that is tapered in thickness from tip to base (Bernard, 1972). In the United States, there are 13 recognized maturity groups (MG) for cultivated soybeans, which range from MG 000, 00, 0, and I through X. Varieties with the lowest number designation (000 to IV) are considered indeterminate while maturity groups V through X are determinate varieties. Early maturing varieties (000 to IV) are adapted to the more northern climatic regions while later maturing varieties are more adapted to the south.

A single gene *dt1* was identified to be responsible for the stem growth type, with *dt1dt1* for the determinate, *Dt1Dt1* for the indeterminate, and *Dt1dt1* for the semi-determinate (Bernard, 1972).

A second locus *dt2* and a third allele (*dt1-t*) at the *dt1* locus were also determined to have effects on the stem growth phenotype (Bernard, 1972; Thompson *et al.*, 1997). In studying the effect of soybean stem growth habit on height and node number after beginning bloom in the midsouthern United States, Heatherly and Smith (2004) discovered that determinate MG V cultivars are capable of producing significant increases in height and node number after R1.

Leaves of soybean are alternate, pinnately trifoliate, with pulvini, stipels, and stipules (Lersten and Carlson, 1987). The nodulated root system is intermediate between a tap root type and a diffuse type. The soybean flower is a standard papilionaceous flower with a calyx of five united sepals; zygomorphic corolla of carina, alae, and vexillum; androecium of 10 diadelphous (9 + 1) stamens; and gynoecium of a single carpel. Twenty to sixty percent of the total number of flowers set pods in the cultivated type of soybean (Yoshida *et al.*, 1983). The reproductive success of flowers produced early in the period is usually greater than those produced later (Egli, 2005). However, more complete understanding is needed for the regulation of the temporal distribution of flower production or its potential role in determining pod and seed number at maturity (Egli, 2005). Although the higher production of flowers looks wasteful, data show that the flower production potential is more important for higher seed yield than the rate of reproductive abortion (Saitoh *et al.*, 1998). Generally, two to four seeds develop in the pods (Carlson and Lersten, 1987). The seeds have two large cotyledons and scant endosperm (Lackey, 1981), and are unique in their ability to accumulate high levels of both protein (40%) and oil (20%) (Fehr, 1987). Soybean seed has a short time frame for high germination and vigor (TeKrony *et al.*, 1987). Although the remaining seed from one crop is capable of germinating the following season, fresh soybean seed is produced annually for the next season in commercial operation.

Soybean descended from a diploid ancestor ($n = 11$) that underwent an aneuploid loss to $n = 10$ and subsequent polyploidization to yield the present genome with $2n = 2x = 40$ (Lackey, 1980). Genetic evidence of gene duplication suggests that soybean is a stable tetraploid with a diploidized genome. The soybean genome

comprises about 1.1 Mbp/C (Arumuganathan and Earle, 1991). This is about seven and a half times larger than the *Arabidopsis* genome, but less than one half the size of the maize genome. Singh and Hymowitz (1988) reported a complete karyotype of soybean based on pachytene analysis (<http://www.crops.cuiuc.edu/faculty/hymowitz/genlab/karyo.html>). About 40–60% of the soybean genome contains repetitive sequences (Goldberg, 1978; Gurley *et al.*, 1979), which was confirmed by the restriction fragment length polymorphism (RFLP) analysis. Approximately 90% of all RFLP probes detected duplicated loci and nearly 60% detected three or more loci in soybean (Shoemaker *et al.*, 1996). Hybridization-based mapping has resolved many duplicated regions of the genome. These homologous regions reflect segmental and whole-genome duplication events that can be used for the studies of the evolution of the genome. Analysis of bacterial artificial chromosome (BAC) end sequences suggests that although there are gene-rich and repetitive-rich regions, most regions contain a mixture of repetitive and genic sequences (Marek *et al.*, 2001). There is relatively low polymorphism in the soybean genome. Zhu *et al.* (2003) found that the rate of single nucleotide polymorphism (SNP) is 0.5 SNP/kb and 4.7 SNP/kb in coding and noncoding DNA, respectively, whereas maize has as much as 10-fold higher levels of nucleotide diversity (Ching *et al.*, 2002).

1.3 Economic Importance

Although soybeans are native to China, 40% of production is in the United States (Table 2). The United States produced 86 million metric tons of soybeans in 2004, of which more than one-third was exported. Other leading producers of soybeans are Brazil, Argentina, China, and India. Brazil became the world's largest soybean exporter in 2004. Much of the US production is used for animal feed, though consumption of soy food products has been increasing. In 2004, the United States planted 75.2 million acres (30.4 million hectares) of soybean, producing a record 3.141 billion bushels (86 million metric tons). The total 2004 crop value exceeded \$17.7 billion. The United States exported 1.1 billion bushels (29.94 million metric tons) of soybeans, which accounted for

Table 2 World soybean production in 2004^(a)

	Million metric tons	%
USA	86	40
Brazil	53	24
Argentina	39	18
China	18	8
India	6	3
Other	15	7
Total	217	100

^(a)Source: USDA (2005)

48% of the world's soybean trade and created \$8.0 billion revenue in 2004. China is the largest buyer of US soybean, importing \$2.3 billion in 2004. Japan was the second largest market for US soybean with purchases of \$1.0 billion. Other significant buyers included the European Union and Mexico. Canada was the largest customer for US soybean meal at \$300 million, followed by Mexico and the Philippines. Mexico was the largest customer for US soybean oil with purchases of \$63 million, followed by Canada with purchases of \$62 million.

China is one of the main soybean producing countries in the world. At the beginning of the 1990s, China operated as a pure soybean-export country, exceeding 1 million tons annually. Now, however, China has become one of the biggest soybean importing countries. According to the data provided by Chinese Customs, China's soybean import began to increase in 1999 and exceeded domestic production in 2003 for the first time. As China's soybean demand continues to grow, its import will keep rising. China's soybean consumption has outpaced increases in domestic production during the last 25 years mainly because of increases in consumption of soybean oil and meal induced by income and population growth, particularly in large urban areas. China has also been a major importer of soy oil for about 20 years.

Soybean can be utilized in a variety of ways, which can be divided into two groups: one based on the utilization of the whole seed, and the other based on products of soybean fractionation into oil and meal. Whole soybeans and their flour are used as ingredients in traditional confectionery products and snacks in China, Japan, Korea, and Indonesia. Soy flour and grits are used in the commercial baking industry. They aid in

dough conditioning and bleaching. Their excellent moisture-holding qualities also help to retard staleness in baked products. Immature whole green seedlings are also consumed as a vegetable. Dried soybeans and bean sprouts are also consumed frequently in western countries and oriental cooking. Processed soybeans are also found in various food products including soymilk, soybean curd (tofu), tofu-based ice cream, soybean yogurt, soybean paste (miso), soy sauce (sho-yu), tempeh, and fermented soybean curd (su-fu).

Processing soybean results in two fractions: oil and meal. Different grades of oil can be produced through a series of refining processes. More complete hydrogenation of soybean oil is the basis for the manufacture of shortenings, margarines, and tailor-made fats used by various food industries.

After removal of the soybean oil, the remaining flakes can be processed into various edible soy protein products or used to produce soybean meal for animal feed. The largest portion of the soybean meal and cake production is used as a protein source in animal feed. The different types of soybean meal are characterized mainly by their protein content and the extent of heat treatment applied during their production to inactivate antinutritional factors. If the soybeans are extracted without dehulling, or if the hulls are added back after extraction, the meal will contain about 44% protein. Meals produced from dehulled beans contain approximately 50% protein.

There are also soybean meal products for human use. Eight essential amino acids are found in soybeans, which are necessary for human nutrition because they are not produced naturally in the body. These amino acids can be added to the human diet in products including soybean protein concentrates, soybean protein isolates, and extrusion-textured soybean protein. Soybean protein can also be spun into a silklike fabric.

Soybean oil has also recently been employed for industrial purposes, opening up a large new market for soybeans. More than 600 million pounds of the soybean oil produced annually in the United States are used for nonedible applications including fatty acids, soaps, paints, varnishes, resins, printing inks, lubricants, surfactants, cleaning solvents, and biodiesel (Marking, 1998). Soy biodiesel represents the largest potential industrial use of soybeans. It is the most flexible, lowest cost alternative fuel

option for diesel fleets. Soy-based biodiesel is made by a chemical process called transesterification. This process results in biodiesel and a by-product called glycerine that has more than 1600 commercial applications from toothpaste to environmental-friendly antifreeze. Biodiesel fuel does not require expensive vehicle modifications or fueling equipment. B20, a mixture of 20% soy-based diesel and 80% petroleum diesel, has been approved by federal, state, county, and utility company vehicles under the Energy Policy Act of 1992 (EPACT) in the United States. Soybean also has the potential for making soy-based diesel fuel additives, which can provide superior lubricating properties. It was estimated that to realize a national goal of 4% renewable fuel use by 2016, soybean production would have to increase from 51 million bushels in 2002 to 318 million bushels by 2016. Commercial production of biodiesel in the United States is mainly through transesterification of soybean oil. A number of biodiesel manufacturing plants have been constructed in Austria, France, Italy, Germany, Denmark, Hungary, Sweden, Ireland, and the United States (National Renderers Association, 1992; Biodiesel Alert, 1993). The National Biodiesel Board reported production of 500 000 gallons in 1999. It is estimated that the demand for biodiesel will be at least 6.5 million gallons in 2010. The demand could be up to 470 million gallons in 2010 if combined with the demand for biodiesel as a lubricity additive (<http://www.eia.doe.gov/emeu/plugs/plbiodsl.html>). Brazil is currently using a new biofuel known as H-Bio. It is a mixture of cotton, castorbean, sunflower, and soybean oils (<http://www.blonnet.com/2006/07/02>).

The surfactant market has reached more than \$10 billion worldwide. Soybean oil is a good candidate for producing environment-friendly surfactants (Longhini, 1996). Soy ink has been on the market since 1987 in the United States. It is produced by slightly refining soybean oil and then blending in pigments, resins, and waxes. It has better resolution and improved mileage performance compared to the petroleum oil-based ink. Inks produced from soybean oil have reduced volatile organic compounds and do not contain polycyclic aromatic compounds, which are regulated by Environmental Protection Agency (EPA) due to the health risks associated with them

(Tao, 1994). Soy ink is also a helpful component in paper recycling. The soy ink can be removed more easily than regular ink, allowing the recycled paper to have less damage to its paper fibers and have a brighter appearance.

Soybean protein can be used to form flexible or more rigid plastics, which are used for food packaging purposes. These soybean-based plastics are biodegradable and are therefore safer for the environment than petroleum-based plastic, which are more persistent in the environment after disposal. The quickly degrading plastic may even help fertilize the soil as it naturally biodegrades. Another benefit of soybean-based plastic is the fact that it is made with a renewable resource. Recycling alternatives are also available for soybean plastic. It can be ground up after its first use and used in animal feed, or mixed together with waste paper to make composite building material that has the appearance of polished granite. This material could also be used in alternative wood products, such as a product called ENVIRON that is already available in the United States.

Soybean hulls also have high potential value. Each year, US soy processing generates 10–15 billion pounds of hulls that are typically sold to animal feed supplement producers for around \$40 a ton. Rather than using the hulls as a feed ingredient, scientists are working to convert them into ion exchange resins for use in adsorbent filters used to capture metals in solutions. Most commercial ion exchange resins cost between \$2 and \$20 per pound while the cost of the soy-based resins would drop to 53 cents per pound.

Soybean-based adhesive resin for the wood composite material industry has been developed (Kuo and Stokke, 2000). This product consists of defatted soybean flour cross-linked with synthetic phenol formaldehyde, the former typically at a ratio of 70% by weight of the adhesive resin. Testing has shown that the adhesive has performance characteristics between urea formaldehyde and phenol formaldehyde resins, which are the two most commonly used synthetic resins in the wood industry (Kuo *et al.*, 1998; Stokke and Kuo, 1999).

Some industrial applications of soybean require genetic enrichment of specific fatty acid components, for example, use of soybean oil as a renewable chemical feedstock. Researchers

at DuPont used genetic engineering approaches to create high oleic acid soybean (Kinney and Knowlton, 1998). High oleic oils are generally considered to be healthier than conventional soybean oil, and have potential uses in industry as discussed in Section 2.4.

1.4 Traditional Breeding

Traditional breeding has been used to modify the genetic make-up of plants and animals for many years. Using crosses to transfer the existing genetic variability or create new variability has been the major venue for traditional breeding (Liu, 1999). The goals of traditional soybean breeding are to develop varieties and germplasm with enhanced yield potential, resistance to biotic and abiotic stress, and improved chemical composition. Some specific objectives may include

1. Developing soybean varieties with resistance to soybean cyst nematode, *Phytophthora* root rot, sudden death syndrome, white mold, etc.
2. Developing varieties with tolerance to herbicides such as glyphosate, glufosinate, and sulfonyleurea.
3. Developing varieties with enhanced yield potential and adaptations to different environments.
4. Developing special varieties such as low linolenic acid, mid oleic acid, low phytate, high protein, etc.

Yield has been the single most important trait for breeders as it is the trait that has great impact on the farmer's bottom line. Typically, breeders use crosses between the parents of choice to create segregating populations. These populations are advanced either locally or at off-site nurseries with or without selection. Breeders use winter nurseries in Puerto Rico, Mexico, Central and South America, and elsewhere to obtain two, three, or more generations in a single season/year, advancing their breeding materials faster. Plant rows are then selected and are evaluated for desired traits primarily in the field; however, some disease traits are evaluated in greenhouses. A breeding cycle ends when the best pure lines are released.

Parent selection is the first and most important step in the breeding process. Parent selection can

be based on a number of different factors: traits of interest, genetic diversity, breeding objectives, complementarity of lines, and resources. Generally elite parents with diverse origin are more likely to produce superior progeny (Burton, 1997). Combining ability assessment of parents is also useful in identifying the best combination to exploit heterosis or combine fixable favorable genes that may lead to the development of superior genotypes (Agrawal *et al.*, 2005). By using a 5×5 diallel set of soybean crosses, Agrawal *et al.* (2005) showed that most plant characteristics might be governed by additive gene effects, however, the complexity of additive and nonadditive gene effects plays an important role in the expression of yield attributes.

Selection in breeding populations can be done during either early or late generation. These selection methods include: pedigree selection, single seed descent, and bulk breeding. Research has shown that the single seed descent coupled with concurrent selection for early maturity is the most efficient for development of early maturing cultivars (Byron and Orf, 1991). Bravo *et al.* (1999) compared family based and line-based pedigree methods in breeding for palmitate, and concluded that the line method is more effective. However, the bulk breeding method has been proven effective in improving yield in environments with disease pressure (Degago and Carviness, 1987).

Backcrossing has also been practiced widely to introgress favorable genes. Sometimes, *G. soja* has been used as a donor parent for small seed size, high protein content, or disease resistance traits. Recurrent selection has also been used in soybean to improve the frequency of favorable alleles in a population. It is a cyclic selection with each cycle having three steps: intermating, evaluation, and selection. A number of researchers have used recurrent selection to improve yield (Holbrook *et al.*, 1987; Piper and Fehr, 1987; Guimaraes and Fehr, 1989; Burton *et al.*, 1990; Werner and Wilcox, 1990; Rose *et al.*, 1992; Uphoff *et al.*, 1997). In general, recurrent selection is effective in soybean (Lewers and Palmer, 1997).

In order to create new genetic variation, mutagens such as ethylmethane sulfonate (EMS), x-rays, and fast neutron have been used to alter the soybean genome. Rahman *et al.* (1994) obtained a high oleic acid mutant line, M23, by using

x-rays on soybean variety Bay. M23 showed a twofold increase in oleic acid content compared with the original variety. The oleic acid content in this mutant was controlled by a single recessive allele, designated as *ol* at the *Ol* locus (Rahman *et al.*, 1996). Chemical mutagenesis was also used successfully to generate mutants with reduced linolenic acid levels (Hammond and Fehr, 1983; Bubeck *et al.*, 1989). Mutants A5 and A23 each had a single gene responsible for reducing linolenic acid content in the seed (Rennie and Tanner, 1991; Fehr *et al.*, 1992). Segregants with even lower linolenic acid content than the parents were identified in the crosses of A5 \times A23. These segregants were considered to have mutant genes from both A5 and A23 (Fehr *et al.*, 1992).

Mutagenesis has also been used to study root nodulation. A soybean supernodulation mutant, designated FN37, which lacks the internal autoregulation of the nodulation mechanism, was generated by fast neutron (Men *et al.*, 2002). Further characterization revealed that this mutant contains a chromosomal deletion. When inoculated with microsymbiont *Bradyrhizobium japonicum*, FN37 formed at least 10 times more nodules than the wild-type *G. soja* parent. Molecular markers were used to isolate BAC contigs delineating both ends of the deletion in FN37 mutant. Deletion break points were determined physically and placed within flanking BAC contigs.

Plant breeding has been practiced for hundreds of years via artificial selection. Selection has led to the highly improved performance of crop species. However, selection for quantitative traits has been difficult because the traits are affected by multiple genes (G), environments (E), and G \times G and G \times E interactions. In order to overcome these problems, marker-assisted selection (MAS) was developed as first described by Sax (1923). Further efforts identified associations of markers and quantitative trait loci (QTLs) in a number of species (reviewed by Paterson *et al.*, 1991). However, the lack of suitable markers hampered further development of this classical method. With the advent of molecular markers including RFLP, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), and SNP, systematic and accurate mapping of QTLs has become possible by the construction of molecular linkage maps.

Mapping of QTLs allows rapid identification of linked molecular markers, which then can be used to tag desirable alleles in segregating breeding populations. More directed genotypic selection of breeding lines should result in supplementing and possibly even eliminating phenotypic selection, especially for quantitative traits which are complicated, time consuming, and expensive to analyze.

To facilitate molecular breeding and other genomics research, the soybean community has constructed a composite genetic map, which contains 1845 markers (1010 SSRs, 718 RFLPs, 73 RAPDs, 23 classical traits, and 10 others) (P. Cregan, unpublished data). Over 319 QTLs for various agronomic and disease resistance traits have been mapped to the soybean genetic maps (Orf *et al.*, 2004). These identified QTLs and their linked molecular markers have greatly facilitated MAS in soybean. For example, using a patented molecular marker approach that combines biotechnology, robotics, and information management technologies, Pioneer Hi-Bred International, Inc. developed soybean cyst nematode (SCN) resistant soybean varieties effectively. MAS based on the location of SCN resistance genes mapped on specific chromosomes is used to confirm the presence of these genes in experimental varieties (Soper *et al.*, 2003). Markers have also been used to detect the presence of transgenes during the selection process.

In addition to the soybean genetic map, there is also a significant amount of soybean genomics resources available that will enhance soybean traditional breeding efficiency and ultimately increase soybean productivity. Over 300 000 soybean expressed sequence tags (ESTs) are in GenBank (<http://129.186.26.94/soybeanest.html>). Two soybean physical maps are constructed, one from soybean cv. Forrest (Wu *et al.*, 2004) and the other from cv. Williams (Warren, 2006). A wide variety of mapping populations and a variety of functional genomics tools (including DNA microarrays and proteomics resources) are also available. In addition, the United States Department of Agriculture/Agricultural Research Service (USDA/ARS) germplasm collection at the University of Illinois houses over 16 000 plant introduction lines from China and other parts of the world. Another collection of southern MG exists at the USDA/ARS facility in Stoneville, MS.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Breeders have been using conventional breeding methods to select favorable phenotypes for many years and have been making steady progress in improving soybean productivity. This method primarily utilizes the existing genetic variability to create new variability. There are a couple of limitations of the conventional breeding method: (1) linkage drag, i.e., often undesirable genes are transferred along with the desired ones in each cross, (2) the barriers for gene transfer through incompatibility, and (3) limited genetic variations available. With the increasing world population and increasing demand for soybean production, more advanced technology is necessary to meet the future needs. Research has been transformed by technologies that are allowing us to understand how genes work, and are providing a new level of understanding of how plants develop and function in their environments. The ability to isolate genes and transform them back into living organisms has greatly facilitated genetic improvement processes by directly modifying genetic make-up of important crops. The transgenic approach has made it possible to introduce desired foreign gene(s) into elite backgrounds. Transgenic breeding offers several advantages: (1) overcoming problems of sexual incompatibility, (2) introducing only the desirable genes without any linkage drag, (3) manipulating transgenes to create novel genetic variation. Transgenic soybean varieties carrying herbicide resistance gene have already been developed. The global transgenic soybean planting area reached 42 million hectares in 2003, which accounts for more than 55% of total soybean planting area and 61% of the planting area of transgenic crops (James, 2003). These transgenic soybeans have greatly improved soybean production efficiency and soybean value for end users.

Transgenic development is an extension of traditional breeding. Together, the two methods will enhance and improve the efficiency of plant breeding. Scientists can genetically engineer soybean plants with novel genes in the laboratory, but plant breeding is necessary to transfer the new transgenes through crossing into elite germplasm.

Genetically modified (GM) crops and the techniques of biotechnology offer huge benefits

in improving world food and nutrition supply, however, awareness must be created among the farming and consumer communities by the scientific community regarding their benefits in order for them to be more widely accepted.

2. DEVELOPMENTS OF TRANSGENIC SOYBEAN WITH IMPROVED TRAITS

With the rapid world population increase and the increasing demand for nutritious soybean products, it is imperative to increase soybean productivity and efficiency. Some major soybean producing countries have increased their production acreage; however, acreage increase alone will not be able to address this challenge due to the land availability. Biotechnology offers great potential by providing superior soybean products through genetic engineering. As illustrated by the examples in the following sections, biotechnology has changed the fundamentals of soybean production practices. Transgenic breeding has significantly increased soybean production efficiency and soybean product value through enhanced resistance to pests and herbicides, healthier and more nutritious soy foods and feeds, and new industrial opportunities for a more sustainable global environment. Genetic transformation is a key step in the transgenic breeding method; therefore, we will summarize the advancement of transformation methodology before illustrating the transgenic traits.

2.1 Soybean Transformation

Since the first reports of soybean transformation in 1988 (Hinchey *et al.*, 1988; McCabe *et al.*, 1988), advances in tissue culture and gene transfer have helped extend the technology to a number of academic and industrial laboratories around the world (reviewed in Dinkins *et al.*, 2003; Simmonds, 2003; Trick *et al.*, 2003; Clemente and Klein, 2004). Although soybean transformation is now more routine than in the immediate years following the first successful transformation reports, the technology is by no means standard and fully reproducible. Soybean transformation, and grain legume transformation in general, is considered more difficult than the transformation of other

crop species, such as rice and maize. Protocols for soybean transformation must be adapted and modified for development of a functional system in a particular laboratory. This is because efficient transformation systems require coupling three interlocking components. The first is the generation of tissues that are receptive to delivered DNA and capable of subsequent proliferation and regeneration to fertile plants. This requires growth and proliferation of cells and tissues in axenic cultures. Therefore, some expertise in plant tissue culture is required for soybean transformation. There are three routes for proliferation of soybean tissues *in vitro* that have been coupled with gene transfer procedures. Shoot organogenesis from mature cotyledons (Cheng *et al.*, 1980; Wright *et al.*, 1986), multiple shoot formation from apical meristems derived from mature seed (Kantha *et al.*, 1981), and somatic embryogenesis from immature cotyledons (Phillips and Collins, 1981; Lippmann and Lippmann, 1984). The second component of the transformation system is the method for delivering genes to the target tissue. Currently, soybean transformation relies on gene transfer mediated by *Agrobacterium* (Gelvin, 2003; Tzfira and Citovsky, 2006) or particle bombardment (Klein and Jones, 1999; Twyman and Christou, 2004). *Agrobacterium*-based systems rely on the organism's natural ability to transfer DNA to plant cells, while bombardment (or biolistics) uses DNA-coated particles to physically deliver genes directly into cells. The third component involves the means used to select transgenic tissue following gene transfer to the chosen target (Miki and McHugh, 2004). Genes that confer tolerance to chemical agents such as antibiotics or herbicides are generally co-introduced with trait genes (Miki and McHugh, 2004). Transgenic tissue can then be recovered after incubation in tissue culture medium containing the selective agent.

Each of these three components encompasses a number of variables, adding to the complexity of the transformation process and difficulties when attempting to replicate published reports. Currently the two most widely employed transformation approaches are delivery of DNA to cotyledonary node explants via *Agrobacterium* or particle bombardment for gene transfer to embryogenic suspension cultures. This review will discuss these approaches and describe how the components of transformation have been brought

together to develop alternative transformation systems.

2.1.1 Transformation using *Agrobacterium* and cotyledonary explants

Particular tissues from germinated seed provide suitable targets for transformation. The junction between the cotyledon and hypocotyls provide a source of axillary meristems when placed on the appropriate tissue culture medium (Cheng *et al.*, 1980). Wright *et al.* (1986) described procedures for regenerating fertile plants by organogenesis from cotyledons excised from seedlings. Following seed germination on tissue culture medium containing the cytokinin benzyladenine (BA), cotyledon explants were placed on medium containing BA. Preexisting meristems present at the base of the cotyledon, close to the hypocotyl junction, form multiple buds that go on to give rise to shoots. These shoots can be excised and placed onto root-inducing tissue culture medium. In one of the first reports of soybean transformation, *Agrobacterium* with transfer DNA (T-DNA) carrying a kanamycin or glyphosate resistance gene and a β -glucuronidase (GUS) gene for visual identification was co-cultivated with cotyledonary node (cot-node) explants. To accomplish this, the explants were simply incubated in a suspension of *Agrobacterium*. Transgenic shoots were recovered from regeneration medium containing sublethal concentrations of kanamycin and regenerated tissue was screened for GUS expression. About 6% of the recovered shoots were transgenic. Glyphosate tolerance and GUS expression was observed in T₂ progeny, demonstrating proper inheritance of the transgenes. The efficiency of the cot-node system as described in this first report is considered low (Olhoft and Somers, 2001), which is in part caused by low rates of DNA transfer to target cells, inefficient selection resulting in large numbers of escapes, and low rates of regeneration. Apparently the gene used to detoxify kanamycin by neomycin phosphotransferase (NPTII) provided a small growth advantage to transgenic shoots making it necessary to carefully screen all shoots surviving the selection. Di *et al.* (1996) also noted very low rates of transformation when employing kanamycin as the selection agent. The herbicide glyphosate was also used as a selection

agent in conjunction with a bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) synthase gene that is insensitive to glyphosate (Clemente *et al.*, 2000), but rates of transformation were also low in this cot-node system.

Several groups have attempted to optimize the cot-node system to increase efficiency and extend the range of suitable genotypes. For example, Zhang *et al.* (1999, 2000) optimized the conditions used for co-cultivating the cot-node tissue with *Agrobacterium* and improved the media used for shoot elongation. These researchers also utilized the *BAR* gene for resistance to the herbicide glufosinate, which allowed for more effective selection of transgenic shoots than kanamycin resistance. The authors presented important details concerning the process, including the method used to prepare the explant for co-cultivation with *Agrobacterium*. Generally, efficient *Agrobacterium*-mediated gene transfer requires mechanical wounding of the explant, both to provide access to the sub-epidermal meristematic cells and as a means to stimulate production of inducer molecules that trigger the virulence genes of *Agrobacterium*. In this experiment, a scalpel was used to produce 7–12 vertical slices on the cotyledon very close to the cotyledon/hypocotyl junction. Following wounding, the explant was placed in a small petridish along with a suspension of *Agrobacterium*. Although the selection scheme with glufosinate was effective at culling the number of wild-type escapes, a number of the transgenic plants were chimeric, consisting of both transformed and nontransformed tissue. Transgenic plants were produced from about 3% of the treated cotyledon explants.

Further improvements in transformation efficiency were achieved by supplementing co-cultivation media with compounds that ameliorate the plant defense-response triggered by *Agrobacterium*. Thiol compounds, such as L-cysteine, increased rates of gene transfer to cot-node tissue (Olhoft and Somers, 2001; Olhoft *et al.*, 2001). This group employed L-cysteine in combination with hygromycin selection to achieve high rates of transgenic plant recovery (Olhoft *et al.*, 2003). About 16 transgenic plants were recovered from 100 explants infected with *Agrobacterium*. Other researchers have noted improved rates of soybean transformation caused by antioxidant supplements during *Agrobacterium*

transformation when using glufosinate as the selection agent (Paz *et al.*, 2004; Zeng *et al.*, 2004).

Other important factors influencing the efficiency of the cot-node system include genotype (Donaldson and Simmonds, 2000; Paz *et al.*, 2004) and *Agrobacterium* strain (Meurer *et al.*, 1998). Soybean genotypes differ in their ability to respond to tissue culture conditions and form shoots from the cot-node region. Soybean genotypes also vary in their capacity to interact and accept DNA from *Agrobacterium*. The heritability of susceptibility to *Agrobacterium* infection has been studied (Bailey *et al.*, 1994; Mauro *et al.*, 1995). One means to study the interaction of genotype and *Agrobacterium* is to infect cotyledons with *Agrobacterium* carrying a marker gene that can be scored visually. Meurer *et al.* (1998) found large differences in GUS expression between genotypes 45 days after co-cultivation with between 0% and 18% of the infected explants expressing GUS. Paz *et al.* (2006) noted a 4.5-fold difference between genotypes in their capacity to produce transgenic plants.

Early research (Byrne *et al.*, 1987; Delzer *et al.*, 1990) found significant strain-by-genotype interactions. Normally, gene transfer by *Agrobacterium* requires induction of bacterial virulence (*vir*) genes by phenolic compounds produced by wounded plant tissue. The *vir* genes reside on the Ti-plasmid and they function to excise the T-DNA region of the plasmid and promote its transfer and integration into the plant genome. Constitutive expression of *vir* genes in particular *Agrobacterium* strains can enhance gene transfer to soybean (Ke *et al.*, 2001).

Even the source of seed and the protocol used to sterilize the seed can have a profound impact on transformation efficiency. Paz *et al.* (2004) found that batches of seed from different suppliers produced seedlings that varied in their response to tissue culture conditions. These researchers also reported that the time used for sterilizing seed with chlorine gas could impact rates of transformation.

Of particular importance and difficult to relate in print is the technique used to wound the cot-node tissue prior to incubation with *Agrobacterium*. Precise wounding at the correct position at the cot-node junction is critical, but producing the correct wound in a consistent manner is very difficult. To overcome certain limitations associated with the cot-node system,

Paz *et al.* (2006) developed a new method that employs cotyledons derived from seed imbibed overnight as opposed to cotyledons derived from seedlings in the traditional cot-node process. This method eliminated the need for precise mechanical wounding, apparently because the wound produced from removal of the embryo axis was sufficient to permit access to meristematic target tissue by *Agrobacterium*. Rates of transformation using this split seed approach were about 50% higher than the rates observed using the more traditional cot-node system. However, only about 3% of the treated explants gave rise to transgenic plants.

Other organogenic tissue culture systems are being developed and could be useful as targets for transformation. Adventitious shoots can be formed from hypocotyl explants on media containing appropriate levels of BA (Dan and Reichert, 1998). Franklin *et al.* (2004) found that the cytokinins 6-benzylaminopurine (BAP) and thidiazuron (TDZ) act synergistically to induce organogenic nodules and shoot buds from both mature and immature cotyledons. Shan *et al.* (2005) also used TDZ to successfully produce multiple bud tissue from cotyledons. These buds can be proliferated and maintained for extended periods in tissue culture, suggesting that the bud cultures can be suitable targets for transformation.

2.1.2 Transformation using somatic embryogenesis

Another tissue culture approach is to induce a process termed somatic embryogenesis. Somatic embryogenesis involves the development of embryolike structures from vegetative (nongametic) cells. For soybean, somatic embryogenesis is most often induced by placing immature cotyledons on medium containing an auxin, usually 2,4-D (Ranch *et al.*, 1985). Clusters of globular stage somatic embryos can be excised from cotyledons, placed in liquid proliferation medium from which the cultures can be subcultured and expanded (Finer and Nagasawa, 1988). Regeneration is accomplished by transfer to media lacking auxin, histodifferentiation of mature somatic embryos, and a subsequent brief drying process that is necessary to permit germination. Recent advances in liquid maturation media have greatly simplified

the regeneration process (Walker and Parrott, 2001; Schmidt *et al.*, 2005). As is the case for the cot-node tissue culture system, the embryogenic response of soybean is also genotype dependent (Bailey *et al.*, 1993; Simmonds and Donaldson, 2000; Tomlin *et al.*, 2002).

Particle bombardment of embryogenic cultures is commonly used to produce transgenic soybean. In the first reported example (Finer and McMullen, 1991), embryogenic material was co-transformed with plasmids harboring the visual marker gene GUS, and the selectable marker gene AphIV, which detoxifies the antibiotic hygromycin. Clumps of embryogenic tissue were bombarded with DNA-coated particles. Following DNA delivery, the tissue was cultured in liquid medium supplemented with 50 mg l⁻¹ hygromycin for 6–8 weeks with media changed at regular intervals. One or two small yellowish-green clusters of hygromycin-resistant tissue were recovered from each bombardment experiment. The surviving tissue was removed from selection and allowed to grow for an additional 3–4 months. The subsequent differentiation, maturation, desiccation, and germination steps were conducted without further selection. The first attempts to transform these embryogenic cultures proved successful but were hampered by low efficiency and frequent recovery of infertile plants. Others have noted low rates of transformation and poor regeneration frequency of plants from transgenic cultures (Parrott *et al.*, 1994; Droste *et al.*, 2002), averaging less than one event per bombarded sample, while other researchers report more efficient levels of transformation (Sato *et al.*, 1993; Simmonds and Donaldson, 2000). Poor rates of transformation and regeneration may be due to the long periods of time in tissue culture needed to produce cultures capable of transformation (Singh *et al.*, 1988). Optimizing levels of nitrogen and sucrose in the liquid proliferation medium could enhance growth and morphology of embryogenic cultures (Samoylov *et al.*, 1998). Since transformation is tied to the accessibility of mitotic cells (Hazel *et al.*, 1998), cultures derived from the optimized medium should be more readily amenable to transformation than those maintained in the original medium developed by Finer and Nagasawa (1988). Bombarding embryogenic cultures at a specific time after subculture may enhance rates of transformation (Hazel *et al.*, 1998). This is

related to a burst of cell division that peaks about 5 days after subculture. Different cell cultures derived from the same genotype can develop distinct morphologies during repeated subcultures, with some cultures having a denser arrangement of globular structures and capable of supporting higher rates of transformation. These morphologies can also be related to the number of cells undergoing division in the outer layers of the tissue. Other treatments can be used to enhance rates of stable transformation. For example, Vain *et al.* (1993) found that pretreatments that decrease the turgor pressure of embryonic tissue can increase rates of transformation.

Agrobacterium has also been used to deliver genes to embryogenic culture but with limited success. Trick and Finer (1998) utilized sonication to wound embryogenic suspensions. Following co-cultivation with an *Agrobacterium* strain carrying a hygromycin resistance gene, a large number of transgenic cell lines were recovered. Plants regenerated from these events were infertile, probably because of the extended age of the cultures. Droste *et al.* (2000) used a combined particle bombardment and *Agrobacterium* technique for transformation of embryogenic suspensions, but did not report plant recovery.

Particle bombardment or *Agrobacterium* can be used to deliver genes directly into immature cotyledons with subsequent recovery of transgenic somatic embryos. Parrott *et al.* (1989) recovered several transgenic events from immature cotyledons treated with *Agrobacterium*. Yan *et al.* (2000) also found extremely low rates of transformation of cotyledon explants with only 0.03% of treated cotyledons giving rise to transgenic plants. In a series of publications, Ko *et al.* (2003, 2004; Ko and Korban, 2004) optimized various parameters including the size and orientation of the cotyledon, *Agrobacterium* strain and concentration, time of co-cultivation and hygromycin concentration to achieve relatively high rates of transformation. About half of the cotyledons treated with the hyper-virulent strain of *Agrobacterium*, KYRT1, formed embryogenic clusters resistant to hygromycin. Lim *et al.* (2005) extended this work and found that expression of a soybean mosaic virus helper component-protease (Hc-Pro) enhances rates of transformation and stabilizes transgene expression in resulting transformants. Hc-Pro acts as a suppressor of post-transcriptional gene

silencing. It is known that transgenes can be silenced soon after transfer to plant cells (Johansen and Carrington, 2001). Apparently, Hc-Pro acted to prevent silencing of the introduced hygromycin-resistance gene, leading to a 2.4-fold increase in transformation rates.

2.1.3 Gene transfer to apical meristems and other approaches with minimal tissue culture

As is evident from the above discussion, transformation methods for soybean are often dependent on proliferating and maintaining tissue in culture and most laboratories practicing soybean transformation rely on these approaches. Transformation technologies that minimize or eliminate tissue culture can potentially reduce time and labor requirements. Gene delivery to apical meristems or to seed or seedlings has been used in developing effective transformation systems for a number of species. For example, *Arabidopsis* can be transformed by simply inoculating flowers with an *Agrobacterium* suspension. A small proportion of seed from these plants is transgenic (Desfeux *et al.*, 2000). A similar system for soybean would be of great utility. De Ronde *et al.* (2001a) attempted to develop a soybean transformation system based on the *Agrobacterium* infiltration method used for *Arabidopsis*. These authors claimed that 30% of the seed from the *Agrobacterium*-treated plants were transgenic, however, replicated results have not yet been published. Hu and Wang (1999) summarized research concerning soybean transformation by techniques using direct injection of DNA into ovaries or by application to pollen tubes. However, this result has not been reproduced and attempts to produce transgenics by floral tube pathway have been unsuccessful (Li *et al.*, 2002).

Agrobacterium has also been used to transfer DNA directly into meristematic cells within the shoot apex, minimizing tissue culture steps. Chee *et al.* (1989) inoculated the area around the apex by inoculating the plumule, cotyledonary node, and adjacent cotyledon tissues of germinating seed. Seedlings were screened for the presence of the transgene by an enzyme assay for NPTII activity. About 0.07% of the recovered plants were transgenic and transformation was confirmed by Southern blot in both primary transformants

and progeny. Although transformation frequencies were extremely low, the study showed that tissue culture is not always necessary for transformation. Efendi *et al.* (2000) carried out similar experiments with NPTII, hygromycin phosphotransferase (HPT), and GUS genes.

Liu *et al.* (2004) also utilized apical meristems as targets for transformation. Meristems were excised from imbibed seed, co-cultivated in an *Agrobacterium* suspension, and placed on a medium containing hormones that promote rapid proliferation of multiple shoots. Relatively high rates of transformation were noted, matching those reported by Olhoft *et al.* (2003). A relatively long co-cultivation period of 20 h was determined to be important for success of this technique.

Particle bombardment can also be used to deliver genes directly to cells within the apical meristem (McCabe *et al.*, 1988). These researchers developed a custom designed gene gun that utilized capacitance discharge between two electrodes to produce a shock wave. This shock wave provided the force necessary to accelerate a mylar sheet, whose front surface was coated with DNA-bearing particles, into a metal screen. The screen stopped the mylar sheet and allowed the passage of the accelerated particles into the target tissue. Following bombardment the meristems were placed on medium that induces multiple shoot formation. The cultures were then maintained until shoots from either the primary or axillary meristem reached 0.5–1.0 cm in length. This tissue culture regime permits the formation of 5–10 shoots from each bombarded meristem. The visual marker gene, GUS, was used to screen for transgenic plantlets arising from bombarded meristems (Christou *et al.*, 1989). Not all GUS-expressing shoots transmitted the transgene to the next generation. In this first report, only one shoot from about 400 that were sampled expressed GUS activity in both the primary and second-generation plants. Many plants exhibited sectors of GUS expression, but not all of these sectors encompassed a cell lineage that gave rise to a gamete (Christou and McCabe, 1992). After observing a series of GUS-expressing shoots, these researchers were able to identify GUS expression patterns that could be used to predict the likelihood of inheritance of the transgene in the next generation. Plants with GUS sectors in the stem that extended from the epidermis through

the cortex and pith usually produced seed carrying the transgene. This system has the advantage of minimal *in vitro* culture time, thus limiting the probability of generating mutations during tissue culture. The applicability of this system to a number of commercially important genotypes was also demonstrated. However, the system is very inefficient and labor intensive.

Aragao *et al.* (2000) employed a similar bombardment system for transformation of apical meristems, delivering a gene that provides tolerance to imidazolinone herbicides. By generating shoots from bombarded meristems on medium containing imazapyr, these researchers were able to use selection to enhance recovery of transgenic shoots from bombarded meristems. These researchers reported that up to 7% of the regenerated shoots were transgenic and transmitted the transgene to the next generation.

2.1.4 Nature of transgenic loci

Knowledge of the number and structure of transgenes in the host genome is important for subsequent analysis of function and phenotype. The presence of multiple transgene copies can lead to unpredictable levels of gene expression and inheritance of phenotype (Meza *et al.*, 2001; De Buck *et al.*, 2004; Schubert *et al.*, 2004). *Agrobacterium* was once thought to produce transgenic loci with very simple insertions of DNA, often consisting of single copies lacking sequence from the plasmid backbone. However, Olhoft *et al.* (2004) found that 37% of soybean transformants recovered from the cotyledonary node system harbored vector backbone. *Agrobacterium*-derived transformants also often harbor multiple copies of the transgene. Olhoft *et al.* (2003) conducted molecular characterization on 72 individual lines of transgenic soybean derived from *Agrobacterium*-mediated transformation. The data revealed that of the 72 lines, 29 (40%) possessed hybridization patterns suggestive of complex T-DNA integration. The majority (48%) of the transgenic lines analyzed transmitted the transgenes as a single functional locus. Thus, complex loci containing multiple copies of the transgene can be inherited as a single locus. Olhoft *et al.* (2004) conducted detailed analysis of 270 independent transgenic plants. Only about 30% of these had single copies of

the transgene cassette. A significant proportion (about 30%) of the transgene insertions was truncated.

Particle bombardment can also produce transgenics with complex transgene integrations, and the gene of interest must be physically purified from the vector backbone by electrophoresis to avoid its delivery into the host genome. The introduction of vector backbone DNA can be circumvented by coating particles with linearized DNA elements that only contain the gene(s) of interest. Transgenic rice produced by bombardment with these linearized gene cassettes had very simple patterns of transgene insertion (Fu *et al.*, 2000). In addition, merely reducing the amount of DNA delivered per shot can decrease insert complexity in microprojectile-derived soybean transformants and promote insertion of single transgene copies into the soybean genome (Simmonds, 2003).

2.2 Agronomic Input Traits

Soybean grain yield and quality are controlled by genetic elements and environmental factors that regulate the growth, development, and reproduction of soybean plants. Soybean scientists are applying biotechnology to prevent yield losses through transgenic control of pests, diseases, herbicides, and abiotic stress. In this section, we will discuss the improvements of agronomic input traits including excellent examples of how biotechnology and transgenic breeding benefit soybean grain production.

2.2.1 Nematode resistance

Over 100 species of nematodes can infect soybeans. All of them are soil dwelling and live in or on the soybean roots. Among the soybean nematode parasites, SCN, *Heterodera glycines*, is the most destructive soybean pathogen worldwide, causing billions of dollars in yield loss worldwide each year. Some of the root-knot nematodes (RKNs), *Meloidogyne* spp., are important problems in southern America (Niblack *et al.*, 2004). The SCN population has been separated into 16 different races or homogenistic (HG) types (Niblack *et al.*, 2002) based on their ability to infect different soybean lines. During infection, soybean roots are

damaged by nematodes causing symptoms, such as retarded growth, wilting, low seed yield, and altered root morphology.

Second-stage juveniles (J2) are the infective stage of the nematodes, which penetrate the soybean roots. Once inside the roots, the J2 secrete many compounds, which transform a group of root cells to form a specialized feeding structure, known as a syncytium (SCN) or giant cell (RKN). The feeding structure is the only channel through which nematodes can ingest nutrients from the soybean cells, and is therefore imperative for nematode growth and reproduction. The nematode accesses the feeding structure by piercing the cell with a hollow stylet. A feeding tube leads the nutrients to the stylet and into the nematode (Davis *et al.*, 2004). Current nematode control relies on the use of resistant soybean varieties, crop rotation, and nematicidal chemicals, which are hazardous to the environment. The use of resistant varieties is the most effective and economical strategy.

Natural SCN resistance genes (R genes) have been mapped with molecular markers (Webb *et al.*, 1995; Vierling *et al.*, 1996; Concibido *et al.*, 2004), and some of them have been cloned by map-based cloning methods (Jung *et al.*, 1998; Lightfoot and Meksem, 2002). Both *Rhg1* and *Rhg4* genes encode the typical NBS-LRR type of R gene, containing a transmembrane domain and a protein kinase domain similar to the rice *Xa21* gene. *Rhg1* is located on soybean linkage group G (Concibido *et al.*, 1997), whereas *Rhg4* is found on soybean linkage group A2 (Concibido *et al.*, 2004). *Rhg1* is the major R gene for SCN resistance; it is necessary but not sufficient for resistance against all known races. *Rhg4* is a partner of *Rhg1* for resistance against SCN race 3 (Meksem *et al.*, 2001). A sequence homolog of the sugar beet nematode resistance gene *Hs1* has been cloned (Jessen and Meyer, 2001). Resistance conferred by R genes is not durable due to race shifting in the field. CystX is a commercial line exhibiting resistance to several SCN races (<http://www.cystx.com>). However, resistant varieties in general show yield penalty when grown in the absence of SCN. In attempt to avoid this obstacle, transgenic approaches have been pursued in both academic and industrial sectors, allowing genes to be quickly introduced into susceptible elite lines. This method may also allow for stacking of different modes of resistance, i.e., transgenes with R genes (Gheyson

and Fenoll, 2002; Atkinson *et al.*, 2003), leading to more durable nematode tolerance.

Transgenic plants are most commonly used to evaluate the effects of transgenes. Soybean hairy roots, induced by *Agrobacterium rhizogenes* strain K599, have also become a useful tool for evaluating candidate SCN resistance genes. Narayanan *et al.* (1999) found that the nematode life cycle can be completed on hairy root cultures and that resistance phenotypes are conserved. The primary method of measuring SCN susceptibility or resistance is by counting the number of mature females that have developed on a given root sample. Because SCN cysts are so small and their coloring is similar to the root, counting can be cumbersome and inaccurate. Lu *et al.* (2005) invented a fluorescence imaging system that has greatly reduced the effort needed to count cysts. Fluorescence imaging improves accuracy by allowing the operator to visualize cysts that are small or obstructed from view. Another assay has been developed for evaluating double-stranded RNA (dsRNA)-induced silencing that involves inducing feeding in J2 with chemicals, such as octopamine or resorcinol, enabling nematodes to feed without the formation of a feeding site. This type of testing provides a quick way to introduce dsRNA molecules into the nematodes and conduct molecular analyses without the establishment of a nematode feeding site under *in vitro* conditions.

Several strategies toward engineered SCN resistance are being explored including expression of antinematode proteins (or peptides), blocking feeding site formation, and knocking off essential nematode genes. Proteinase inhibitors (PIs) are often part of a plant's wounding response. Because they are not toxic to the plant, they can be constitutively expressed, possibly leading to resistance to other soybean pests (Jung *et al.*, 1998). Urwin *et al.* (1997a) studied a mutated oryzacystatin-I gene from rice, *Oc-IAD86*, expressed in *Arabidopsis* plants. The *Oc-IAD86* gene was expressed under CaMV 35S promoter and Nos terminator (Table 3) in a binary vector, which was transformed into *Arabidopsis* by *Agrobacterium*-mediated transformation. Selected events contained a single copy of the *Oc-IAD86* gene, as confirmed by Southern blot analysis. Quantitative Western blot analysis revealed that the expression of *Oc-IAD86* was 0.4% of the total

protein. Ingestion of the modified cystatin from the plant was correlated with a loss of cysteine proteinase activity in the intestine, preventing female nematodes of both *Meloidogyne incognita* and *Heterodera schachtii* (beet-cyst nematode) from becoming large enough to produce eggs. Urwin *et al.* (1997b) also identified genes for two cysteine proteinases in *H. glycines*, hgcp-I and hgcp-II. The same engineered *Oc-IAD86* cystatin as described above was able to inhibit the activity of Hgcp-I, but not Hgcp-II in cryosections (Lilley *et al.*, 1996). Urwin *et al.* (1998) achieved resistance to *M. incognita* and *H. schachtii* in transgenic *Arabidopsis*. Translational fusion constructs containing the genes for PIs CpTI and *Oc-IAD86* joined by a noncleavable linker was generated by polymerase chain reaction (PCR). Individual *CpTI* and *Oc-IAD86* sequences, as well as the fused sequence, were driven by the pBI121 CaMV 35S promoter and Nos terminator (Table 3) for analysis in plants. The constructs were introduced into *Arabidopsis thaliana* via *Agrobacterium*-mediated root transformation. Expression analysis was conducted by Western blot using antibodies for both *Oc-IAD86* and *CpTI*. In both *Escherichia coli* and *Arabidopsis*, expression of the noncleavable construct resulted in a protein of ~23 kDa that contained both PIs. Relative-inhibition assays were also carried out to ensure that the proteins were still active in their various forms. Western blot analysis of *M. incognita* and *H. schachtii* females recovered from T₁ roots revealed that the nematodes had ingested *Oc-IAD86* or *CpTI* when recovered from plants expressing single PI constructs. The 23 kDa fusion proteins were detected in *M. incognita*, but not in *H. schachtii*. Results of infection assays showed that expression of *CpTI* in either form changed the *H. schachtii* sex ratio, evident by the reduced number of saccate females at 20 dpi on T₁ plants. At 45 dpi fewer mature females were observed on the *CpTI*-transformed roots than on the controls. Plants expressing *Oc-IAD86* or the fusion protein had a greater number of females with delayed development compared to the controls. The data showed that the expression of the two proteins fused together had an additive effect that can confer resistance to the RKN. The data also indicate that *M. incognita* is able to take up proteins up to 23 kDa, despite the small opening in the stylet. Although there is no direct evidence yet,

Table 3 Published transgenes for improving soybean traits

Trait	Transgene	Gene source	Promoter	Terminator	Transformation plant	References
Nematode	<i>Oc1AD86</i>	<i>O. sativa</i>	CaMV 35S	Nos	<i>Agrobacterium</i> <i>Arabidopsis</i>	Urwin <i>et al.</i> , 1998
Nematode	<i>MSP dsRNA</i>	<i>H. glycines</i>	Actin-2	Actin-2	Particle bombardment Soybean	Trick <i>et al.</i> , 2004
Nematode	<i>pat-10 dsRNA</i>	<i>H. glycines</i>	Super	Nos	<i>Agrobacterium</i> Soybean (hairy roots)	Ren <i>et al.</i> , 2005
Nematode	<i>16D10</i>	<i>M. incognita</i>	CaMV 35S	Nos	Floral dip	Hussey and Huang, 2006
Sclerotinia	<i>gf-2.8</i>	<i>T. aestivum</i>	CaMV 35S	pFF19 3'	<i>Arabidopsis</i> <i>Agrobacterium</i> Soybean	Donaldson <i>et al.</i> , 2001
Phytophthora	<i>Rps1-k</i>	<i>G. max</i>	Rps1-k	Rps1-k	<i>Agrobacterium</i> Soybean	Gao <i>et al.</i> , 2005
Insect	<i>cry1Ab</i>	<i>B. thuringiensis</i>	CaMV 35S	AMV polyA	Particle bombardment Soybean	Parrott <i>et al.</i> , 1994
Insect	<i>cry1Ac</i>	Synthetic <i>B. thuringiensis</i>	35S	Orf25	Particle bombardment Soybean	Stewart <i>et al.</i> , 1996
Insect	<i>cry1A</i>	<i>B. thuringiensis</i>	Rubisco	NA ^(a)	<i>Agrobacterium</i> Soybean	Macrae <i>et al.</i> , 2005
Insect	<i>TcdA</i>	<i>P. Luminescens</i>	CsVMV	Orf25	<i>Agrobacterium</i> <i>Arabidopsis</i>	Liu <i>et al.</i> , 2003
Herbicide	<i>EPSPS</i>	<i>Agrobacterium CP4</i>	CaMV 35S	NA	Particle bombardment Soybean	Padgett <i>et al.</i> , 1995
Herbicide	GAT	<i>B. licheniformis</i>	Ubiquitin	PinII	<i>Agrobacterium</i> Maize	Castle <i>et al.</i> , 2004
Virus	<i>CP</i>	<i>SbDV</i>	CaMV 35S	Nos	Particle bombardment Soybean	Tougou <i>et al.</i> , 2006
Virus	<i>PCP</i>	<i>BPMV</i>	Dup. FMV 35S	RbcS	Particle bombardment Soybean	Reddy <i>et al.</i> , 2001
Salt stress	<i>CAX1</i>	<i>G. max</i>	CaMV 35S	Nos	Vacuum infiltration	Luo <i>et al.</i> , 2005
Drought/heat stress	<i>P5CR</i>	<i>A. thaliana</i>	HS	NA	<i>Arabidopsis</i> <i>Agrobacterium</i> Soybean	Simon-Sarkadi <i>et al.</i> , 2005
Iron deficiency	<i>FRO2</i>	<i>A. thaliana</i>	CaMV 35S	NA	<i>Agrobacterium</i> Soybean	Vasconcelos <i>et al.</i> , 2006

Oil	<i>FAD2-1 antisense</i>	<i>G. max</i>	Phaseolin	CaMV 35S	<i>Agrobacterium</i> Soybean	Buhr <i>et al.</i> , 2002
Oil	<i>FatB + FAD2-1 antisense</i>	<i>G. max</i>	Phaseolin	CaMV 35S	<i>Agrobacterium</i> Soybean	Buhr <i>et al.</i> , 2002
Oil	<i>FAD2-1</i>	<i>G. max</i>	β -conglycinin	Phaseolin	Particle bombardment Soybean	Kinney and Knowlton, 1998
Oil	<i>dapA (DHDPs)</i>	<i>E. coli</i>	CaMV 35S	Trypsin inh.	Particle bombardment Soybean	Kinney and Knowlton, 1998
Oil	<i>FADB</i>	<i>G. max</i>	β -conglycinin	Phaseolin	Particle bombardment Soybean	Cahoon, 2003
Oil	δ -6 desaturase	<i>Borage</i>	β -conglycinin	CaMV 35S	<i>Agrobacterium</i> Soybean	Sato <i>et al.</i> , 2004
Oil	δ -6 desaturase	<i>Borage</i>	β -conglycinin	CaMV 35S	<i>Agrobacterium</i> Soybean	Eckert <i>et al.</i> , 2006
Oil	δ -15 desaturase	<i>A. thaliana</i>	β -conglycinin	CaMV 35S	<i>Agrobacterium</i> Soybean	Eckert <i>et al.</i> , 2006
Protein	7S Conglycinin	<i>G. max</i>	β -conglycinin	Phaseolin	Particle bombardment Soybean	Kinney and Fader, 2002
Protein	11S Glycinin	<i>G. max</i>	β -conglycinin	Phaseolin	Particle bombardment Soybean	Kinney and Fader, 2002
Protein	β -casein	<i>Bovine</i>	Lectin	Lectin 3'UTR	Particle bombardment Soybean	Maughan <i>et al.</i> , 1999
Protein/allergy	P34	<i>G. max</i>	β -conglycinin	Phaseolin	Particle bombardment Soybean	Herman <i>et al.</i> , 2003
Flavor	<i>FAD2-1</i>	<i>G. max</i>	β -conglycinin	Phaseolin	Particle bombardment Soybean	Kinney and Kowltion, 1998
Amino acid	<i>AK (lysC)</i>	<i>E. coli</i>	Phaseolin	Phaseolin	Particle bombardment Soybean	Falco <i>et al.</i> , 1995
Amino acid	<i>DHDPs</i>	<i>Corynebacterium</i>	Phaseolin	Phaseolin	Particle bombardment Soybean	Falco <i>et al.</i> , 1995
Amino acid	2S albumin	<i>Brazil nut</i>	NA	NA	NA	Townsend and Thomas, 1994
Amino acid	15 kDa zein	<i>Z. mays</i>	β -conglycinin	Nos	Particle bombardment Soybean	Dinkins <i>et al.</i> , 2001

(continued)

Table 3 Published transgenes for improving soybean traits (*continued*)

Trait	Transgene	Gene source	Promoter	Terminator	Transformation plant	References
Amino acid	Modified β -conglycinin	Synthetic	β -conglycinin	β -conglycinin	<i>Agrobacterium</i>	Rapp <i>et al.</i> , 2005
Amino acid	27 kDa <i>zlen</i>	<i>Z. mays</i>	β -conglycinin	β -conglycinin	Soybean Particle bombardment	Li <i>et al.</i> , 2005
Vitamin E	<i>HGGT</i>	<i>H. vulgare</i>	CaMV 35S	Nos	Soybean <i>Agrobacterium</i>	Cahoon <i>et al.</i> , 2003
Vitamin E	<i>At-VTE3/At-VTE4</i>	<i>A. thaliana</i>	β -conglycinin	Pea 3' UTR	<i>Arabidopsis</i> <i>Agrobacterium</i>	Van Eenennaam <i>et al.</i> , 2003
Phytate	<i>Phytase</i>	<i>Aspergillus niger</i>	CaMV 35S	Nos	Soybean Particle bombardment	Denbow <i>et al.</i> , 1998
Phytate	<i>Phytase</i>	<i>G. max</i>	β -conglycinin	β -conglycinin	Soybean Particle bombardment	Chiera <i>et al.</i> , 2004
Phytate	<i>GmMIPSI hairpin</i>	<i>G. max</i>	CaMV 35S	OCS	Soybean NA	Nunes <i>et al.</i> , 2006
Protein industry	<i>Dragline silk</i>	Synthetic	β -conglycinin	Phaseolin	Soybean Particle bombardment	Barr <i>et al.</i> , 2004
Unusual fatty acid	<i>MomoFadX/ImpFadX</i>	<i>M. charantia/L. balsamina</i>	β -conglycinin	Phaseolin	Soybean Particle bombardment	Cahoon <i>et al.</i> , 1999
Unusual fatty acid	<i>CoFAD2-1/CoFAD2-2</i>	<i>C. officinalis</i>	β -conglycinin	Phaseolin	Soybean Particle bombardment	Cahoon <i>et al.</i> , 2001
Unusual fatty acid	<i>CoA/FAE1</i>	<i>L. douglasii</i>	β -conglycinin	Phaseolin	Soybean Particle bombardment	Cahoon <i>et al.</i> , 2006
Unusual fatty acid	<i>CYP726A1</i>	<i>Asteraceae/Euphorbaceae</i>	β -conglycinin	Phaseolin	Soybean Particle bombardment	Cahoon <i>et al.</i> , 2002
Unusual fatty acid	<i>DsFAD2-1/DsFAD2-2</i>	<i>D. sinuate</i>	β -conglycinin	Phaseolin	Soybean Particle bombardment	Cahoon and Kinney, 2004
Unusual fatty acid	<i>LuFADBA</i>	<i>L. usitatissimum</i>	Flax 2S conlinin	NA	Soybean <i>Agrobacterium</i>	Vrinten <i>et al.</i> , 2005
LCPUFA	<i>SdΔ6-desaturase</i>	<i>S. diclina</i>	β -conglycinin	Phaseolin	<i>Flax</i> Particle bombardment	Kinney <i>et al.</i> , 2004
LCPUFA	<i>MaΔ6-elongase</i>	<i>M. alpine</i>	Glycinin Gy1	Pea leguminA2	Soybean Particle bombardment	Kinney <i>et al.</i> , 2004
LCPUFA	<i>MaΔ5-desaturase</i>	<i>M. alpine</i>	Trypsin inhibitor	Trypsin inhibitor	Soybean Particle bombardment	Kinney <i>et al.</i> , 2004
LCPUFA	<i>SdΔ15-desaturase</i>	<i>A. thaliana</i>	Trypsin inhibitor	Trypsin inhibitor	Soybean Particle bombardment	Kinney <i>et al.</i> , 2004
LCPUFA	<i>SdΔ17-desaturase</i>	<i>S. diclina</i>	Annexin	BD30	Soybean Particle bombardment	Kinney <i>et al.</i> , 2004

^(a)NA, not available

these PIs may also confer resistance in soybean for both SCN and RKN.

A relatively new approach to nematode resistance is RNA interference (RNAi). This phenomenon was first identified by Guo and Kemphues (1995) and subsequently by Fire *et al.* (1998) in *Caenorhabditis elegans*. Urwin *et al.* (2002) used a J2 forced feeding assay to investigate cysteine proteinase gene silencing in *H. glycines* and *G. pallida*. Reduced transcript abundance was evident; however, subsequent infection did not lead to a reduction in the number of parasites, but resulted in a shift from the normal male/female ratio of 75% to 50% after RNAi. Forced feeding SCN J2 with C-type lectin dsRNA was found to reduce SCN reproduction on roots by 41% (Urwin *et al.*, 2002). RNA soaking assays with RKN revealed that the dual oxidase gene (Bakhetia *et al.*, 2005a), polyglacturonase gene, calreticulin gene, (Rosso *et al.*, 2005), and chitin synthase gene (Fanelli *et al.*, 2004) are all important genes for RKN development and reproduction. These genes are potential candidates for engineering soybean for SCN and RKN resistance through the RNAi approach (Bakhetia *et al.*, 2005b).

Expression of nematode dsRNA in plants has already been demonstrated. In order to obtain deleterious effects on the nematode *in planta*, SCN-specific dsRNA I is expressed in the syncytium. The nematode is then able to draw the dsRNA into the gut cells and RNA silencing is induced. Trick *et al.* (2004) and Steeves *et al.* (2006) expressed dsRNA corresponding to the nematode major sperm protein (MSP) gene in soybean plants. Degenerate primers were used to amplify a *MSP* gene fragment from *H. glycines* genomic DNA. The fragment was then used to isolate the *MSP* complementary DNA (cDNA) from a *H. glycines* J2 juvenile cDNA library. Approximately 250 bp sense and antisense sequences of the *MSP* cDNA were linked by a loop of ~112 bp. The fragment was flanked by the *Arabidopsis* actin-2 promoter (An *et al.*, 1996) and terminator for transformation. Particle bombardment was used to transform soybean somatic embryos with the hairpin construct. The resulting transgenic plants were confirmed by hygromycin selection and subjected to bioassay testing. The first greenhouse bioassay was used to determine SCN resistance in the transgenic plants. Events were transplanted into SCN-infested soil and after 6 weeks, the

number of cysts, eggs per cyst, and root weight data were recorded. Cysts from replicates of each event in the first experiment were pooled independently and eggs were collected. The eggs were then used to inoculate Flyer plants (a soybean line with no resistance to SCN) in sterile soil. After growing in the infected soil for 6 weeks, data on cyst number, eggs per cyst, and root weight were collected. The number of cysts per plant was slightly reduced in the transgenic line compared to the nonengineered control during the first bioassay. Results of the second bioassay revealed that a much higher reduction occurred in the second generation of cysts exposed to the *MSP* RNAi. The effects of the *MSP* transgene were present in the first generation and may have been carried over into the second generation. Bioassay 1 had a 64% reduction in the number of *H. glycines* eggs per plant. In the second experiment, even though the eggs from the transgenic and nontransgenic control lines were applied at the same rate, the number of eggs per transgenic plant was significantly reduced (71%).

Ren *et al.* (2005) reported resistance to race 3 SCN in soybean hairy roots. Four SCN target genes were identified through GenBank SCN EST and *C. elegans* databases. Targets were checked for low homology to soybean genes at the DNA sequence level. Fragments of *H. glycines* homologs to *C. elegans* genes were isolated from cDNA for each of the targets: *cct-6* (545 bp), *daf-21* (399 bp), *Y65BR.5a* (499 bp), and *pat-10* (399 bp). In order to validate the targets, a nematode soaking assay was conducted. dsRNA was amplified by T7 *in vitro* transcription for the four targets and a GUS control. Freshly hatched SCN J2s were soaked in a 0.6 mg ml⁻¹ dsRNA solution for 5 days. Observation of nematode mobility versus immobility was assessed in order to determine efficacy of the targets. In a representative experiment, 95% of the J2 soaked in water and 60% of the J2 treated with GUS dsRNA exhibited active motility, showing that some nonspecific activity may be occurring. However, the activity of the targets was still evident above this high baseline. *H. glycines cct-6* showed the highest activity, immobilizing 90% of the nematodes. Similarly *daf-21* immobilized 75% and *Y65BR.5a* immobilized 80%. In another experiment, *pat-10* dsRNA immobilized 53% of the J2, compared to 8% and 26% in water- and GUS-treated samples, respectively. The targets

were also tested in a *C. elegans* feeding assay. After 5 days of feeding, the controls had advanced to gravid adults and produced a second generation, whereas the four targets resulted in developmental arrest. These data indicate that the selected targets play a key role in the development of both *C. elegans* and *H. glycines*. Ren *et al.* (2005) further evaluated the SCN target genes in soybean hairy roots. Sense and antisense fragments of the targets were linked together with an intron, encoding dsRNA. The transgene was driven by the SuperMas promoter (Ni *et al.*, 1995) that drives expression at a high level in many tissues including roots. Binary vectors of the individual targets were made, and then a vector containing three of the targets, *cct-6*, *daf-21*, and *Y65BR.5a* were linked together in order to produce dsRNAs simultaneously. The vectors were transformed into soybean hairy roots via *A. rhizogenes*. Positive transformants were identified using the arsenal herbicide resistance gene driven by the Actin-2 promoter (Table 3). Expression of sense and antisense *pat-10* and *cct-6* DNA was confirmed by PCR analysis. Northern blot analysis also confirmed siRNA expression in the hairy roots. The transgenic roots were subjected to SCN infection and the number of female cysts was determined. Roots derived from the *pat-10* vector showed a statistically significant 23–85% reduction in female cysts compared to the susceptible controls. At this time, data for the other three targets and the concatemer construct have not been published.

Hussey and Huang (2006) and Huang *et al.* (2006) were able to apply the RNAi technology to control root-RKNs in plants. A clone encoding a 43 amino acid secretory signaling peptide, designated *16D10*, was identified during random sequencing of a gland-cell specific library of *M. incognita*. The protein sequence has some similarity to the *Arabidopsis* CLV3-like proteins (Cock and McCormick, 2001) as well as a cAMP/cGMP-dependent protein kinase phosphorylation site. Transformation of *Arabidopsis* *clv3* mutants, however, showed that the 16D10 protein does not have *clv3*-like activity (Hussey and Huang, 2006). Southern blot analysis showed that this clone is present in four agriculturally important species: *M. incognita*, *Meloidogyne javanica*, *Meloidogyne arenari*, and *Meloidogyne hapla*. *In situ* messenger RNA (mRNA) hybridization revealed that *16D10*

was strongly expressed in the two subventral esophageal gland cells of *M. incognita* at early parasitic stages. Stylet secretions were collected and found to contain the 16D10 peptide. An *M. incognita* J2 forced feeding assay was used to test the effect of 16D10 dsRNA. After 4 h of feeding, the J2 were subjected to RNA isolation and real time (RT) PCR. A significant reduction in the nematode *16D10* transcripts in the treated nematodes was observed providing direct evidence for targeting *16D10* in RKN. Binary constructs were prepared containing CaMV 35S promoter driving the hairpin containing *16D10* sense and antisense cDNAs linked together by an intron, followed by the Nos terminator (Table 3). This construct was transformed into *A. thaliana* via floral dip transformation. Transgenic plants were selected by kanamycin, and T₂ events were developed for nematode infection assays. The assays clearly demonstrated that the *16D10* RNAi plants are resistant to the four major *Meloidogyne* species. These plants showed a 63–90% reduction in the number and size of galls, and a 70–97% reduction in the number of RKN eggs per gram of root compared to the vector-transformed control plants. Considering these data, the *16D10* gene is a potent candidate for engineering RKN resistance in soybean.

2.2.2 Fungal resistance

More than 40 fungal pathogens are reported to cause significant plant disease worldwide (Hartman *et al.*, 1999), some of which infect soybean. The importance of the soybean diseases is dependent on the plant tissue type, environmental condition, and soybean genotype. Fungal pathogens can cause damage in seedlings, roots, stems, leaves, and pods (Grau *et al.*, 2004). The important fungal diseases include *Phytophthora* root and stem rot, sudden death syndrome (SDS), brown stem rot, *Fusarium* wilt, anthracnose, *Sclerotinia* diseases (white mold and *Sclerotinia* blight), powdery mildew, stem canker, frog-eye leaf spot, and soybean rust. Grau *et al.* (2004) described the symptoms, importance, and management for these diseases.

The *Phytophthora* diseases are caused by the oomycete *P. sojae*. These diseases cause severe destruction in soybean crops worldwide. This

pathogen can infect root and stem tissues and the infection rates are dependent on the environmental conditions. The interactions between the pathogen and its hosts have been reviewed recently (Tyler, 2002; Ellis *et al.*, 2006). The genome sequence of *P. sojae* is now available (<http://www.pfgd.org>). Native *Phytophthora* R genes (*Rps*) have been identified and isolated (Gao *et al.*, 2005). Resistant varieties are available and even new cultivars containing major resistance genes can show resilience to *P. sojae* for 5–10 years. Breeders are bringing several QTLs together into new varieties to build more durable resistance (Ellis *et al.*, 2006). Among these R genes, *Rps1-k* is the most stable and widely used. Four coiled coil-nucleotide binding-leucine rich repeat (CC-NB-LRR)-type proteins were isolated from the *Rps1-k* locus, and three of them have identical open reading frames and 5' end regions. These genes were designated *Rps1-k-1*, *Rps1-k-2*, *Rps1-k-3*, and *Rps1-k-4* and were transformed into transgenic soybean plants using their own promoters and terminators (Table 3) (Gao *et al.*, 2005). Transgenic plants were generated by *Agrobacterium*-mediated transformation and tested for four generations. The results consistently showed that expression of these R genes conferred enhanced resistance to *Phytophthora* in the transgenic soybean plants.

Sclerotinia sclerotiorum (Lib.) de Barry causes stem rot, also known as white mold, and is also an important soybean pathogen. The fungus colonizes dead tissue on the plant and then invades the host plant when wet soil and canopy conditions at flowering occur (Grau, 1988). Under suitable conditions, *Sclerotinia* initially infects soybean plants by way of ascospores that land on flowers. When moisture is adequate, the ascospores germinate and form mycelium, using the flower petals as a nutrient base. The pathogen causes lesions that completely encircle the stem and disrupt the transport of water, mineral nutrients, and photosynthates to developing pods. Cottony mycelial growth on all diseased plant parts is a characteristic sign of the pathogen. *Sclerotinia* can form inside and outside the infected tissues. Combating this problem with traditional technologies is difficult because few genetic sources of resistance are available. *Sclerotinia* resistance has been shown to be a quantitative trait (Gentzbittel *et al.*, 1998; Zhao and Meng, 2003); however, commercial germplasm has limited

genetic variation for resistance (Cober *et al.*, 2003; Hu and Lu, 2005). Kim and Diers (2000) and Arahana *et al.* (2001) identified several of the QTLs associated with resistance, though they were often associated with avoidance traits rather than physiological resistance. Low correlation between field and laboratory tests also contributes to difficulties in breeding (Boland and Hall, 1987; Wegulo *et al.*, 1998). Management of this disease includes utilization of crop rotation and chemical treatments, but these are expensive and often unsuccessful. Research on *S. sclerotiorum* pathogenicity factors has been the most promising lead toward transgenic resistance. As the fungus infects the host tissue, *Sclerotinia* secretions trigger a complex exchange of molecular signals and responses between the pathogen and its host. *S. sclerotiorum* secretes millimolar concentrations of oxalic acid into infected host tissues (Maxwell and Lumsden, 1970; Marciano *et al.*, 1983; Godoy *et al.*, 1990), acidifying the plant tissue and chelating the Ca^{++} from the cell wall, leaving the tissue susceptible to further breakdown by the fungus (Lumsden, 1979). Oxalic acid (OA) also inhibits *O*-diphenol oxidase (Ferrar and Walker, 1993), which suppresses the oxidative burst (Cessna *et al.*, 2000). Manual injection of oxalate into plants caused the development of *Sclerotinia* diseaselike symptoms (Bateman and Beer, 1965; Noyes and Hancock, 1981). Mutants of *S. sclerotiorum* that are deficient in the ability to synthesize oxalate are nonpathogenic, whereas revertant strains that regain their oxalate biosynthetic capacity exhibit normal virulence (Godoy *et al.*, 1990). The data clearly demonstrate that OA is a pathogenicity factor, and a common strategy is to degrade OA by expressing oxalate-degrading enzymes in plants for combating this fungal pathogen.

Donaldson *et al.* (2001) demonstrated that the wheat germin protein, oxalate oxidase (OXO), degrades oxalic acid and confers resistance to *Sclerotinia* in growth chamber inoculation tests. *Agrobacterium tumefaciens* was used to transform a binary vector containing a fragment of the wheat genomic clone *gf-2.8*, including its signal peptide sequence (Dratewka-Kos *et al.*, 1989; Lane *et al.*, 1991); linked to the CaMV 35S enhanced promoter (Odell *et al.*, 1985; Kay *et al.*, 1987) and the 3' terminator region from pFF19 (Table 3). Kanamycin resistance was used to select transgenic shoots. Expression was confirmed

through an OXO histological assay, and was detected in all tested tissues. Western blots showed that the transgenic soybeans expressed a 130 kDa protein similar to the native germin. Line 80-30-1, which was selected for further investigation of *Sclerotinia* resistance, was positive for OXO activity, but negative for the marker gene. Progeny of this line have shown stable transmission of the OXO transgene up to the T₈ generation. Events from the homozygous T₃ generation were tested for *Sclerotinia* resistance in growth chamber assays by cotyledon and stem inoculation. Both assays consistently showed that the transgenic line exhibited a higher level of resistance to *Sclerotinia* infection and higher survival rate when compared to the null and parental lines. During the cotyledon infection assays, *Sclerotinia* lesions on the controls and transgenics extended similarly, however when the lesion reached the hypocotyl, infection stopped and the plants were able to recover. Stem inoculations revealed a delayed infection, significantly reducing the lesion size. Field studies to characterize the 80(30)-1 transgenic soybean line described above were conducted by Cober *et al.* (2003). The study was completed over 3 years at three different sites. Infected plants were rated for disease severity index (DSI) at the beginning of leaf senescence (0 = no infection; 100 = all plants killed by white mold). Results showed that the transgenic line was significantly less infected with *Sclerotinia* than the negative sib line. At one site, plants were artificially infested with *S. sclerotiorum* sclerotia. The transgenic line had an average DSI of 7 while the negative sib line had an average DSI of 46 across the 3-year period. Two resistant cultivars, Maple Arrow and OAC Salem, had a DSI of 2 over 2 years. The OXO transgene was not able to confer complete resistance to white mold infection; however, in a susceptible genetic background its resistance level was quite comparable to the current resistant short-season cultivars.

Sclerotinia blight in soybean, also known as southern blight, occurs primarily in southern America. This is a soybean-specific disease caused by *Sclerotinia rolfisii*, which produces oxalic acid as the major pathogenic factor (Bateman and Beer, 1965). Livingstone *et al.* (2005) reported that overexpression of barley OXO conferred enhanced resistance to *Sclerotinia* blight in peanut, which is caused by *Sclerotinia minor*. Both *S. minor* and

S. rolfisii produce oxalic acids. Direct application of oxalic acid to stem or leaf tissue causes tissue injury and wilting in soybean, similar to plant responses to fungal infection by *S. rolfisii* (Bateman and Beer, 1965) and *S. sclerotiorum* (Noyes and Hancock, 1981). Therefore, oxalate-degrading enzymes may also confer enhanced resistance to soybean *Sclerotinia* blight. However, OXO is a potential allergen as indicated by the results from Jensen-Jarolim *et al.* (2002). In addition to OXO, there are two other classes of oxalate degrading enzymes, oxalate decarboxylase and oxalyl-CoA-decarboxylase, which also have potential for engineering *Sclerotinia* resistance (Lu, 2003; Hu and Lu, 2005; Lu and Hoeft, 2008).

Gene flow has been reported and is a concern for transgenic crops such as sunflower (Snow *et al.*, 2003). To address this issue, Burke and Rieseberg (2003) backcrossed the OXO transgene from OXO-sunflowers to wild species and carried out case and field experiments using the progeny. The OXO transgene was shown to enhance resistance to *S. sclerotiorum*, but did not significantly impact seed production and reproductive fecundity of the wild species. These results indicate that genetically enhanced wild plants will not become more “weedy” since they do not produce more seeds than unmodified controls (Lu and Hoeft, 2008). In addition, there are no wild relatives of soybean in the United States and soybeans are self-pollinated crop, therefore if a transgene were released in these areas, no comparable weeds or wild relatives would be receptive to the gene through pollination, eliminating the risk of gene flow in the United States. This is a big advantage for developing transgenic soybean products.

Asian soybean rust, caused by *Phakopsora pachyrhiz* or *Phakopsora meibomia*, was first observed in Japan in 1902 and was found throughout most Asian countries and Australia by 1934. Rust was identified in South America in 2001, and the first US cases were discovered in November 2004. Currently, there are no commercial US soybean cultivars that are known to be resistant to soybean rust. Annual losses in future years could average \$240 million to \$2 billion worldwide, depending on the severity and extent of outbreaks (www.ers.usda.gov). Current fungicides and tolerant varieties as well as crop rotation are the main means of controlling the rust disease (McGinnis and Suszkiw, 2006).

Glyphosate was proposed to inhibit rust disease in the Roundup Ready-soybean fields (Feng *et al.*, 2005). The technology developed while studying other diseases in soybean and other crops (Gurr and Rushton, 2005) may help to create a transgenic strategy for the control of rust and other diseases.

No transgenic crops with enhanced fungal disease resistance are available or even close to being commercialized yet (Castle *et al.*, 2006). However, several potential approaches are emerging for disease control (Hammond-Kosack and Parker, 2003; Narayanan *et al.*, 2004; Gurr and Rushton, 2005). One of the approaches is to express antifungal proteins (or peptides) for combating fungal diseases. Li *et al.* (2004) transformed genes encoding chitinase and ribosome-inactivating protein into transgenic soybean plants. The expression of both genes was confirmed in the transgenic lines. However, there is no further report of the effects of these antifungal proteins against fungal pathogens.

2.2.3 Virus resistance

There are more than 100 viruses capable of infecting crops. Soybean mosaic virus, bean pod mottle virus (BPMV), and soybean dwarf virus (SbDV) are the most important viruses affecting soybean (Conner *et al.*, 2004).

SbDV severely decreases the soybean yield by damaging the leaves and greatly reducing the number of seed pods. The virus is commonly found in Australia, Japan, and New Zealand. It can only transfer from plant to plant by female aphids. Plants infected with SbDV grow only to one-third of the normal size, and leaves become dark green and curl downward. Tougou *et al.* (2006) were the first to report transgenic resistance to SbDV conferred by RNA-induced post-transcriptional gene silencing. A transformation cassette was developed using sense and antisense orientations of the SbDV *CP* gene separated by a 670 bp loop of *GUS* gene sequence. The hairpin was flanked by the CaMV 35S promoter and Nos terminator (Table 3), and the HPT gene was used as a selectable marker. Both *CP* hairpin and hygromycin whole plasmid constructs were transformed into Jack by particle bombardment. One plant showing hygromycin resistance survived to produce T₁ seeds. PCR and Southern blot

analyses were used to confirm integration of the transgene. Of the T₁ plants, 68% showed the presence of the *CP* gene. Fragments representing the intact hairpin construct were also detected by Southern blot analysis of three T₁ plants; however, the same three plants did not have SbDV-*CP* expression as confirmed by Northern blot analysis. T₂ plants were tested against SbDV infection. Three out of seven plants showed no symptoms of infection compared to one out of seven in the control. Northern analysis showed that all seven of the tested T₂ plants had siRNA accumulation. When tested for virus accumulation, only the three without symptoms lacked SbDV-specific RNAs. These three events were considered to be resistant to SbDV and may be further evaluated for conferring SbDV-resistance in soybean lines.

BPMV commonly infects fields in the southern regions of the United States, however, in recent years infection has spread to northern regions as well. BPMV is a member of the genus comovirus, and is spread by many species of leaf-feeding beetles. The virus not only decreases seed yield, but also causes the seeds that form to be predisposed to other infection (Stuckey *et al.*, 1982). Reddy *et al.* (2001) was able to confer resistance in transgenic soybean expressing the BPMV pCP (coat protein precursor) gene. The BPMV-*pCP* gene was removed from pMON1900 (Di *et al.*, 1996) and inserted into a bluescript-based plasmid containing a duplicated Figwort mosaic virus (FMV) 35S promoter and *rbcs* terminator (Table 3). This construct was then ligated into binary vector pHIG (Yan *et al.*, 2000). Somatic embryos of Jack were transformed by particle gun bombardment. Transformed material was selected by growing embryos on media containing hygromycin. Ninety-five percent of the events showed integration of the hairpin transgene (confirmed by Southern blot analysis). Probing Northern blots with the loop region of the hairpin revealed the presence of the transcript in all plants. GUS staining was also used to confirm presence of the transcript. Expression levels of the 63 kDa pCP were estimated by enzyme-linked immunosorbent assay (ELISA) to be as high as 0.2% of the total soluble protein in the leaves. Detached leaves of the primary transformants showed lower levels of BPMV accumulation compared to the nontransformed control. T₁ and T₂ plants were subjected to rub-in inoculation

with a purified preparation of Hopkins, the severe strain of BPMV. All inoculated plants inheriting the *pCP* gene are resistant to BPMV. Use of this gene in soybean lines may help to alleviate some of the viral problems occurring in fields today. RNAi technology may play important roles in engineering broad-spectrum virus control (Prins, 2003). In addition, BPMV can be used as a vector for expression of recombinant proteins in soybean, and a viral vector for gene silencing (Zhang and Ghabrial, 2006).

2.2.4 Insect resistance

Many insect species have been reported to infect soybean (Kogan and Turnipseed, 1987). These insects can be classified into three groups by the tissues they target. These include pod, stem, and seed feeders; foliage feeders; and root and nodule feeders. The important soybean insects include southern green stink bug, bean leaf beetle, corn earworm, soybean looper, grasshopper, soybean aphids, and soybean nodule fly. Boethel (2004) has reviewed soybean insects and insect management.

Bacillus thuringiensis (*Bt*) is a soil bacterium that produces insecticidal crystalline proteins. Insoluble crystalline proteins, also known as δ -endotoxins, are produced during sporulation. The *Bt* crystalline proteins kill the insect by binding to a receptor on the epithelial brush border membrane of the midgut, causing a disruption of osmotic balance, and leading to cell lysis. More than 50 toxin genes have been cloned from *Bt* (Zhu *et al.*, 1999). *Bt* is currently the most effective strategy for insect resistance in soybean and other crops (Castle *et al.*, 2006). Mazier *et al.* (1997) summarized the expression of δ -endotoxin genes from *Bt* in plants including soybean. Parrott *et al.* (1994) first reported successful transformation of a native *cryIAb* gene in soybean plants. This gene was isolated from *Bt* var. *Kurstaki* HD-1 and is specific for soybean and cabbage loopers. The promoter used for expression of *cryIAb* is the CaMV 35S promoter and the polyadenylation and leader sequences of alfalfa mosaic virus Orf25 were used as terminator (Table 3) and 5'UTR enhancer, respectively. This construct was transformed into soybean by particle bombardment of embryogenic cultures, and the transformants were selected on hygromycin. Mature somatic embryos were

recovered from hygromycin-resistant cell lines, and were converted into plants. Transgenic plants were further confirmed by Southern blot and hygromycin resistance analysis. A T_0 leaf-feeding assay indicated that overexpression of the *cryIAb* gene in the soybean lines conferred resistance to velvetbean caterpillar feeding, development, and survival at a level comparable to the traditional insect resistant line GatIR81-296. The resistance was correlated to the expression level of the *cryIAb* gene in the transgenic soybeans.

Stewart *et al.* (1996) transformed a synthetic *Bt* gene, designated *cryIAc*, into soybean cv. Jack for engineering insect resistance. The truncated *cryIAc* gene was originally isolated from *Bt* var. *Kurstaki* and was synthesized based on the methods described by Adang *et al.* (1993). The modified *cryIA* gene has the codon usage pattern of an average dicot gene and does not contain AT-rich nucleotide sequences typical of native *Bt* *cry* genes. The CaMV 35S promoter and Orf25 terminator (Table 3) (Murray *et al.*, 1991) were used to express the *cryIAc* gene. This construct was transformed into somatic embryos of Jack using particle bombardment. Approximately 10 g of tissue were bombarded, and three transgenic lines were identified by hygromycin selection. Fertile transgenic soybean plants were regenerated from the transgenic tissues. One of the lines was confirmed to accumulate *cryIAc* protein up to 46 ng mg⁻¹ soluble protein. In leaf-feeding bioassays, the transgenic plant leaves showed resistance to damage by corn earworm, soybean looper, velvetbean caterpillar, and tobacco budworm. The insect resistance level of these transgenic plants is higher than that of the insect-resistant line GatIR81-296. In field studies, the *Bt*-Jack line showed threefold to fivefold less defoliation by corn earworm and eightfold to ninefold less defoliation from velvetbean caterpillar compared to the nontransformed Jack (Walker *et al.*, 2000). The *Bt*-Jack plants also showed relatively lower resistance to soybean looper and exhibited fourfold greater resistance than Jack to natural infestations of lesser cornstalk borer. The truncated *cryIAc* gene is very similar to the *Bt* gene used for transforming commercialized *Bt* cotton and corn plants (All *et al.*, 1999b). These results indicate that expression of this *cryIAc* gene in soybean can provide a significant level of resistance to several soybean pests (Walker

et al., 2000). Some soybean varieties are known to have native insect resistance (All *et al.*, 1999a). Walker *et al.* (2002) first reported a multiple resistance gene pyramiding strategy using MAS. The *cry1Ac* gene in Jack was crossed with native resistance lines, combining both modes of resistance into a single soybean line. Field trials were conducted to evaluate resistance to corn earworm and soybean looper, and detached leaf bioassays were used to test for resistance to *Bt*-resistant and *Bt*-susceptible strains of tobacco budworm. Transgenic lines with significantly more resistance to the lepidopteran pests were identified. Stacking the *Bt* gene with native resistance genes may enhance the resistance further and provide multiple modes of insect resistance.

Macrae *et al.* (2005) evaluated transgenic soybean lines expressing a synthetic *cry1A* gene (*tic107*) in screenhouse and conventional field trials. The *cry1A* gene was isolated from *Bt*. A synthetic *cry1A* construct similar to *cry1Ac* (Fischhoff and Perlak, 1995) was constructed and transformed into soybean line A5547 by *Agrobacterium* (Hinchey *et al.*, 1988). The *cry1A* gene was expressed under *Arabidopsis* Rubisco small subunit promoter (Table 3). The precursor protein was directed to the chloroplasts under the guidance of the N-terminal stromal-import sequence of *ArabSSU1A*. Three marker-free lines were identified by Southern blot analysis. Progeny from the three transgenic soybean lines were evaluated in greenhouse trials against velvetbean caterpillar and soybean looper in the United States and against velvetbean caterpillar, *Epinotia aporema* (Walsingham), *Rachiplusia nu* (Guenée), and *Spilosoma virginica* (F.) in Argentina. All transgenic soybeans containing the *cry1A* gene exhibited almost complete control against each of the lepidopteran pests. Transgenic plants from two of the lines demonstrated season-long high dose against *A. gemmatilis* and *P. includens* in both field and greenhouse assays. These lines have a single copy of the *cry1A* gene and no marker gene. The results clearly demonstrate that *cry1A* can confer highly efficacious control of several lepidopterans in soybeans. The *Bt* soybean line is under further characterization including field performance, yield performance, and efficacy confirmation. A regulatory approval application has been filed for commercial release.

The bean leaf beetle, *Cerotoma trifurcata* (Forster), is one of the most important soybean pests in the United States. This insect was once an infrequent soybean pest in the Midwest; however in the 1970s, soybean growers began reporting increased incidence of bean leaf beetle damage (Boethel, 2004). Since 2000, this pest has become a more important problem partly because it also acts as a vector for BPMV. The insect feeds on infected plants and transmits the virus particles to the next plant on which it feeds. Another important soybean insect is soybean aphid (*Aphis glycines*) (Marking, 2001). Soybean aphid is a leaf pest, which damages soybean plants by reducing photosynthesis and yield. The aphid transmits soybean mosaic virus (Boethel, 2004). Soybean plants can be infected simultaneously by the insect pests and the viruses, which causes even more plant damage. Currently insecticides are the main management for these pests (Boethel, 2004).

In addition to *Bt* genes, scientists have evaluated PIs for insect control in soybean and other crops (Ryan, 1990; Schultze *et al.*, 1998; Su *et al.*, 1999). Protein TcdA is an example of a non-*Bt* insecticidal protein (Liu *et al.*, 2003). This protein was isolated from *Photobacterium luminescens*, a soil nematode parasite. This gene was synthetically codon optimized according to dicot-preferred codon usage and expressed under constitutive CsVMV promoter (Verdaguer *et al.*, 1996) and Orf25 terminator (Baker *et al.*, 1983) (Table 3). The construct was transformed into *Arabidopsis* by *Agrobacterium*-mediated transformation and transgenic plants were identified by kanamycin selection. The *TcdA-Arabidopsis* plants showed significant resistance to a number of feeding insects including southern corn rootworm (*Diabrotica undecimpunctata howardi*). The insect resistance was maintained for at least five generations in the transgenic *Arabidopsis* plants. The *TcdA* gene could be a candidate for insect control in soybean. Other strategies against insects have been reviewed by Ferry *et al.* (2004).

2.2.5 Herbicide resistance

Herbicides are the primary means of weed control in soybean fields. Historically, weed control in soybean has relied on herbicides applied directly to the soil before weed emergence. Foliar-applied

herbicides have gained popularity more recently, which allow farmers to assess the nature of the weed infestation before treatment (Buhler and Hartzler, 2004). The first cases of resistance selection in soybean involved acetolactate synthase (ALS) inhibitor herbicides (Horak and Peterson, 1995; Sprague *et al.*, 1997), however, ALS-resistant weeds caused a major problem for soybean producers in the 1990s (Heap and Lebaron, 2001). Because of these problems, the introduction of transgenic glyphosate-resistant soybeans in 1996 resulted in a rapid shift toward glyphosate usage among soybean growers (Owen, 2000). Integration of glyphosate resistance is one of the best examples of a modern world agricultural technology altering the path of a world commodity crop. Unlike previous traits in soybean, the rapid commercial adoption of glyphosate-tolerant soybeans provided enhanced productivity and tangible benefits to the grower that fueled the trait adoption (James, 2001). Today herbicide tolerance is the most widely planted transgenic trait. In fact, over 85% of US soybeans and 56% of soybeans globally are now herbicide resistant (Castle *et al.*, 2006). The rapid rate of adoption in the United States would likely have been mirrored in other soybean-producing countries if the regulatory, intellectual property, and equity criteria for deployment of agricultural technology had been achieved (Conner *et al.*, 2004).

Glyphosate, under the brand name Roundup (registered trademark of Monsanto Company, St. Louis, MO), is the most widely used postemergent herbicide. This herbicide targets a broad spectrum of plant species and is nonselective. Glyphosate binds specifically to EPSPS, which is localized in the plastids (Steinrucken and Amrhein, 1980). Blocking this enzyme interrupts the shikimic pathway and prevents aromatic amino acid production necessary for protein synthesis and some secondary metabolites (Padgett *et al.*, 1996), thus killing the plant within days. Because the EPSPS gene is found in all plants, bacteria, and fungi but not in animals, glyphosate is harmless to animals. In addition, the chemical has favorable environmental qualities including strong adsorption to soil. Due to its nonselective nature, glyphosate cannot be used to prevent weeds in nontransgenic crops because it causes severe plant injury (Liu, 1999).

Glyphosate-tolerant crops have been pursued since the early 1980s (Duke, 1996). Two strategies have been employed to develop glyphosate tolerance in crop plants: identification of glyphosate-tolerant EPSPS and metabolic detoxification of the glyphosate molecule.

Glyphosate-tolerant EPSPS genes have been identified (Stalker *et al.*, 1985; Klee *et al.*, 1987; Barry *et al.*, 1992) for use in conferring transgenic glyphosate tolerance. The Roundup Ready trait was developed using this technology by Monsanto Company. The *aroA* gene from *Agrobacterium* sp. strain CP4 was isolated and found to have high tolerance to glyphosate when targeted to the chloroplasts in higher plants (Barry *et al.*, 1992). Two CP4 EPSPS constructs were created for transformation. Both consisted of pPV-GMGT04 (Padgett *et al.*, 1995), but one contained the CP4 EPSPS gene driven by the CaMV 35S promoter, and the other contained the CP4 gene controlled by the FMV 35S promoter (Table 3) (Gowda *et al.*, 1989). The gene was also fused to the petunia (*Petunia hybrida* Vilm.) EPSPS transit peptide in both constructs. The *GUS* gene was used as a selection marker (Jefferson *et al.*, 1986) and kanamycin resistance was included for bacterial selection on each plasmid. The plasmids were introduced into Asgrow cv. A5403 by particle bombardment as previously described by McCabe *et al.* (1988). Transformants with a positive germline were predicted by GUS expression patterns (Christou and McCabe, 1992). More than 300 lines were selfed and the T₁ seeds were screened for Roundup tolerance in the greenhouse (Padgett *et al.*, 1995). One line, 40-3, showed a high level of tolerance and was advanced by selfing. Four lines were generated and of these, line 40-3-2 demonstrated consistent glyphosate tolerance in the field (Padgett *et al.*, 1995). Line 40-3-2 was then used as the parental source of the CP4 EPSPS gene in the pedigrees of all subsequent Roundup Ready (RR) germplasm. Characterization of line 40-3-2 indicated that the transgenic locus is inherited as a single dominant gene and contains one functional insert of the CaMV 35S:CP4 cassette, an additional 254 bp fragment of the CP4 gene adjacent to its 3' untranslated region (Windels *et al.*, 2001), and a 72 bp segment of the CP4 gene that co-segregates with the functional CP4 cassette. The *GUS* gene and the FMV:EPSPS cassette were segregated out

in earlier generations and are not present in the final product.

To ensure that the CP4 protein is expressed correctly *in planta*, expression of the transgene was compared in plants and *E. coli*. Molecular weight, immunoreactivity, N-terminal sequence, glycosylation, and functional activity were compared from the two sources. In accordance with standard regulatory requirements, nutritional and compositional characteristics of the RR soybean were compared to those of the conventional soybean (Harrison *et al.*, 1996). Results showed that there were no significant differences in total protein, total oil, amino acid, and fatty acid concentrations.

GM RR soybean was subjected to rigorous safety assessment prior to regulatory approval and release. Food and Drug Administration (FDA) regulations mandate testing for allergenicity. The CP4 protein was subjected to *in vitro* digestibility studies in order to assess its ability to withstand degradation in the intestinal tract (Harrison *et al.*, 1996). One characteristic of potential allergens is their ability to resist degradation in the gastrointestinal tract (Astwood *et al.*, 1996). During analysis, the CP4 protein degraded within 15 s in simulated stomach fluid and within 10 min in simulated intestinal fluid. Furthermore, the CP4 protein is only present in the whole soybean seed at approximately 0.04% (Padgett *et al.*, 1995) and levels are significantly reduced during processing, whereas all known food allergens are present in the ingested product at a rate of 1–80% (Taylor, 1992). These data indicate that RR soybeans are as safe as conventional soybeans. With application of glyphosate to soybean fields, chemical residue on the plants is unavoidable. The EPA (2000) has identified acceptable glyphosate residue levels for seed: 20 mg kg⁻¹, hulls: 100 mg kg⁻¹, forage: 100 mg kg⁻¹, and hay: 200 mg kg⁻¹.

Initial studies reported that the RR transgene in soybeans had no negative impact on agronomic performance (Delannay *et al.*, 1995). However, several reports have been released claiming the contrary, including overdeposition of lignin leading to stem splitting (Gertz *et al.*, 1999) and yield loss (Benbrook, 1999; Carpenter, 2001; Elmore *et al.*, 2001b). True nearly isogenic lines with and without the RR trait are not available, making it impossible to distinguish if the yield loss effects are due to linkage drag or effects

caused by the transgene. Elmore *et al.* (2001a) conducted a field experiment studying the response of several RR soybean cultivars to glyphosate, ammonium sulfate, and water. They found that the glyphosate did not affect the grain yield. A study by Lappe *et al.* (1998) reported that the level of phytoestrogens is lower in RR soybeans, but the American Soybean Association (2002) countered that claim, stating that considering the environmental impacts on isoflavone levels, the phytoestrogen levels reported (Lappe *et al.*, 1998) were within the normal variation known to exist in soybean.

Glyphosate can be detoxified by various microbes (Barry *et al.*, 1992), which often occur in soils (Nomura and Hilton, 1977; Rueppel *et al.*, 1977). Transforming soybean with genes that detoxify glyphosate adds another mode of action against herbicide. Barry *et al.* (1992) isolated a glyphosate oxidoreductase gene from *Achromobacter* sp. Strain LBAA. They found that glyphosate tolerance could be induced in plants by expression of an optimized version of the gene. The glyphosate detoxification strategy has also been explored by Castle *et al.* (2004) and Siehl *et al.* (2005). Glyphosate acetyltransferase (GAT) enzyme was isolated in *Bacillus licheniformis* (Weigmann) Chester, a common saprophytic bacterium that has weak GAT activity. The enzyme detoxifies glyphosate by N-acetylation. Acetyl coenzyme A acts as an acetyl donor for the reaction and hydroxyl groups and primary amines are typically the acceptors. Kinetic properties of the enzyme were insufficient to confer glyphosate tolerance in transgenic plants. Through directed evolution, they were able to improve its activity 7000-fold and apply it toward transgenic glyphosate resistance in soybean. *B. licheniformis* genomic DNA fragments were expressed in *E. coli* and screened for N-acetylation activity. All active fragments contained a common region including an open reading frame of 438 bp coding for a 17 kDa protein. From this sequence, primers were constructed and used to identify three major alleles of GAT genes (93% identical) from several closely related strains (Castle *et al.*, 2006; Siehl *et al.*, 2005). DNA shuffling, a method for molecular recombination between genes (Stemmer, 1994; Ness *et al.*, 2002) has successfully been used to alter protein properties such as kinetics, substrate specificity, pH optimum, and thermal stability

and solubility (Cramer *et al.*, 1996; Zhang *et al.*, 1997). Castle *et al.* (2004) and Siehl *et al.* (2005) used this technology to improve the activity of the isolated *GAT* genes. The parental *GAT* genes were subjected to fragmentation-based, multigene shuffling. The development of synthetic shuffling in which synthetic oligonucleotides are used to add diversity to specific positions in a backbone template (Ness *et al.*, 2002) greatly improved the variants. Variants were tested by introduction into tobacco, *Arabidopsis*, and maize via *Agrobacterium*-mediated transformation. For the transgenic maize, maize ubiquitin promoter and potato PINII terminator (Table 3) were used to express the optimized *GAT* gene. Spray tests on T₀ maize plants transformed with *GAT* genes from the 5th to the 11th iteration of shuffling were also performed. A range of tolerance that correlated with the kinetic properties of the enzyme was observed. Most of the 11th iteration variants were asymptomatic at application rates of 4.5 lb AE A⁻¹ (5.45 kg AE ha⁻¹). Field trials are already underway to evaluate commercial potential of this type of glyphosate tolerance trait (Castle *et al.*, 2004; Siehl *et al.*, 2005). Currently Pioneer Hi-Bred is developing this trait in maize, soybean, and other crops, and they plan to offer *GAT* glyphosate resistance in combination with resistance to sulfonylurea herbicides (Castle *et al.*, 2006). This trait will provide an alternative form of glyphosate tolerance with several benefits including removal of herbicidal residue and possible spraying during reproductive development.

Glufosinate is a broad-spectrum grass and broadleaf contact herbicide. Usually sold under the name Liberty (Aventis Company) or Basta (Hoechst AG), it contains the active ingredient phosphinothricin (PPT), a compound similar in structure to the amino acid glutamine. Because of this similarity, PPT blocks the plant enzyme glutamine synthetase (GS) (Bayer *et al.*, 1972; Lea *et al.*, 1984), which is needed for nitrogen fixation and ammonia detoxification (Droge *et al.*, 1992). The plant death associated with glufosinate apparently results from the disruption of nitrogen assimilation metabolism (Wild and Wendler, 1990) and its indirect effect on photosynthesis (Wendler *et al.*, 1990, 1992; Wild and Wendler, 1990, 1993). The gene encoding L-phosphinothricin-N-acetyl transferase (*PAT*) was characterized and isolated from *Streptomyces viridochromogenes* Tu494

(Strauch *et al.*, 1988; Wohlleben *et al.*, 1988). The *BAR* gene encodes the same enzyme as *PAT* and was isolated from *Streptomyces hygroscopicus*. Both *PAT* and *BAR* convert PPT or bialaphos-based herbicides to the nonphytotoxic metabolite L-N-acetyl-PPT (Droge-Laser *et al.*, 1994) and prevent it from blocking the GS enzyme. Microbes readily metabolize PPT, making the half life in soil approximately 7 days. As a result, glufosinate has a very good environmental safety profile (Ahrens, 1994). Bayer CropScience developed commercial transgenic crop products using the trade name LibertyLink[®] (Castle *et al.*, 2006). Based on the assessment report from Food Standard Australia New Zealand (http://www.foodstandards.gov.au/_srcfiles/A481_GM_soy_FAR.pdf), Liberty-tolerant soybeans were developed by expression of the *PAT* gene under CaMV 35S promoter and CaMV 35S terminator (Table 3). The gene was synthesized with reduced GC content in order to optimize the plant expression and it encodes an amino acid sequence identical to the native *PAT* gene. The *PAT* transgenic lines, A2704 and A5547-127, were generated by particle bombardment and transgenic plants were selected by glufosinate. The two transgenic soybean lines demonstrated commercial levels of glufosinate resistance, passed all of the regulatory criteria, and were approved for commercialization in Canada in 1996 and United States in 1998. However, there is no commercial cultivation of the transgenic soybeans in these countries because of the wide acceptance of RR soybean. In addition, the European Union (EU) has not approved the LibertyLink soybean product yet.

2.2.6 Tolerance to abiotic stress

Generating transgenic crops with tolerance to abiotic stress is a big challenge for plant biotechnologists. The stresses include drought, cold, salinity, heat, and heavy metals. For soybean, drought is the most important stress inhibiting production. Progress is being made in soybean for engineering tolerance to abiotic stresses.

Luo *et al.* (2005) reported that a putative soybean plasma membrane cation/proton antiporter (CAX1) confers salt tolerance in *Arabidopsis*. The CAX (cation/proton exchanger)

family is potentially an important member for ion regulation in plant cells. *CAX1* was identified from a cDNA library containing sequences related to drought stress. The CaMV 35S promoter and Nos terminator were used to overexpress the *CAX1* gene in binary vector pBIN438 (Table 3). This construct was transformed into *Arabidopsis* by vacuum infiltration. Transgenic plants were identified by kanamycin selection on medium. T₃ *Cax1-Arabidopsis* plants accumulated less Na⁺, K⁺, and Li⁺, and were more tolerant to Na⁺ and Li⁺ stress compared to the control. Although the role that *CAX1* played in the drought stress response may be subtle, further studies should reveal its roles in overall plant stress response. This *CAX1* gene may be a good candidate for soybean salt tolerance during the germination stage.

Proline plays an important protective role on crops during drought stress since its accumulation can cause a decrease in osmotic potential (Good and Zaplachinski, 1994) and detoxify reactive oxygen species that are generated during drought stress (Guerrier *et al.*, 2000; Hong *et al.*, 2000). Simon-Sarkadi *et al.* (2005) reported that genetic manipulation of proline accumulation may confer tolerance to drought and heat in soybean. The gene encoding L-Δ¹-pyrroline-5-carboxylate reductase (*P5CR*), a key enzyme of the proline biosynthesis pathway, was isolated from *Arabidopsis*. The *P5CR* gene was overexpressed or antisense expressed under a heat shock-inducible promoter HS in an expression cassette (Table 3) (Ainley *et al.*, 1990). The construct was transformed into soybean by *Agrobacterium*-mediated transformation (De Ronde *et al.*, 2000). Transformed cells and plants were selected on kanamycin medium. Simon-Sarkadi *et al.* (2005) observed that antisense expression of the *P5CR* gene in T₃ plants reduced the level of proline and increased water loss, whereas overexpression of *P5CR* significantly increased the proline concentration in the plants and resulted in less water loss. The plants overexpressing the *P5CR* gene exhibited a higher photosynthesis capacity compared to the control and *P5CR*-silenced plants (De Ronde *et al.*, 2004). These results indicate that manipulation of proline level can confer tolerance to water stress in soybean plants (De Ronde *et al.*, 2000, 2001b, 2004; Simon-Sarkadi *et al.*, 2005). Engineering drought tolerance in model plants has made excellent progress (Umezawa *et al.*, 2006). The technology and

materials developed in other plants can be used to improve soybean's tolerance to drought stress.

Iron deficiency is a significant problem for soybean production in the upper Midwest region of the United States due to iron-limiting calcareous soils. In response to iron-limiting environments, soybean plants induce an active proton pump, a ferric iron reductase, and an iron transporter. Vasconcelos *et al.* (2006) reported that heterologous expression of the *A. thaliana* ferric chelate reductase gene, *FRO2*, in transgenic soybean significantly enhances Fe³⁺ reduction in roots and leaves. The *FRO2* gene was expressed under the CaMV 35S promoter in pPZP binary vector with the *BAR* gene as the selection marker (Table 3). This vector was transformed into soybean tissue by *A. tumefaciens* strain EHA101 (Zhang *et al.*, 1999; Xing *et al.*, 2000). Soybean transformation was conducted with genotypes Thorne (Ohio State University) and A3237 (Asgrow Seed Company). Transformants were selected on glufosinate-containing medium. An assay comparing the transgenic plants in Fe-treatments and control plants grown under hydroponics that mimic Fe-sufficient and Fe-deficient soil environments was performed. In the transgenic soybean plants, the ferric reductase activity significantly increased (threefold to 10-fold), leading to reduced chlorosis, increased chlorophyll concentration, and less biomass loss in the transgenic plants. However, the data indicate that constitutive expression of *FRO2* under noniron stress conditions may cause decrease in plant productivity. This research suggests that expression of an iron chelate reductase in soybean is a potential approach to control iron deficiency chlorosis.

With the advancement of functional genomics research in *Arabidopsis*, soybean, and other crops, a number of stress-regulation genes have been discovered and are being evaluated in plants (Vinocur and Altman, 2005; Umezawa *et al.*, 2006). Transgenic soybean plants with enhanced tolerance to abiotic stresses will be available in the future for soybean farmers.

2.3 Quality Output Traits

Soybean is a staple food worldwide mainly due to its high protein and oil contents. Soybean

seeds contain 20% oil, 40% protein, 22–23% carbohydrate, 3–6% fiber, and 3–6% minerals based on dry weight (Wang, 1997). The efforts of improving soybean seed quality have been focused on modifications of oil, protein, amino acid, flavor, and other compositions. We will discuss the transgenic improvement of these seed compositions toward increasing food and feed value of soybean products.

2.3.1 Oil modification

The soybean oil biosynthesis pathway is well understood (Ohlrogge and Browse, 1995; Yadav, 1996; Voelker and Kinney, 2001). Soybean oil, similar to other vegetable oils, contains five common fatty acids: palmitic acid (16:0, ~11%), stearic acid (18:0, ~4%), oleic acid (18:1, ~22%), linoleic acid (18:2, ~53%), and linolenic acid (18:3, ~8%) (Parrott and Clemente, 2004). Various desaturases have important roles in the conversion and balance of the five different fatty acids, which have distinct oxidative stabilities, physical properties, and health benefits. In addition to these five common fatty acids, there are also more than 200 chemically diverse uncommon fatty acids found in different rare plant species that are controlled by specific desaturases (Van de loo *et al.*, 1993; Yadav, 1996). Soybean scientists have been focusing on three objectives in the metabolic engineering of oil: producing more of the desirable fatty acids; producing less of the undesirable fatty acids; and producing natural but novel fatty acids in soybean seeds. The approaches taken to achieve these objectives include overexpression or co-suppression of soybean desaturase genes or desaturase genes from other plant species. As illustrated below, great progress has been made and unique soybean oils have been developed for various uses. There is great potential for improving soybean oil for food applications and industrial uses through soybean biotechnology (Mazur *et al.*, 1999; Drexler *et al.*, 2003; Kinney, 2006), we will focus on the food and feed application in this section.

Monounsaturated oleic acid (18:1) is preferred by consumers due to its health benefits and stability compared to the polyunsaturated linoleic (18:2) and linolenic (18:3) acids. Linolenic acid has low oxidative stability, which causes reduction in the performance, rancidity, and poor soybean oil

flavor over time. A key goal of improving soybean oil quality has been to increase its oxidative stability by reducing its linolenic acid (18:3) content. Hydrogenation can significantly improve oil stability and flavor; however, the process is expensive, and produces *trans*-fatty acids, which have human health risks. Oil containing low levels of saturates and polyunsaturates and high levels of monounsaturated fats would provide significant human health benefits and improved oil stability, as well as economic benefit to oil processors. Both mutation breeding and transgenic breeding have been used to identify soybean lines producing high oleic soybean oil (Takagi and Rahman, 1996; Mazur *et al.*, 1999). Transgenic soybean has been found to produce oleic acid in the oil as high as 85%, which is much higher than products developed through other methods.

A transgenic high oleic acid soybean line developed by DuPont Company was first commercialized in 1998 and the detailed information on the product was described by Kinney and Knowlton (1998) and Australia New Zealand Food Authority (ANZFA, 1991). Two constructs, pBS43 and pML102, were transformed into the shoot apex of the Asgrow cv. A2396 by particle bombardment. The plasmid pBS43 contained two transgene units: seed-specific promoter β -conglycinin:*FAD2-1*:phaseolin terminator (Table 3) and CaMV 35S promoter:*GUS*:Nos terminator. This construct was designed to introduce post-transcriptional gene silencing, also known as co-suppression or RNAi (Cerutti, 2003) of the *FAD2-1* gene (Matzke and Matzke, 1995; Singh *et al.*, 2000). The second construct, pML102, contained the *Corynebacterium* *dapA* gene encoding a lysine-insensitive version of dihydrodipicolinic acid synthase (DHDDS) flanked by the CaMV 35S promoter and the terminator of Kunitz trypsin inhibitor gene 3 (Table 3). This construct was designed to enhance the lysine level in the soybean seeds. High oleic progeny were developed from a single selfed transgenic line (260-05) derived from the co-transformation of pBS43 and pML102. The high oleic acid plants showed that the original transgenic line contained two loci, one locus possessed an element from pBS43 only, which is actually the silencing allele, and the other locus contains inserts from both plasmids. Further molecular studies revealed that this silencing allele contains two copies of pBS43 in an inverted repeat

orientation (Kinney and Knowlton, 1998; Parrott and Clemente, 2004).

Regulatory analysis of the high oleic soybean products included a comprehensive Northern blot and protein profiling. Protein profiling indicates that no new proteins are expressed in the transgenic soybean compared to the parental line; however, minor changes occurred in β -conglycinin due to co-suppression of the β -conglycinin promoter sequence (Kinney *et al.*, 2001a, b). To determine if the alterations in protein profile affect the allergenicity compared to the parental line, radioallergosorbent reactivity was measured using control sera and sera from 31 individuals who are known to have allergies to soybean extracts or other foods. No alteration in allergenicity was observed compared to the parental line. The analyses of amino acid content, total oil content, and oil compositions demonstrated that the high oleic acid soybean seeds are equivalent to the conventional soybean seeds except for the enhanced oleic acid level and alteration in protein profile. Data from feeding trials indicate that the meal from transgenic soybean is equivalent to that of the conventional soybean lines in term of its ability to support the growth and development of chickens and pigs.

Conversion of oleic acid to linoleic acid is controlled by a δ -12 desaturase encoded by the *FAD2-1* gene (Heppard *et al.*, 1996). Down-regulation of this gene causes an accumulation of oleic acid in transgenic seed oil up to 85% (Kinney, 1996; Mazur *et al.*, 1999; Buhr *et al.*, 2002). Palmitic acid is released from palmitoyl-acyl carrier protein (ACP) by palmitoyl-ACP thioesterase (*FAD3*). Buhr *et al.* (2002) silenced the embryo-specific *FAD2-1* and *FAD3* genes using ribozyme-terminated antisense constructs in soybean plants. Both genes were isolated from soybean by PCR (Heppard *et al.*, 1996). Seed-specific phaseolin promoter and CaMV 35S terminator were used to express *FAD2-1* in a binary vector (Table 3). Seed-specific β -conglycinin promoter and CaMV 35S terminator were used in the binary vector containing the fusion of *FAD2-1* and *FAD3* (Table 3). The CaMV 35S promoter-driven *BAR* gene cassette was used as a selection marker. These constructs were transformed into soybean (A3237) by *A. tumefaciens*-mediated transformation as previously described (Hood *et al.*, 1986; Hinchee *et al.*, 1988; Zhang *et al.*, 1999; Clemente *et al.*,

2000). Positive transformants were identified and selected by glufosinate during shoot initiation and elongation. Two of eight transgenic lines showed over 75% oleic acid content. Transformation with the dual constructs, simultaneously knocking off *FAD2-1* and *FAD3* genes, generated five events. These five events had oleic acid levels greater than 85% and saturated fatty acid levels less than 6%. The increase of oleic acid and decrease in saturated fatty acid was correlated to the reduction of the *FAD2-1* and *FAD3* mRNAs. These results clearly demonstrated that silencing desaturase and thioesterase genes can modify the oil compositions in soybean seeds.

Linolenic acid is an ω -3 fatty acid that is essential in mammalian diets. Romieu *et al.* (2005) reported that ω -3 fatty acids prevent heart rate variability reductions associated with particulate matter; therefore there is increasing interest in high linolenic oil from the nutritional standpoint. High linolenic oil has desirable properties for use as drying oils in coating applications such as paints, inks, and varnishes (see detail in the Section 2.4). Although high linolenic oil has low oxidative stability, the heart-healthy ω -3 fatty acid can be consumed in the form of soybean seeds, sprouts, or as tofu. High linolenic soybean lines have been generated through mutation breeding methods (Burton *et al.*, 2004). Seed-specific overexpression of the *FAD3* gene under β -conglycinin promoter and phaseolin terminator (Table 3), which encodes the enzyme that converts linoleic to linolenic acid, significantly increased linolenic acid content from 10% in the control to 50% in transgenic soybean lines (Cahoon, 2003). An increase in stearic acid (18:0) content in soybean oil is desirable for certain food-processing applications since it offers the potential for the production of solid fat products without hydrogenation (Spencer *et al.*, 2003). This would help to reduce the current health concern surrounding foods containing *trans*-fatty acids. Stearic acid concentration in soybean is determined by alterations in the δ -9 stearoyl-ACP desaturase (*SACPD*) gene, which encodes a soluble enzyme that converts stearic acid to oleic acid (Rahman *et al.*, 1997). Six soybean germplasm lines carrying the mutant *SACPD* alleles contain increased levels of stearic acid. Using *SACPD* genes to manipulate stearic acid content in oil has been demonstrated by Bidney *et al.* (2002). Antisense expression of a *SACPD* gene under

the Napin promoter and Nos terminator in sunflower significantly increased saturated fatty acids. The transgenic sunflower lines increased seed stearic acid content fourfold, producing over 40% saturated fatty acids. Similar antisense expression technology for increasing saturated fatty acid has also been used in other crops (Knutzon *et al.*, 1992; Singh *et al.*, 2000). Byfield *et al.* (2006) identified two *SACPD* genes in soybean that may provide the means to achieve stable production of high stearic acid soy oil. This oil has great potential for industrial use in products such as coating fat, soap, and margarine (Bidney *et al.*, 2002).

Medical studies have shown that diets high in saturated acyl compounds such as palmitic and stearic acids may increase blood serum cholesterol levels and the risk of coronary heart disease. New FDA labeling regulations require that a vegetable oil characterized as “low saturated” must contain less than 70 g kg⁻¹ total saturates. Conventional soybean oil contains 120–140 g kg⁻¹, therefore a reduction is required for the new market. The key enzyme in the biosynthesis of palmitic acid is 16:0-ACP thioesterase, which is encoded by the *FATB* gene. *FATB* gene mutants were identified by conventional breeding and a low palmitic acid soybean line was developed through mutation breeding (Burton *et al.*, 2004). As discussed previously, *FatB* gene silencing can significantly reduce palmitic acid levels in transgenic soybean (Buhr *et al.*, 2002). Blocking the exit of palmitoyl-ACP and stearyl-ACP from the plastid by silencing acyl-ACP thioesterase (*GmFatB1* and *GmFatB2*) genes resulted in a reduction in total saturated fatty acids from 14% to 3% (Kinney, 1996). Overexpression of a castor seed δ -9 stearyl-ACP desaturase in sunflower under a seed-specific promoter significantly reduced the stearic acid level in sunflower oil (Rousselin *et al.*, 2002). From these data, one can deduce that soybean *SACPD*-like genes or desaturase genes from other crops will be useful in manipulating stearic acid levels in soybean oil.

Many bioactive fatty acids have a significant impact on human health, but are not normally present in soybean oil. These unusual polyunsaturated fatty acids provide heart health benefits independent from soybean proteins (Martin and Valeille, 2002; Kelley and Erikson, 2003; Knapp *et al.*, 2003). It is possible to engineer soybean

with these useful polyunsaturated fatty acids to further improve the health benefits of soybean. For example, γ -linolenic acid and arachidonic acid are important for human health and nutrition (Napier and Michaelson, 2001). Genes encoding the desaturases that are responsible for the production of these fatty acids are available (Michaelson *et al.*, 1998; Hong *et al.*, 2002). Liu *et al.* (2001) expressed an 18:1 δ -12 desaturase either alone or in combination with 18:2 δ -12 desaturase in canola. The 18:1 δ -12 desaturase alone can increase linoleic acid by 46%, and the two enzymes together dramatically enhance the level of γ -linolenic acid up to 43% in the transgenic canola seeds. These genes could enable engineered soybean to produce high levels of γ -linolenic acid in soybean seed, improving its nutritional value.

Stearidonic acid (STA) has been considered as a valuable alternative source for ω -3 fatty acids since it can be more efficiently converted to eicosapentaenoic acid in animals compared to α -linolenic acid. Sato *et al.* (2004) expressed a borage δ -6 desaturase gene in soybean under seed-specific β -conglycinin promoter and CaMV 35S terminator (Table 3). The tobacco etch virus translation enhancer element was used to enhance the expression of the desaturase enzyme. The Nos promoter driving *BAR* gene cassette was used as a selection marker. The construct was transformed into somatic embryos by particle bombardment. Four δ -6 desaturase events were free of the *BAR* gene and one of the events, designated 420-5, was further evaluated in field studies (Sato *et al.*, 2004). Progeny from this event consistently produced γ -linolenic acid (up to 30%) and STA (up to 4%) under various environmental conditions. In an attempt to maximize the STA content of the soybean oil, Eckert *et al.* (2006) stacked a borage δ -6 desaturase and an *Arabidopsis* δ -15 desaturase through either sexual crossing of transgenic events, re-transformation, or co-transformation of both genes. Both genes were overexpressed under the control of the seed-specific soybean β -conglycinin promoter and CaMV 35S terminator (Table 3). The constructs were transformed into soybean by particle bombardment and the *BAR* gene was used as a selection marker. Overexpression of *Arabidopsis* δ -15 desaturase in soybean significantly enhanced the α -linolenic acid content. Notably co-expression of δ -15 and δ -6 desaturases together dramatically increased the

accumulation of STA, which can be as high as 29% of the total fatty acids in the transgenic soybean seeds. In terms of ω -3 activity, 29% STA is equal to about 60% of γ -linolenic acid. These transgenic soybean events are under further evaluation for product development.

Long-chain polyunsaturated fatty acids with 20 and 22 carbon atoms containing a few *cis*-double bonds (C20- and C22-LCPUFA) are very important for human development and health. However, humans cannot obtain C20- or C22-LCPUFA from vegetables. The major source of ω -3 LCPUFA in the human diet is from fish and fish products. Recently great progress has been reported in transgenic production of LCPUFA in oilseed crops (Domergue *et al.*, 2005). Kinney *et al.* (2004) co-expressed five genes under different seed-specific promoters and terminators in transgenic soybean embryos and seeds (Table 3). These five genes were isolated from *S. diclina* for Sd Δ 6-desaturase and Sd Δ 17-desaturase, *M. alpine* for Ma Δ 6-elongase and Ma Δ 5-desaturase, and *A. thaliana* for Sd Δ 15-desaturase. These genes were transformed into soybean by co-bombardment of two DNA inserts. One of the inserts contained three transgene units: (β -conglycinin promoter:Sd Δ 6-desaturase:phaseolin terminator), (glycinin Gy1 promoter:Ma Δ 6-elongase:pea leguminA2 terminator), and (trypsin inhibitor promoter:Ma Δ 5-desaturase:trypsin inhibitor terminator). The other insert contained two transgene units: (annexin promoter:Sd Δ 17-desaturase:BD30 terminator) and (trypsin promoter:Sd Δ 15-desaturase:trypsin terminator). Transgenic plants were selected by hygromycin B. These five genes established a biosynthesis pathway of eicosapentaenoic acid and resulted in the production of 19.6% eicosapentaenoic acid in the transgenic soybeans (Kinney *et al.*, 2004). The LCPUFA levels in the transgenic soybean seeds were as high as 35%, which is 59% higher than the level in salmon steak (Domergue *et al.*, 2005). Transgenic approaches in *Arabidopsis* and other oilseed plants have achieved very similar results (Abbadì *et al.*, 2004; Qi *et al.*, 2004). These are good examples of engineering a biosynthetic pathway in soybean seeds to produce nutritious oil products in oilseed crops.

New technology for gene expression and gene silencing in crop plants has been developed to manipulate the specific desaturase mRNA/enzyme

levels to desired concentrations. However, the expected effects on fatty acid compositions may not always be achieved. Kinney *et al.* (2001) summarized and discussed the challenges in genetic engineering for improving oil quality. The main factors that impact genetic manipulation efficacy include fatty acid flux, desaturase gene families, and intracellular transport of desaturase products in soybean. Other factors may also affect the ability to modify unsaturated fatty acid content in the oil of transgenic soybean plants. The expression or silencing of desaturase transgenes needs to be closely timed with oil biosynthesis for maximum effectiveness. Once the embryo begins to make oil, fatty acids become incorporated into the triglycerol. Any fatty acids made before the transgene becomes effective will be stored in their unaltered state. Therefore, the expression cassette should include the proper seed-specific promoter in combination with the right desaturase transgene. The recent reports on transgenic production of LCPUFA in oilseed crops clearly indicate that biotechnology skills have been advanced to meet some of these challenges (Kinney, 2006).

2.3.2 Protein modification

More than 70% of the protein consumed by humans is derived from legumes and cereals. Among the most commonly consumed food sources, soybean is the most protein-rich food (about 40% proteins) (Krishnan, 2005). Soybean isoflavones have been linked to reducing the risk of cancer and heart diseases, and soybean protein consumption can diminish chronic ailments such as osteoporosis, atherosclerosis, and renal diseases (Messina, 1999). Soybean is also extensively used as a high protein feed ingredient in livestock and poultry production. The health benefits of soybean consumption has increased consumer demand for new soybean products such as energy bars, breakfast cereals, meat alternatives, and beverages in the United States (Kinney, 2003).

The major soybean storage proteins are globulins including 2S, 7S, and 11S. The names were designated based on the sedimentation coefficients of the proteins in sucrose gradient centrifugation (Thanh and Shibasaki, 1976). The 7S and 11S globulins are also known as conglycinin

and glycinin and account for about 30% and 40% of the total seed protein, respectively (Nielsen, 1996). Genes encoding conglycinins and glycinins have been isolated (Krishnan, 2005). Conglycinin exists as a trimer consisting of α (76 kDa), α (72 kDa), and β (53 kDa) subunits (Thanh and Shibasaki, 1976, 1978; Coates *et al.*, 1985; Tierney *et al.*, 1987). Glycinin is present in hexamers and consists of acidic and basic subunits from a same precursor (Barton *et al.*, 1981; Beachy *et al.*, 1981). Glycinin contains significantly more sulfur amino acids, such as cysteine and methionine, than β -conglycinin (Krishnan, 2005). Approaches to enhancing soybean protein quality include conventional breeding in combination with mutation breeding with the purpose of increasing seed storage protein levels such as glycinin and β -conglycinin in soybean seeds (Krishnan, 2005), expression of sulfur-rich proteins with well-balanced amino acid compositions (see detail in the amino acid modification), and increasing the levels of abundant and low abundant proteins by transgenic engineering.

Both glycinin and conglycinin proteins are encoded by large gene families (Harada *et al.*, 1989; Beilinson *et al.*, 2002). Kinney and Fader (2002) isolated the soybean genes encoding glycinin and conglycinin. To determine whether β -conglycinin in developing soybean cotyledons could be targeted by co-suppression, truncated cDNA fragments of the α - and α -subunits of β -conglycinin were prepared using RT-PCR. The fragments were then cloned into an antisense expression cassette under β -conglycinin promoter and phaseolin terminator (Table 3). Similarly the five glycinin genes were cloned from genomic or cDNA clones and then subcloned into an antisense expression vector under the β -conglycinin promoter and phaseolin terminator. These constructs were transformed into soybean somatic embryos by particle bombardment. Hygromycin B was used as a selection agent. The conglycinin and glycinin proteins were visualized by SDS-PAGE, and transgenic soybean lines that produce no 7S or no 11S protein were identified. In these lines, the reduction of one protein class is compensated by an increase in the other class since the total protein level was maintained in the transgenic seeds. These transgenic lines provide excellent material for studying protein functionality and providing potential new soybean protein products with

unique functional properties. Soybean storage proteins such as 7S can reduce blood low-density lipoprotein (LDL) cholesterol concentration in humans (Sirtori and Lovati, 2001; Weggemans and Trautwein, 2003). In addition, soybean proteins together with isoflavones may reduce bone loss in postmenopausal women (Potter *et al.*, 1998). The transgenic lines with no 7S or 11S protein (Kinney and Fader, 2002) will be useful in studying the mechanism of reducing LDL cholesterol reduction.

Protein engineering technology enables artificially designed proteins with desirable functional properties to be introduced into soybean. It is possible to express novel proteins in soybean seeds and develop novel and desirable soybean protein products. Glycinins are encoded by at least five genes (Nielsen *et al.*, 1989), including a hypervariable protein domain. Scientists have been trying to insert sulfur amino acid residues into this domain (Nielsen *et al.*, 1990). It is possible to engineer extra methionine residues into the variable glycinin domain and the modifications have little impact on its stability (Gidamis *et al.*, 1995; Takaiwa *et al.*, 1995). These modified versions of glycinin genes are good candidates for improving the protein value of soybean seeds.

Some low abundant soybean proteins also have potential to be used for improving the protein value. Examples include directly expressing methionine-rich proteins (Galvez *et al.*, 1997) and overexpressing PIs to induce altered levels of amino acids (Kennedy, 1998). Overexpression of genes encoding Bowman-Birk chymotrypsin and trypsin inhibitors significantly increased the sulfur amino acid level in soybean seeds, and the inhibitor protein may be beneficial to humans since it may have anticarcinogenic properties (Kennedy, 1998). However, PIs have been shown to have a negative impact on the performance of nonruminants such as poultry and swine. Therefore, increasing sulfur amino acids by expressing PIs is not desirable.

Soybean seeds store a large amount of protein during their maturation phase in specific bodies known as protein storage vacuoles (PSV). In order to alter the nutritional quality of seed proteins and to enable production of recombinant proteins in the soybean seeds, it is necessary to localize transgenic proteins to the PSV (Kusnadi *et al.*, 1997; Horvath *et al.*, 2000). Maughan *et al.* (1999) cloned a gene encoding the bovine

milk protein, β -casein, into a seed-specific lectin promoter expression cassette (Table 3), and it was introduced into soybean somatic embryos via particle bombardment. A chimeric lectin β -casein gene was fused to the N-terminal signal peptide (32 amino acids) of soybean seed-specific lectin, and the lectin 3' UTR was used as a terminator. Transgenic plants were selected by hygromycin. Subsequent analyses on the transgenic plants were completed by Philip *et al.* (2001). Southern blot assay indicated that there are 4–8 copies of the β -casein gene in a single locus and Northern blot assay detected an enhanced level of casein mRNA in the developing cotyledons. Bovine β -casein protein was detected by Western blot assay in the cotyledons. The β -casein protein was purified from the immature soybean seeds by immunoaffinity chromatography and analyzed by two-dimensional gel electrophoresis, blotting, and N-terminal sequencing. The results demonstrated that the 32 amino acid signal peptide at the N-terminus of the chimeric β -casein was cleaved precisely from the protein. The recombinant soybean β -casein protein was not phosphorylated, causing a size difference compared to the soybean and native bovine β -casein protein on SDS–PAGE. Immunolocalization experiments demonstrated that the recombinant casein protein was localized in the PSV and accumulated to a high level in developing and mature soybean seeds. The addition of the β -casein milk protein into soybean seeds has the potential to increase soybean value. This is the first experiment showing the expression of a milk protein in soybean, showing that it is possible to improve protein quality in soybean by introducing foreign proteins controlled by seed-specific promoters. These results also suggest that this system could possibly be used for growing pharmaceutical or other valuable recombinant proteins in soybean seed, such as monoclonal antibodies (Zeitlin *et al.*, 1998).

Food allergies can cause nutritional problems in children and adults. Any food that contains protein has the potential to elicit an allergic reaction. Increased awareness of the many health benefits of soy protein, along with improved isolation techniques resulting in better flavor and increased functionality, has resulted in widespread use of soybean proteins in food products such as soymilk. This food development provides benefits to human health; however, the increasing use of

soybean products in processed foods brings a potential threat to soybean-sensitive individuals (Vidal *et al.*, 1997; Herman, 2005). The abundant storage proteins and a few less-abundant seed proteins are potential allergens. One of these low abundance proteins, P34 (also known as Gly m Bd 30K), is a major soybean allergen (Herman, 2005). Herman *et al.* (2003) isolated the *P34* gene from soybean by PCR amplification. Seed-specific β -conglycinin promoter and phaseolin terminator were used to express the *P34* gene (Table 3), and the hygromycin B phosphotransferase gene under control of CaMV 35S promoter was used for selection of transformants. This construct was transformed into soybean embryos by particle bombardment and transgenic tissue was selected on medium containing hygromycin B. Western blot assays using the P34 monoclonal antibody were used to identify homozygous *P34*-transgenic soybean plants, which showed dominant Mendelian inheritance and stability of the trait for three generations. The *P34*-transgenic soybean plants exhibited normal agronomic performance such as seed size, shape, protein content, and oil content as compared to the controls. Analysis of seeds from T₃ homozygous lines revealed that the protein profile is the same as the parental lines except for significantly reduced levels of the P34 protein. These data provide evidence that one of the dominant allergens of soybean seeds can be reduced while maintaining substantial equivalence of composition in transgenic seed. Joseph *et al.* (2006) identified 12 P34 null lines that contain P34 protein at a low level, likely due to a site mutation in the *P34* open reading frame that destabilizes the P34 protein. These mutant genes are candidates for reducing P34 protein in transgenic soybean seeds. In addition, backcrossing the silencing allele in the transgenic line generated by Herman *et al.* (2003) into the P34 null lines may further reduce the level of P34 protein in soybean seeds. Soybean products with low levels of P34 protein can benefit people with soybean allergies.

2.3.3 Flavors

Many people wish to consume more soybean products due to the recent reports on their health benefits. However, the flavor of conventional soybeans has been described by many as painty,

grassy, beany, metallic, and bitter. Although some people like the natural soy flavors, these negative flavors are the limiting factor for the widespread development of soybean food products. Improving soybean flavors can be achieved by conventional breeding and transgenic methods through reducing the bad flavors and/or increasing the good flavors. For example, the beany flavor can be reduced or removed by reducing lipoxygenase activity in transgenic soybean plants (Kitamura, 1995). Physically changing functional properties including solubility, water absorption, viscosity, and flavor binding can also impact soybean flavor (Adler-Nissen, 1978; Kinsella, 1979; Kitamura, 1995).

The driving force behind soybean food products is the health properties of soybean food compositions. Soybean isoflavones have benefits to human health (Fader *et al.*, 2000; Liu *et al.*, 2002). They also have natural roles in plant defense and root nodulation (Subramanian *et al.*, 2004). Soybean isoflavone synthase genes were isolated through a yeast expression assay for screening soybean ESTs encoding cytochrome P450 proteins. Isoflavone synthase genes were also isolated from several other legume plants using the soybean isoflavone synthase gene as a probe (Jung *et al.*, 2000). When expressing soybean isoflavone synthase in the nonlegume plants *A. thaliana* and rice, genistein was able to accumulate (Jung *et al.*, 2000; Sreevidya *et al.*, 2006). This result indicates that beneficial isoflavones can be produced in nonlegume crops that may not have the taste of soybean. In addition, isoflavone synthase may elevate the capacity of defense and nodulation in crops (Subramanian *et al.*, 2004; Sreevidya *et al.*, 2006). Isoflavone synthase genes are useful in the transgenic efforts of enhancing the nutritional value in crops.

Many of the unfavorable flavors associated with the soybean are due to oxidation of polyunsaturated fatty linoleic and linolenic acids (Frankel, 1987). High oleic soybeans have reduced polyunsaturated fatty acid levels (Kinney, 1996; Kinney and Knowlton, 1998). By silencing the *FAD2-1* gene (see detail in the Oil Modification section), Kinney and Knowlton (1998) decreased the linolenic acid content from 70% to 5%, leading to increased oxidative stability of the high oleic soybean oil. In addition, the transgenic soybean seeds produce significantly reduced levels (five fold to 10-fold) of lipid oxidation products, such as

2-pentyl furan and trans 2-hexanal in flours and protein isolates. This leads to reduced grassy and beany flavors in the various products from the high oleic soybean, however, some grassy flavor is still present. Combining the high oleic lines with other transgenic lines that have reduced or removed lipoxygenase (Hildebrand, 1989) and/or hydroperoxide lyases (Noordermeer *et al.*, 2001) can further reduce the grassy flavor (Kinney, 2003).

The nonvolatile compounds that contribute to bitter and astringent flavors of soybean proteins are less understood. These flavors may be involved in the biosynthesis of small peptides, flavonoids, phenols, and saponins (Drewnowski, 2001). Reduced levels of these flavors may be achieved by transgenically reducing the synthesis of these compounds (Kinney, 2003); however, some of these compounds may have health benefits in soybean products (Messina *et al.*, 2002). Transgenic products with less bitter flavor may be achieved with better understanding of biosynthesis pathways of the secondary compounds.

2.3.4 Amino acid

Mammals obtain essential amino acids such as lysine from foods. Lysine is an essential amino acid that is limiting in soybean food and feed. Two of the key enzymes in the lysine biosynthesis pathway, aspartokinase (AK) and DHDPS, have been isolated from bacteria or other sources for increasing lysine levels in seeds. These two enzymes are feedback inhibited by lysine (Mazur *et al.*, 1999). Falco *et al.* (1995) isolated a gene encoding a lysine-insensitive AK mutant, *lysC*, from an *E. coli* genomic library, and a DHDPS *dapA* gene from *Corynebacterium* by PCR amplification. The AK enzyme encoded by a gene from *E. coli* gene has a substitution of isoleucine for threonine at amino acid position 352. This site mutation is responsible for the enzyme's insensitivity to lysine. Plant deregulated enzymes were also studied, but they showed inconsistent results (Falco *et al.*, 1995). The DHDPS and AK enzymes were linked to a chloroplast transit peptide and expressed in transgenic canola and soybean seeds. The seed-specific phaseolin promoter and its terminator were used in the construct expressing the *AK* and *DHDPS* genes (Table 3). These constructs were transformed into soybean by particle bombardment. GUS

visualization and PCR analysis were used to select the positively transformed soybean plants. Expression of DHDPS resulted in more than a 100-fold increase in the accumulation of free lysine in the seeds of canola; total seed lysine content approximately doubled. Expression of both DHDPS and the lysine-insensitive AK in soybean transformants similarly caused a several 100-fold increase in free lysine, and increased total seed lysine content by as much as fivefold. These data indicate that microbial genetic materials are useful in plant engineering.

Zhu and Galili (2003, 2004) expressed the bacterial feedback-insensitive DHDPS in a seed-specific manner in wild-type *Arabidopsis* as well as in an *Arabidopsis* knockout mutant with increased levels of lysine. Transgenic plants expressing the *DHDPS* gene contained 12-fold and fivefold higher levels of seed free lysine, respectively. Their results show that lysine catabolism plays a major regulatory role in balancing lysine levels in *Arabidopsis* seeds. To increase the lysine level in soybean seeds, we should consider increasing the synthesis and decreasing the catabolic rate. In addition, lysine content can be enhanced by the expression of lysine-rich protein for increasing methionine content as illustrated below.

The low sulfur content of conventional soybean seed protein is not optimal for ration formulations in the human diet and animal feed. Therefore, increasing the methionine and cysteine content of soybean seed proteins can increase the nutritional value of the soybean seeds. Methionine-rich 2S albumin from Brazil nut was introduced into transgenic soybean plants. Overexpression of Brazil nut 2S albumin (containing 18% methionine and 8% cysteine) significantly increased the methionine content, potentially increasing nutritional value (Townsend and Thomas, 1994). In the transgenic soybean lines, the albumin protein can accumulate to as much as 10% of the total seed protein and increase the overall methionine content by 15–40% (Townsend and Thomas, 1994). However, the Brazil nut 2S albumin protein is a major allergen (Nordlee *et al.*, 1996) and this transgenic approach was not developed further. Sunflower albumin is also a sulfur-rich protein and has potential in engineering high sulfur soybean products (Wang *et al.*, 2001); however, it also has potential to be an allergen (Kelly *et al.*, 2000).

Dinkins *et al.* (2001) demonstrated that overexpression of maize 15 kDa zein protein could

confer higher levels of methionine and cysteine in soybean seed. The zein gene encoding the 15 kDa zein protein along with a portion of the zein 3'UTR was isolated from maize. The seed-specific β -conglycinin promoter and Nos terminator were used to express the 15 kDa zein protein (Table 3). The construct was transformed into soybean somatic embryo cultures by particle bombardment and hygromycin was used to select the transgenic tissues. Transgenic soybean plants from two transgenic lines were characterized by Southern blot analysis, and multiple copies of zein genes were revealed in the transgenic plants. Amino acid analysis indicated that methionine and cysteine levels in the transgenic lines increased 12–20% compared to the control. Kim and Krishnan (2004) reported the expression of an 11 kDa methionine-rich δ zein in soybean. They isolated the *zein* gene from maize inbred line W23a1, and cloned the gene between β -conglycinin promoter and potato protein inhibitor II (*PINII*) terminator for seed-specific expression in transgenic soybean seeds (Table 3). This construct was transformed into soybean cotyledons by *Agrobacterium*-cotyledon node transformation (Hinchee *et al.*, 1988) utilizing glufosinate as a selection agent (Zhang *et al.*, 1999). Overexpression of the zein protein gene significantly enhanced the methionine content in the alcohol-soluble protein fraction even though overall methionine content of the seed protein remained the same. Recently, Li *et al.* (2005) expressed the maize 27 kDa γ -zein gene (Prat *et al.*, 1985) in transgenic soybean plants under β -conglycinin promoter and β -conglycinin terminator (Doyle *et al.*, 1986) (Table 3). The transgene construct was transformed into somatic embryogenic cultures by particle bombardment and transgenic plants were selected on medium containing hygromycin B. Transgenic T₁ and T₂ seeds consistently showed enhanced expression of the γ -zein protein. Up to 6.49% of the alcohol-extractable proteins from the transgenic seeds were the γ -zein protein. In the transgenic seeds, the content of cysteine and methionine increased about 30% compared to the nontransformed control (Li *et al.*, 2005). These results indicate that sulfur amino acid composition in soybean seeds can be modified by overexpressing a plant sulfur amino acid-rich protein gene.

Krishnan (2005) reviewed the research efforts on enhancing sulfur amino acid content in transgenic soybean seeds. Traditional breeding has been

primarily utilized to increase the total protein content but not to enhance the sulfur amino acid content of soybean. Compared to the conventional breeding method, genetic engineering appears to be a more realistic approach. As discussed above, introduction of methionine-rich heterologous proteins has resulted in a modest increase of this amino acid in soybean. A better understanding of the sulfur assimilatory pathway in soybean will provide additional information to enhance the research on increasing sulfur amino acid level in soybean seeds. Expression of feedback-insensitive forms of serine acetyl transferase and *O*-acetylserine (thiol) lyase, two key enzymes in the sulfur assimilatory pathway, could lead to an increase in the availability of sulfur amino acids (Krishnan, 2005). An alternative approach is to express a synthetic protein such as MB1 or APS1 that has well-balanced sulfur amino acids (Chassy *et al.*, 2004). *ASP1* is such a synthetic gene that encodes 80% of essential amino acids and has four 20-amino acid helical-repeating monomers. These synthetic genes have been successfully expressed in crops (Kim *et al.*, 1992; Beauregard *et al.*, 1995; Zhang *et al.*, 2002; Potrykus, 2003) and are excellent candidates for engineering soybean.

In addition to the sulfur amino acids, isoleucine, tryptophan, valine, and arginine have been identified as “second tier” limiting amino acids in animal feed (Krishnan, 2005). Studies on increasing these amino acids have been pursued. Rapp *et al.* (2003) transformed soybean plants to produce 10–20 residues of isoleucine, tryptophan, methionine, or arginine. The synthetic genes were expressed under β -conglycinin promoter and terminator, and the construct was introduced into soybean via *Agrobacterium*-mediated transformation. Expression of the extra amino acids had no impact on the β -conglycinin structure and protein folding but conferred increased levels of the “second tier” amino acids (Rapp *et al.*, 2005).

2.3.5 Vitamin E

The nutritional value of vitamin E in the human diet was recognized long ago (Evans and Bishop, 1922). Vitamin E is a group of compounds (vitamers) including eight major forms of α -, β -, γ -, and

δ -tocopherol and four corresponding unsaturated derivatives, α -, β -, γ -, and δ -tocotrienol (Van Eenennaam *et al.*, 2003). Medical evidence shows that daily vitamin E supplementation of 400 IU (250 mg of RRR- α -tocopherol) can significantly reduce the risk for cardiovascular disease and cancer, in addition to preventing or slowing degenerative diseases (Buring and Hennekens, 1997; Tangney, 1997; Bramley *et al.*, 2000). Soybean seeds synthesize tocopherols and have significant amounts of γ -tocopherol (60–65% of the total) and δ -tocopherol (20–26% of the total) (Tan, 1989), however, these tocopherols have low activity. Conversion of the less active tocopherols to the active α -tocopherol form by manipulating the seed tocopherol biosynthetic pathway can increase soybean food value and benefit human health.

Cahoon *et al.* (2003) isolated a barley cDNA encoding homogentisic acid geranylgeranyl transferase (HGGT), which catalyzes the key step of tocotrienol biosynthesis. The barley *HGGT* gene was cloned between the CaMV 35S promoter and *Nos* terminator (Table 3). This construct was transformed into *Arabidopsis* by *Agrobacterium*-mediated transformation. Transgenic *Arabidopsis* plants were identified by kanamycin selection. Overexpression of the barley *HGGT* in *Arabidopsis* resulted in a 10–15-fold increase in total vitamin E antioxidants. Similar results were obtained in transgenic maize seeds. This result shows that vitamin E content can be improved by manipulation of a single transgene that redirects metabolic flux.

Van Eenennaam *et al.* (2003) isolated the *Arabidopsis* gene (*At-VTE3*) encoding the tocopherol biosynthetic enzyme 2-methyl-6-phytylbenzoquinol methyltransferase by map-based cloning. *At-VTE4* cDNA was obtained by PCR amplification. Three expression constructs were made: two of them express either *At-VTE3* or *At-VTE4* under the control of the β -conglycinin promoter (Chen *et al.*, 1986; Wang and Dubois, 2003) with a 3' untranslated region from pea SSU (Table 3). The third construct contains the two expressing units. The *At-VTE3* and *At-VTE4* expression cassettes were cloned individually and jointly into a plant binary expression vector containing a CaMV 35S promoter driving CP4 cassette as a selectable marker (Martinell *et al.*, 2002). *A. tumefaciens*-mediated transformation

was carried out as described previously (Martinell *et al.*, 2002) using freshly germinated soybean meristems that were induced to form shoots. Seed-specific expression of *At-VTE3* in transgenic soybean reduced seed δ -tocopherol from 20% to 2%. When *At-VTE3* was co-expressed with *At-VTE4* in soybean, the seed accumulated more than 95% α -tocopherol, a dramatic change from the normal 10%. The Vitamin E activity increased fivefold in the transgenic soybean seeds. These results show the utility of genes from other plants in altering soybean tocopherol composition toward improving soybean nutritional quality. DellaPenna (2005) and Kinney (2006) reviewed the progress of manipulation of vitamin E synthesis in plants. Technology developed in other plants can be applied in soybean.

2.3.6 Phytate

Soybean meal contains a high level of phosphorus in the form of phytate. Unfortunately, phytate is largely unavailable to poultry and swine (Denbow *et al.*, 1998). Nelson *et al.* (1997) reported that mixing phytase enzymes with soybean feed formulations can increase the availability of phosphorus. Denbow *et al.* (1998) also pursued a transgenic approach to increase the availability of phosphorus. They isolated a *phytase* gene from *Aspergillus niger* by PCR based on the published sequence and expressed the gene under CaMV 35S promoter and Nos terminator (Table 3) (Depicker *et al.*, 1982). The tobacco leader sequence was used to enhance the expression level of the *phytase* gene (De Loose *et al.*, 1991). This construct was transformed into soybean plants (Asgrow A5403) by particle bombardment and transgenic plants were identified by kanamycin (nptII) selection as described by McCabe *et al.* (1988). The seeds from these events were evaluated using a chicken feeding assay. The results indicated that *phytase*-transgenic soybean seeds were comparable to fungal phytase-treated diets in term of increased growth rate, feed intake, toe ash weight, energy, and phosphorus digestibility. This study indicated that phytase can improve broiler performance when provided either as a commercial supplement or in the form of transformed soybeans.

Chiera *et al.* (2004) was able to recover fertile transgenic soybean plants transformed with

the soybean *phytase* gene. The *phytase* gene was cloned from soybean and overexpressed under the seed-specific β -conglycinin promoter and terminator (Table 3). The construct was transformed into soybean suspension cell cultures by co-bombardment with a hygromycin resistance gene construct. Transgenic cells were selected by hygromycin (Finer and McMullen, 1991). Phosphorus availability increased almost threefold and a 25% reduction of phytate level was observed in the transgenic soybean seeds (Chiera *et al.*, 2004).

Nunes *et al.* (2006) reported that RNAi-mediated silencing of myo-inositol-1-phosphate synthase gene (*GmMIPS1*) in soybean can reduce the phytate content. In soybean, myo-inositol-1-phosphate is synthesized from glucose 6-phosphate in a reaction catalyzed by the enzyme GmMIPS. INositol can be converted into phytic acid through a sequential phosphorylation of iNositol phosphates, and/or in part through phosphatidyliNositol phosphate. To understand the role of the *GmMIPS1* gene, transgenic soybean lines were produced using a hairpin construct (Wesley *et al.*, 2001) with the CaMV 35S promoter and octopine synthase (OCS) terminator (Table 3). Transgenic progenies were identified by PCR assay and confirmed by Southern blot assay. Expression levels of the *GmMIPS1* gene were detected by RT-PCR analysis. Phytic acid was determined by high performance liquid chromatography (HPLC) using a modification of the procedure described by Sandberg and Ahderinne (1986). The phytate level in the transgenic soybean seeds reached levels as high as 94% without any negative effect on the plant phenotype. Completely silencing this gene, however, may be lethal to seed development since iNositol plays a role in membrane trafficking and signaling in addition to regulating cellular metabolism and controlling growth.

2.4 Industrial Uses

Soybean seeds have a long history of industrial use (Tao, 1994). They contain approximately 40% protein and 20% lipids, both of which are used in industry. More than 600 million pounds of soybean oil are used for nonedible applications annually, including industrial material production

in the United States (Cahoon, 2003). Recently soybean's industrial usage has been highlighted by biofuel since it is a renewable energy source. The physical and chemical properties of conventional soybean oil limit its use in many industrial applications. Genetic engineering provides a great opportunity to improve the properties of soybean oil for industrial usage (Duffield *et al.*, 1998; Cahoon, 2003).

2.4.1 Biodiesel and other oil-based usages

Biodiesel (or biofuel) consists of mono alkyl ester-based fuels produced through *trans*-esterification of vegetable oils or animal fats. Glycerine is a by-product of the process which has more than 1600 commercial applications ranging from toothpaste to environmental-friendly antifreeze. The concept of using vegetable oil in engines started in 1895 when Rudolf Diesel developed the first engine to run on peanut oil (<http://www.cyberlipid.org/glycer/biodiesel.htm>). Pure biodiesel can be used in modified engines or it can be blended with petroleum diesel for use in standard engines. It has the same energy efficiency as petroleum diesel but provides many environmental benefits including low toxicity, high biodegradability, and high cetane number. Biodiesel has also been found to extend the life of engines. The main challenge preventing biodiesel from becoming mainstream is the relatively high cost of raw vegetable oil feedstock and the costs involved with the *trans*-esterification process. Many countries have initiated biodiesel-related projects and have supported the development and application of the technology since the late 1990s.

Conventional soybean oil contains a large quantity of linolenic acid and therefore has relatively low oxidative stability that impacts biodiesel quality (Knothe, 2004; Ferrari *et al.*, 2005). Scientists at DuPont have developed transgenic soybeans that produce oil with high oleic (80%) and low saturated fatty acid (6%), as well as low polyunsaturated fatty acid (5%) by knocking off the *FAD2* gene (Kinney, 1997; Kinney and Knowlton, 1998; Parrott and Clemente, 2004). This type of soybean oil has high oxidative stability

and is useful for biodiesel. This is an example of modifying soybean oil profile for improving the biodiesel blends (Kinney and Clemente, 2005). Another approach involves producing high concentrations of industrially useful fatty acids in plant seeds using the transgenic approach (Cahoon, 2003; Jaworski and Cahoon, 2003) (see detail in Section 2.4.3). This is an excellent example of how biotechnology links agriculture and industry.

Polyols are chemical compounds that contain multiple hydroxyl groups. Petroleum-based polyols are commonly used in manufacturing polyurethane plastic products. Soybean oil can also be used to produce polyols (Crandall, 2002). At least two types of soybean polyols have been prepared from soybean oil by chemical epoxidization. The properties of epoxidized soybean oil can be improved by using more homogenous fatty acids such as oleic acid. Natural plant lipids provide excellent lubrication and surface coating; however, their viscosity and oxidative reactivity limit their usage in modern high temperature applications. This is mainly due to the presence of multiple double bonds and ester linkages in the soybean oil. Transgenic high oleic soybean produces oil that contains more than 80% oleic acid with high oxidative stability, which is ideal for lubricant uses and polyol production (Kinney and Knowlton, 1998; Cahoon, 2003). Currently, the DuPont high oleic soybeans are commercially used for biodegradable lubricant formulations (Cahoon, 2003).

Rubber can be produced from soybean oil through chemical polymerization (Andjelkovic and Larock, 2006). Preliminary results indicate that these biobased products may be useful alternatives to current petroleum-based polymers for use in rubber products including tires. Plasticizers derived from epoxidized soybean oil are commercially available. They are primarily used to keep plastics and rubber soft and pliable in flooring, upholstery, food packaging, hoses, tubing, blood bags, and other products. The epoxy functionality of soybean oil provides excellent heat and light stability. Transgenic modification of fatty acid composition in soybeans, such as increasing the conjugated fatty acid content, can further improve the properties of the novel polymers toward producing nonpetroleum based rubber.

2.4.2 Plastic and other protein-based usage

Disposable plastic products are a part of every day life in many parts of the world, from drinking cups to food containers. Scientists have found that soybean proteins are a more environment-friendly and biodegradable material for making these products (American Soybean Association and United Soybean Board, 2004). The indestructibility of petroleum-based plastic is a growing concern because of its accumulation in the environment. The development of plant-based plastics, which are biodegradable by humidity and microorganisms, has made significant progress after the pioneering research by Jay-Lin Jan at Iowa State University in the United States (Paetau *et al.*, 1994). There is increasing interest in using natural plant fibers as reinforcements for plastics. Natural oils are useful raw materials in polymer synthesis. In the United States, the major source of vegetable oil for industrial applications is soybean oil. The biodegradable polymers will soon be commercialized in competition with commodity plastics offering lower cost, enhanced performance, and reduced weight compared to the petrochemical-based plastic products. Liu *et al.* (2006) reported “green” composites obtained from a mixture of epoxidized soybean oil and epoxy resin, 1,1,1-tris(p-hydroxyphenyl)ethane triglycidyl ether (THPE-GE), reinforced with flax fiber. The resulting composites have sufficient mechanical properties for agricultural equipment, civil engineering, and the automotive and construction industries. There are examples of producing polyhydroxyalkanoate (PHA) polymers in transgenic plants (Snell and Peoples, 2002). PHAs are polymers of hydroxyacids synthesized by a range of bacteria and can be used as biodegradable plastics. Three to four genes control the PHA or related polymer synthesis and these genes have been isolated from bacteria (Slater *et al.*, 1999; Bohmert *et al.*, 2000). Expression of these enzymes in plants can produce the PHA polymers (Snell and Peoples, 2002). These genes may be used to engineer soybean toward producing novel plastics.

High performance flax fiber made from plants is a potential substitute for glass or carbon fibers (O'Donnell *et al.*, 2004). Soybean protein textile fiber technology is also being pursued to provide alternatives to synthetic fibers. Soybean protein can be made into an inexpensive, washable, silklike

fabric. Research on chemical modification of soybean protein for industrial uses is underway (Tao, 1994; United Soybean Board Bulletin on Soybean Utilization, 1997). Spider dragline silk is a fiber with unique toughness and strength. It has potential to be used in medical materials and industrial fabric (Kaplan *et al.*, 1997). However, it is impossible to produce mass amounts of dragline silk from spiders. Barr *et al.* (2004) reported a method of producing and purifying recombinant silklike protein in soybean somatic embryos. A plant codon-optimized dragline silklike protein gene (*DP1B*) was synthesized based on the spider dragline silk protein sequence. The β -conglycinin promoter and the phaseolin terminator were constructed at the 5' and 3' of the *DP1B* gene to make a seed-specific expression cassette for the *DP1B* gene (Table 3). This construct was introduced into soybean somatic embryos using particle bombardment. The transgenic embryonic cells were determined by hygromycin B selection (Finer and McMullen, 1991; Cahoon *et al.*, 1999). Protein analysis demonstrated that *DP1B* can be expressed and the spider draglinelike protein can be accumulated in the soybean embryos. The results indicate that it is possible to produce silklike protein in transgenic soybean seeds and improve the quality and value of the soybean proteins for the fabric industry.

Production of antibodies, enzymes, and vaccines in transgenic plants is a very attractive research area and has made significant progress in the last decade (Biesgen *et al.*, 2002; Schillberg *et al.*, 2003; Warzecha and Mason, 2003). Soybean seeds have special use in this area because they have the capacity to accumulate large amounts of protein. The soybean glycinin G1 promoter can direct high-level expression of GUS protein and a human basic fibroblast growth factor in transgenic soybean seeds. Ding *et al.* (2006) found that the growth factor protein can accumulate to as much as 2.3% of the total soluble seed protein. The G1 promoter expression cassette is useful in expressing recombinant proteins in soybean seeds. Transgenic plants, such as soybean, provide a cheap system for producing valuable proteins. This system is also beneficial because animal viruses or other contaminations can be avoided. However, clearly separating the food and medical products during the development of this technology is important for molecular phytopharming.

2.4.3 Novel fatty acids

In addition to the five common fatty acids, there are more than 200 chemically diverse uncommon fatty acids found in different rare plant species (Van de Loo *et al.*, 1993; Cahoon, 2003). The biosynthesis of these uncommon fatty acids is controlled by specific genes. Research efforts have been focused on isolation of the genes that are associated with novel fatty acid synthesis and the transformation of these novel genes into oilseeds for producing novel oils in the transgenic crops such as soybean (Yadav, 1996; Cahoon, 2003).

One of the unusual fatty acids contains conjugated double bonds, which are different from the double bonds existing in linoleic and α -linolenic acids where the double bonds are separated by methyl groups (Cahoon *et al.*, 1999). Two of the fatty acids with conjugated double bonds are α -eleostearic acid and α -parinaric acid, which are useful in drying oil applications. Cahoon *et al.* (1999) isolated genes encoding δ -12 oleic acid desaturases (also named conjugases) from developing seeds of *Momordica charantia* and *Impatiens balsamina*. These genes are designated *MomoFadX* and *ImpFadX* and were fused together and expressed under seed-specific β -conglycinin promoter and phaseolin terminator (Table 3). Selection of transgenic soybean plants was achieved by hygromycin B with the phosphotransferase gene under control of the CaMV 35S promoter. The constructs were transformed into soybean somatic embryos via particle bombardment (Finer and McMullen, 1991). Transgenic embryos overexpressing the fused genes resulted in accumulation of as much as 17% of α -eleostearic and α -parinaric acids. These results demonstrate the ability to produce fatty acid components of high-value drying oils in transgenic soybean.

Various plant δ -12 oleic desaturases (*FAD2*s) have been identified from plant species and can catalyze the formation of acetylenic bonds, epoxy groups, and conjugated δ -11, δ -13-double bonds from an existing δ -12-double bond in C_{18} fatty acids (Moto *et al.*, 2004; Whittle *et al.*, 2005; Damude *et al.*, 2006). Cahoon *et al.* (2001) isolated two *FAD2* genes, *CoFADX-1* and *CoFADX-2*, from calendic acid-producing *Calendula officinalis*. Vector pKS67 featuring

the seed-specific β -conglycinin promoter and phaseolin terminator (Table 3) was used to express the *CoFADX* genes. Transgenic plant selection is conferred by a hygromycin B phosphotransferase gene under the control of CaMV 35S promoter. The constructs were introduced into soybean somatic embryos by particle bombardment. Expression of either *CoFADX-1* or *CoFADX-2* led to the production of calendic acid in somatic soybean embryos. *CoFADX-2* conferred 22% calendic acid production in the transgenic embryos. Cahoon *et al.* (2006) further demonstrated that soybeans expressing δ -12-oleic acid-related fatty acid conjugases from other plant species significantly accumulate the conjugated fatty acids in phospholipids, which may not be able to be transported and stored in triacylglycerol. Calendic acid, α -eleostearic, and α -parinaric acids are novel conjugated polyunsaturated fatty acids in transgenic soybean that have low oxidative stability which is a superior quality for use as drying oil in coating applications such as paints, varnishes, and inks.

The seed oils of meadow foam (*Limnanthes alba*) and other *Limnanthes* spp. are enriched in the unusual fatty acid δ -5-eicosenoic acid (20:1). This fatty acid has special physical and chemical properties that fit a number of industrial applications. Cahoon *et al.* (2000) isolated the genes encoding an acyl-coenzyme A (CoA) desaturase and fatty acid elongase 1 (*FAEI*) from developing *Limnanthes douglasii* seeds. These genes were co-expressed in soybean somatic embryos under the seed-specific β -conglycinin promoter and phaseolin terminator (Table 3). The CaMV 35S driving the *hygromycin B phosphotransferase* gene was used as a selection marker. These constructs were transformed into soybean embryos by particle bombardment. The fatty acid compositions were measured by the method from Hitz *et al.* (1994). δ -5-eicosenoic acid (20:1) and δ -docosenoic acid increased to as high as 12% of the total fatty acids in the transgenic embryos. These fatty acids have special use in cosmetics, surfactants, and lubricants.

Seed oils of a number of *Asteraceae* and *Euphorbiaceae* species are enriched in 12-epoxyoctadecacis-9-enoic acid (vernolic acid), an unusual 18-carbon δ -12-epoxy fatty acid with potential industrial value. Somatic soybean embryos are enriched in triacylglycerols and provide a model

system for predicting the functions of transgenes in developing soybean seeds (Kinney, 1996). Cahoon *et al.* (2002) isolated a cytochrome P450 enzyme gene classified as *CYP726A1*. To characterize this gene, *CYP726A1* was expressed in somatic soybean embryos under control of the seed-specific β -conglycinin promoter and phaseolin terminator (Table 3). The constructs were transformed into soybean embryos by particle bombardment. Fatty acid analyses indicated that the somatic soybean embryos accumulated vernolic acid and 12-epoxy-18:2 $\delta^{9,15}$. Vernolic acid-enriched seed oils can be used as plasticizers of polyvinyl chloride, a market that is currently served by petroleum-derived compounds or chemically epoxidized soybean and linseed oils (Perdue *et al.*, 1986; Budziszewski *et al.*, 1996). Vernolic acid-containing oils are also useful in adhesives and coating materials such as paint due to the cross linking ability of the epoxy group (Perdue *et al.*, 1986). Furthermore, vernolic acid can be used as a precursor of monomeric components of nylon-11 and nylon-12 (Ayorinde *et al.*, 1997). Cahoon and Kinney (2004) isolated two genes encoding divergent forms of δ -12-oleic acid desaturase, designated *DsFAD2-1* and *DsFAD2-2*, from developing *Dimorphothea sinuata* seeds and expressed them in soybean. *DsFAD2-1* and *DsFAD2-2* were expressed under the β -conglycinin promoter and phaseolin terminator in the vector pKS67 (Table 3). The fused genes of *DsFAD2-1* and *DsFAD2-2* were also expressed under the β -conglycinin promoter and phaseolin terminator in vector pKS67 (Table 3). These constructs were introduced into soybean somatic embryos by particle bombardment. To test the genes simultaneously, co-transformation was also carried out as described by Finer and McMullen (1991) (Cahoon *et al.*, 2000). Transgenic embryos were selected by hygromycin resistance conferred by the marker gene for HPT in pKS67. Expression of *DsFAD2-1* resulted in significant accumulation of the *trans*- δ -12 isomer of linoleic acid (18: 2- $\delta^{9cis,12trans}$) rather than the more typical *cis*- δ -12 isomer in soybean somatic embryos. Expression of *DsFAD2-2* alone resulted in trace amounts of the *cis*- δ^{12} isomer of dimorphecolic acid (9-OH-18:2- $\delta^{9cis,12cis}$). Co-expression of both genes resulted in accumulation of dimorphecolic acid in soybean embryos.

Overexpression of *FAD3* gene in soybean significantly increased the linolenic acid from 10%

in the control to 50% in the transgenic soybean line (Cahoon, 2003). The transgenically modified soybean oil has low oxidative stability, which is a desirable property of drying oil used in coating applications such as paints, inks, and varnishes. The seed oil of flax (*Linum usitatissimum*) contains a high level of linolenic acid, 45–65% of the total fatty acids, which also makes it useful as drying oil. Vrinten *et al.* (2005) isolated a flax gene *LuFAD3A* encoding microsomal desaturase. This gene is capable of desaturating linoleic acid and can recover the high linolenic acid traits when expressed in low linolenic-acid flax. The flax 2S storage protein conglycinin promoter was used to overexpress *LuFAD3A* gene and the construct was transformed into flax by *A. tumefaciens* strain GV3101. The transgenic flax seeds contained a high level of linolenic acid. *FAD3*-like genes have been isolated from other plant species and are useful gene sources for engineering soybean to produce high levels of linolenic acid.

Transgenic technology can significantly improve soybean oil and protein compositions to develop desirable soybean-based products. The challenge is the productive level and the potential negative impact on the plants (Kinney *et al.*, 2001). Highly active and specific novel desaturases can be achieved by gene shuffling technology (Castle *et al.*, 2004). Expression of novel and diverse desaturases in soybean may enable soybean seeds to be used as a factory for producing valuable soybean oils for various industrial usages.

2.5 Regulations of Transgenic Soybean Plants

All of the regulations pertaining to transgenic plants apply to transgenic soybeans. As illustrated in Section 3.2, transgenic soybean plants with high oleic acid or herbicide resistance traits were subjected to extensive field trials and regulatory studies before their commercialization. Release of all transgenic soybeans is regulated by various regulatory authorities in different countries. In the United States, USDA Animal and Plant Health Inspection Service (USDA-APHIS) permits are required for shipping transgenic soybeans across state lines, performing

greenhouse experiments, and for field evaluations (<http://usbiotechreg.nbii.gov/roles.asp>). All transgenic products require approvals from the EPA (<http://www.epa.gov>) and the FDA before commercialization. These approvals require robust data demonstrating that novel transgenic products are safe for human consumption, safe for the environment, and do not negatively impact nontarget organisms. Field experiments involving transgenic plants are highly regulated by federal and state laws as well as institutional biosafety committees (IBCs). IBCs have been established under the National Institute of Health (NIH) guidelines to provide local review and oversight of transgenic research. For greenhouse research with transgenic plants, NIH has guidelines at four physical containment levels (http://www.isb.vt.edu/greenhouse/green_man.section3.htm) to prevent the transfer of recombinant DNA from transgenic organisms in greenhouse experiments to organisms outside the greenhouse. Regulatory procedures have also been developed in countries other than the United States. In Europe, the European Economic Community (EEC) controls the release of transgenic plants into the environment. Developing countries like China and India are also developing regulatory procedures (Huang and Wang, 2002; Huang *et al.*, 2002; Manjunath, 2005). UNIDO/UNEP/WHO/FAO is contributing to the development and application of common procedures for developing countries (UNIDO, 1991). The Rio de Janeiro Agreement (Taylhardat and Zilinskas, 1992) intends to foster the development of an international set of guidelines on safety in biotechnology.

As summarized in Table 3, soybean scientists are developing various transgenic traits to improve soybean productivity, increase nutritional value, and expand soybean's industrial uses. Currently only RR soybeans and high oleic soybeans are available on the soybean market. *Bt* soybeans are currently at the field-testing stage (Macrae *et al.*, 2005), and novel herbicide resistant products are being developed (Castle *et al.*, 2006). As illustrated by RR soybeans and high oleic soybeans, transgenic crops are becoming more popular each year and unquestionably more exciting transgenic soybean products will undergo regulatory approval and will be on the market in the near future.

3. FUTURE PERSPECTIVES

3.1 Expected Transgenic Products

RR soybean was the first transgenic soybean product that was developed by Monsanto and commercialized in 1996. It went from 17% of US soybean acreage in 1997 to 68% in 2001 and 89% in 2006 (<http://www.ers.usda.gov>). Global production of herbicide-tolerant soybean during the 2006 growing season was estimated at 133 million acres in nine countries including the United States, Argentina, Brazil, Paraguay, Canada, Uruguay, Romania, South Africa, and Mexico. (<http://www.monsanto.com/monsanto/content/investor/financial/reports/2006/Q32006Acreage.pdf>). More than 50% of them (71 million acres) were planted in the United States, followed by Argentina (37 million acres) and Brazil (19 million acres) (Ash *et al.*, 2006). The widespread acceptance of RR soybean is significantly promoting the development of various transgenic traits in soybean.

The second transgenic soybean product to receive clearance for market is the high oleic soybeans developed by DuPont. The high oleic level in soybean can provide health benefits to consumers (Kinney and Knowlton, 1998; Buhr *et al.*, 2002) as well as better properties for use in industrial applications such as biodiesel, lubricant formulations, and polyol (Cahoon, 2003).

According to the field test release permits database authorized by USDA-APHIS, two major industrial companies, DuPont and Monsanto, are the leaders in developing soybean biotech traits including yield enhancement, altered maturity, altered feed property, altered flavonoid level, altered fatty acid metabolism, ω -3 fatty acids, altered oil profile, altered amino acid composition, altered carbohydrate metabolism, protein quality, altered starch metabolism, altered seed composition, altered cell wall, altered fiber, drought tolerance, cold tolerance, high temperature tolerance, salt tolerance, *Sclerotinia* resistance, nematode resistance, lepidoptera resistance, aphid resistance, glyphosate tolerance, dicamba tolerance, PPT tolerance, sulfonylurea tolerance, nitrogen utilization efficiency, and phytate reduction. There are also great efforts in academia (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). We will highlight a few promising future products with quality output

traits and agronomic input traits as well as industrial usages.

The second generation of transgenic soybean products will likely be Optimum™ GAT™ and RReady2Yield™ soybeans for herbicide resistance, *Bt* for insect resistance, and ω -3 and high oleic for healthy oils. The optimized GAT in combination with resistance to sulfonylurea herbicide confers dual herbicide resistance with two modes of action for weed management. The soybean product Optimum™ GAT™ is expected to be on the market in 2008 (<http://www.pioneer.com/pipeline/pipeline.pdf>) (Soper *et al.*, 2003). The lead event of RReady2Yield™ soybean (www.monsanto.com) was confirmed in April 2005, and this new trait is expected to deliver better yield. *Bt* soybeans are in field trials and initial results show significant yield protection. ω -3 soybeans are also under field evaluations and preliminary data show that current transgenic events have improved agronomics and emergence over earlier events (www.monsanto.com). Products with stacked traits, such as herbicide and insect resistances or herbicide resistance and a modified oil trait, could also possibly be on the market in 5–10 years.

As indicated in Table 3 and the current APHIS permit database, biotechnologists are developing various agronomic input and quality output traits as well as industrial products in transgenic soybeans. A few potential long-term products are listed here.

1. Quality output traits: High linolenic acid, high lysine and high sulfur amino acid, ω -3, reduced phytate, modified flavor, vitamin E, LCPUFA, and modified protein compositions.
2. Agronomic traits: High yield, nematode resistance, virus resistance, drought tolerance, and fungal resistance.
3. Industrial uses: High oil, novel fatty acids, and special oils for industrial uses such as biodiesel.

The ultimate purpose of improving resistance or tolerance to biotic and abiotic factors is to increase the productivity of harvestable yield. Yield is the most complex trait, which is the result of various growth and developmental processes. However, accumulated evidence in model plants indicates that crop yield can be improved by manipulating genes that regulate biological processes such as photosynthesis, starch biosynthesis, nitrogen

utilization, plant architecture, and signaling pathways (Good *et al.*, 2004; Camp, 2005; Raines, 2006). Soybean research, such as photosynthesis and nitrogen utilization, could benefit from advances in these areas.

Crops are separated into two classes according to their photosynthetic carbon assimilation pathway. The most common class, known as C_3 includes major crops such as rice, wheat, and soybean. The first product of photosynthesis in C_3 plants is 3-phosphoglycerate, a three carbon compound. It is produced in the Calvin cycle by ribulose 1,5-bisphosphate carboxylase dikinase (Rubisco), which has both carboxylase and oxygenase activities. Its carboxylase activity assimilates CO_2 and produces oxygen in photosynthesis, and the oxygenase activity generates CO_2 in photorespiration. Competitive inhibition of CO_2 fixation by O_2 at the Rubisco active site can result in a loss of up to 50% of the carbon fixed during photorespiration (Ogren, 1984). The other photosynthetic class is known as C_4 plants. During the evolution of higher plants, C_4 crops such as maize have developed a strategy to release CO_2 at the site of the Rubisco, thus concentrating CO_2 and increasing the ratio of Rubisco carboxylation/oxygenation (Leegood, 2002). This mechanism prevents major losses of CO_2 during photorespiration and increases the efficiency of water and nitrogen use compared to C_3 crops. The first step in the C_4 cycle is the carboxylation of phosphoenolpyruvate by phosphoenolpyruvate carboxylase, which produces oxaloacetate, a four carbon compound. In general, C_4 crops have better photosynthesis capacity and produce more biomass under water stress conditions compared to C_3 crops (Häusler *et al.*, 2002; Miyao, 2003). All of the enzymes and transporters involved in the C_4 pathway also exist in C_3 plants. The main differences between the two classes of crops are the activity levels, functions, and localizations of the enzymes involved. To improve the C_3 crop photosynthesis capacity, scientists have pursued overexpression of C_4 enzymes in C_3 plants (Häusler *et al.*, 2002; Miyao, 2003). Since photosynthesis and the downstream carbon and nitrogen metabolisms mainly occur in the mesophyll cells in C_3 plants, transgenes were targeted to these cells. The enzymes evaluated in transgenic plants include phosphoenolpyruvate carboxylase, malic enzymes, malate dehydrogenase,

phosphoenolpyruvate carboxykinase, pyruvate/orthophosphate dikinase, and metabolite transporters. These approaches have mainly been carried out in model plants such as tobacco and rice (Häusler *et al.*, 2002). Ku *et al.* (2001) reported that co-expression of both phosphoenolpyruvate carboxylase and pyruvate/orthophosphate dikinase in rice could increase photosynthetic capacity up to 35% and grain yield by 20% compared to the nontransformed plants. These preliminary observations are to be confirmed in advanced generations. Another approach is to directly improve Rubisco activity. Scientists at Verdia-Pioneer-DuPont (Zhu *et al.*, 2005) have generated a heat-tolerant version of the Rubisco activase by gene shuffling technology. Transgenic *Arabidopsis* plants expressing this optimized Rubisco activase showed significantly improved photosynthetic performance and leaf growth compared to the nontransformed control plants. Other approaches have been reviewed recently (Pellny *et al.*, 2004; Sakamoto and Matsuoka, 2004; Camp, 2005; Raines, 2006). The technology developed with model plants may be used to enhance soybean's photosynthetic performance. The enhanced level of photosynthetic oxygen release may increase lipid synthesis and storage in developing seeds (Rolletschek *et al.*, 2005).

Nitrogen fixation symbiosis on the roots of leguminous plants, such as soybean, reduces the need for nitrogen fertilizer. This symbiosis includes complex interactions between host roots and specific rhizoidal bacteria resulting in the formation of nodules in which the bacteria fix nitrogen. Genetics and genomic studies have significantly advanced our understanding of the mechanisms that control nodulation and nitrogen fixation. Genes with potential to improve nitrogen use efficiency have been discovered and evaluated in model plant systems as recently reviewed (Good *et al.*, 2004; Pauly *et al.*, 2006; Stacey *et al.*, 2006). A number of important factors involving various signaling pathways of nodule formation have been discovered, including soybean genes. Soybean apyrase is an early nodulin that has important roles in nodule formation and can increase nodulation in *Lotus japonicus* (McAlvin and Stacey, 2005). ENOD40 is an important nodulin in *L. japonicus* (Kumagai *et al.*, 2006). These examples indicate that the capacity for nitrogen

fixation in legumes such as soybean could be improved by transgenic manipulation of important nodulins.

Biotechnology has opened a bright future for improving soybean traits. Biodiesel has great positive benefits on the economy and environment (Hill *et al.*, 2006). The increasing demand for soybean oils as a renewable biofuel resource will significantly promote investment in and the advancement of soybean research. Transgenic soybean products will help to meet this demand. However, unintended effects can occur in the transgenic soybeans. It is important to keep "risk" sense in mind during research and product development as well as marketing.

3.2 Risk Assessment

3.2.1 Food safety concerns and issues

Assessing the occurrence and significance of unintended health effects using a range of toxicological, metabolic, and epidemiological sciences is necessary to fully understand the effects of transgenic plants in the environment. Current safety assessments prior to commercialization focus on comparing the transgenic food with its conventional counterpart (a near-isogenic line or variety) to identify unique components. Typically, these comparisons are made on the basis of proximate analysis—an analytical determinant of major classes of food components as well as nutritional components, toxins, toxicants, antinutrients, and any other characterizing components. In addition to compositional comparisons, agronomic comparisons have been routinely conducted as part of the line selection phase in the development of transgenic crops (Chassy *et al.*, 2004). Animal feeding trials are also used to compare the nutritional qualities of a transgenic crop with its conventional counterpart. Any adverse effects on the health of the animals indicate the possible existence of unexpected alterations in the transgenic crop that could adversely affect human health if consumed.

Postmarket stewardship is an approach to verify premarket screening for unanticipated adverse health consequence from the consumption of transgenic food. This approach holds promise

in monitoring potential effects, anticipated and unanticipated, of transgenic foods that are not substantially equivalent to their conventional counterparts or that contain significantly altered nutritional and compositional profiles. To help ensure acceptance, soybeans derived through transgenic breeding must be developed while following stringent safety and regulatory guidelines established by industry and government agencies. These guidelines must be followed from product concept to postmarket stewardship (Soper *et al.*, 2003).

The safety of high oleic soybeans for use as food and livestock feed was established based largely on the fact that there were no new proteins present in these soybean lines. It was determined that the allergenic potential of the high oleic soybeans was the same as for conventional soybeans. The nutritional equivalence of the transgenic soybeans compared to conventional soybeans was demonstrated by the analyses of key nutrients, including proximates (e.g., protein, fat, fiber, ash, and carbohydrates), amino acid and fatty acid composition, as well as antinutrients. High oleic soybean oil contains approximately 10% saturated fats, greater than 80% oleic acid, and low levels of polyunsaturated fatty acids (approximately 2% linoleic acid and 3.5% linolenic acid). Trace amounts (0.5%) of a linoleic acid 9,15 isomer were also detected, which, while absent from nonhydrogenated soybean oil, is present at similar levels in butterfat, and is often found at considerably higher levels (typically 1–3%) in partially hydrogenated vegetable oils. The equivalence of high oleic soybeans to conventional soybeans was confirmed in feeding studies with pigs and broiler chickens. Soybean meal from high oleic soybeans was nutritionally equivalent to processed soybean meal derived from the conventional soybeans (Kinney and Knowlton, 1998; Chassy *et al.*, 2004).

High oleic soybeans are a value-added commodity. These premium soybeans are grown under contract to preserve the identity of high oleic soybeans from the point of planting through delivery of seed to the processing plant. Other than this, management and production practices for growing high oleic soybeans are much the same as growing any regular variety of soybeans.

3.2.2 Environmental issues

High oleic acid soybean lines were tested in field trials in the United States and Canada. Data collected from these trials demonstrated that these lines did not differ from conventional soybeans in agronomic characteristics including seed production and susceptibility to diseases and insects. These tests also demonstrated that the transformed lines did not exhibit weedy characteristics or negatively affect beneficial or nontarget organisms, and were not expected to have an impact on threatened or endangered species.

There has been some concern whether the transgenic herbicide resistance trait would flow into weeds. To date all instances of weeds becoming resistant have resulted from the plant evolving its own biochemical mechanism and not by acquiring resistance genes from the crop. Nonetheless, in certain circumstances it would be possible for herbicide resistance genes to flow from the crop to weeds. The most important variable affecting gene flow is the degree of relatedness between the crop and the weed, making gene flow only possible if close relatives are growing near the crop. As a result, the possibility of gene flow depends first and foremost on the presence of weedy relatives in the environment.

Soybean does not have any weedy relatives with which it can crossbreed in the continental United States and Canada. Cultivated soybean can naturally cross with the wild annual species *G. soja*; however *G. soja*, which occurs naturally in China, Korea, Japan, and the former USSR, is not naturalized in North America. Additionally, soybean plants are almost completely self-pollinated, and reproductive and growth characteristics were unchanged by the genetic modification resulting in these high oleic acid soybean lines. It was, therefore, concluded that the potential for transfer of the trait for high oleic acid from the transgenic line to soybean relatives through gene flow (outcrossing) was negligible in managed ecosystems, and that there was no potential for transfer to wild species in Canada and the continental United States.

Soybean meal is primarily used for animal feed. One of the major challenges facing animal production is the prevention of environmental

pollution from animal production facilities. Environmental pollution concerns have focused primarily on phosphorus, nitrogen, and malodor. In the United States, the EPA as well as state natural resources and conservation agencies, have enacted regulations for the application of animal waste to land, mainly concerning the loading rate of phosphorus and nitrogen onto soil as well as the malodor abatement. The regulatory pressures of nitrogen excretion and malodor abatement present costs associated with feed ingredients that contribute to excess nitrogen excretion or malodor production. Failure to address these issues will negatively affect animal production in the United States and other countries (Kerley and Allee, 2003). Environmental opportunities associated with lowering the levels of nitrogen and phosphorous manure components generated per animal fed may be achieved through biotechnology. Nitrogen can be addressed by improving the amino acid balance, as well as the extent to which soybean meal protein is digested. Phosphorus can be addressed by increasing the extent to which the digestible phosphorus is present in the meal and productively utilized by the animal.

The transgenic high oleic soybeans are a good example of how biotechnologists can develop a safe and useful product, however, this is not always the case. Unintended effects in transgenic crops have been reported (Chassy *et al.*, 2004). For example, the original purpose of expressing glucose oxidase in cotton and tobacco was to engineer plants with enhanced resistance to fungal diseases (Murray *et al.*, 1991). However, in addition to reducing fungal infection the oxidase caused phytotoxic effects in the transgenic plants. Unintended effects have also been observed in conventional breeding programs (Chassy *et al.*, 2004). Therefore, both conventional and transgenic breeding approaches require risk assessment. To meet the challenge, improved and novel technologies are required.

3.3 Technologies that Improve Transgenic Breeding

Development of a transgenic trait in soybean requires a trait gene, a proper promoter, a method of transferring the promoter-gene-terminator unit

into the soybean genome, evaluation of the trait gene in the greenhouse and field, and then breeding the transgene unit into elite lines for product development. Improved and novel technologies are required to accelerate the discovery of trait genes and promoters, improve gene efficacy, express transgenes in desirable patterns, and efficiently stack and transfer the transgenes into elite lines. This section describes a few technologies that have the potential to promote the development of desirable transgenic traits in soybeans.

3.3.1 Genomics

Genomics is an emerging tool in biotechnology for the effective study of the function and structure of genes for almost all organisms. Although single-gene and single-investigator studies will continue to provide detail and resolution to our understanding of biological processes, the high-throughput discovery tools of genomics will become the dominant tools for identification and expression of genes for desired trait development (Shoemaker *et al.*, 2003). The genomes of soybean and other species hold a vast resource of gene blueprints that will aid the development of desired traits such as pest resistance, higher yield, and better grain composition. The knowledge-based tools also help to develop gene markers for trait selection or trait solutions (Soper *et al.*, 2003).

The soybean has a rich repertoire of genomic tools and resources that include a vast EST collection, a densely populated genetic map, developed physical maps, microarray resources, and a relatively efficient transformation system. It also has a large and active research community.

A genome-wide physical map was constructed from more than 78 000 BAC clones. This map consists of approximately 2900 contigs. The total contig length exceeded the predicted size of the soybean genome. More than half of the length of the physical map was anchored to the genetic map using RFLP and SSR markers (Wu *et al.*, 2004). A second physical map of the soybean genome was recently constructed using Williams 82 cultivar, chosen as the model genotype by the soybean community (Warren, 2006). A total of 96 524 fingerprints were generated from two independent BAC libraries, referred to as UM-BstyI and ISU-*Hind*III, utilizing the

high information content capillary fingerprint (HICF) method. The map consists of a total of 1880 fingerprint contigs. Molecular markers were associated with specific contigs by RFLP hybridization, overgo oligo hybridization, RFLP hybridization or SSR screening of BAC DNA pools. This resulted in 4015 clones and 715 contigs with associated markers. One of many potential applications of genome physical maps is to determine locations of transgenes within the genome.

Soybean has amassed more than 300 000 ESTs representing over 80 different cDNA libraries sponsored by the soybean commodity boards (North Central Soybean Research Program (NCSRP) and United Soybean Board (USB)) (Shoemaker *et al.*, 2002). The cDNA libraries giving rise to those ESTs represent a wide range of organs, developmental stages, genotypes, and environmental conditions. This resource provides a great deal of information on differences in gene expression of members of multigene families (Granger *et al.*, 2002). The soybean EST collection provides a large resource of publicly available genes and gene sequences and provides valuable insight into structure, function, and evolution of this model crop legume (Shoemaker *et al.*, 2003).

Genome sequencing is fundamental to understanding the genetic composition of an organism. Generation of whole-genome sequences of soybean is lacking. A prerequisite to reaching this goal is to understand the organization, complexity, and distribution of the gene space of an organism, including the topography of its repetitive sequences. Genomic sampling of nearly 2700 DNA sequences from more than 600 mapped loci (Marek *et al.*, 2001) and 237 000 BAC-end sequences obtained from the Better Bean Initiative (USB) has provided a glimpse of the composition and general structure of the soybean genome. These sequences will be instrumental in defining soybean gene space and in creating a soybean repeat database useful for whole-genome sequencing efforts (Shoemaker *et al.*, 2003). Recently, the Joint Genome Institute of the Department of Energy announced their intention to sequence the whole genome of soybean. The initial plan is to carry out a whole genome shotgun up to 4× coverage to be completed within 2 years.

Using these EST and genomic sequences, the soybean community has developed a large number

of molecular markers (Section 1.4). MAS for native traits has been routinely practiced in soybean. With an increasing number of transgenic traits in soybean, marker-assisted backcrossing will be the next major application of molecular markers. Backcrossing is often used to incorporate transgenes into elite germplasm. In traditional backcross programs, a gene's presence and absence in a backcross individual is often determined by a time-consuming phenotypic assay. Moreover, background selection for the recovery of recurrent parent genome is also a lengthy process. Without selection, Young and Tanksley (1989) found lengths up to 51 cm of the segment attached to a resistance gene after six backcross generations in tomato. With the development of high-density molecular linkage maps in soybean, it became possible to select individuals that not only carry transgenes but also are homozygous for the recurrent parent alleles at the large portion of markers. Using molecular markers in background selection can reduce both the number of backcross generations needed and the size of linkage drag (Frisch and Melchinger, 2000), and thus improve the efficiency of transgenic product development.

Functional genomics is a useful approach for accelerating the studies of function and structure of genes. High-density expression arrays containing 18 000 cDNAs arrayed on a filter have been developed (Vodkin *et al.*, 2002) along with microarray technology. Serial analysis of gene expression (SAGE) captures short 10–20-nucleotide “tags” near the 3' end of individual mRNA molecules. This technology has been used to accurately estimate expression levels in the mRNA source tissue based on the frequency of tag appearances in the library. Initial analysis from 20 SAGE libraries in soybean has resulted in 132 992 SAGE tags, of which 40 121 are unique (Shoemaker *et al.*, 2003, 2004). The applications of microarray technology to soybean are enormous. The future of functional genomics research will include arrays that will distinguish gene family members. Phenotypical functional genomics systems via gene knockout systems are also needed in soybean (Shoemaker *et al.*, 2003). In soybean, transposon tagging projects, viral-induced gene silencing systems, and TILLING (targeted induced local lesions in genomes) populations are under development and will become useful tools for phenotypical

functional genomics. Crop genomics promise a new era of yield maximization through genetic improvement and optimized crop management (Johnson, 1987; Somerville and Somerville, 1999; Huang *et al.*, 2002). Most agronomic and quality traits are genetically complex (Stuber *et al.*, 2003) and often show complex interactions between genotype and environment. Various molecular models such as Decision Support System for Agrotechnology Transfer (DSSAT) (Jones *et al.*, 2003) and Agricultural Production Systems Simulator (APSIM) (Keating *et al.*, 2003) have been reported for linking genetic organizations and whole-organism phenotypic expression. Recently, Messina *et al.* (2006) reported a gene-based model (CROPGRO-Soybean) of simulating soybean development and yield responses to environment. Their model indicates that gene-based approaches can effectively use agricultural genomics data for cultivar performance prediction.

The rich genomic resources available for soybean make it a model legume crop. The gene discovery stemming from structural and functional genomics research in soybean will certainly lead to discovery of trait genes for developing new products and varieties with improved nutritional and agronomic characters. A number of desaturase genes were discovered by EST sequencing and bioinformatics searches and showed potential for novel soybean products (Cahoon, 2003). These are examples of how genomic tools accelerate gene discovery. Functional genomics data from other crops are available and comparative analysis of the genomics data can lead to the discovery of new trait genes (Windsor and Mitchell-Olds, 2006).

3.3.2 Promoter technology

Promoters have critical roles in successful transgenic crop improvement. A broad spectrum of promoters that can regulate the temporal and spatial expression patterns of a transgene when needed can be an effective tool to fine-tuning the transgene expression. Promoters can be divided into three categories: constitutive (with viral and plant origin that drive constitutive expression), tissue- or temporal-specific (fruit, seed, tubers, flowers, pistils, anther and pollen, roots, root

nodules, leaves, and stem tissue), and inducible promoters (their activities can be regulated by biotic and abiotic signals).

The most common promoters used for constitutive overexpression are derived from plant virus sources, such as the CaMV 35S promoter (Odell *et al.*, 1985). Details of other virus promoters can be found in the review by Potenza *et al.* (2004). Concerns of using the virus promoters include the perception of risk to human health due to the infective viruses (Ho *et al.*, 1999; Hull *et al.*, 2000) and transcriptional gene silencing (TGS) of methylation-dependent and methylation-independent inactivation of the transgene and promoter (Scheid *et al.*, 2002). Endogenous plant promoters are used regularly to drive high constitutive levels of transgene expression; actin and ubiquitin promoters are the common ones that are used heavily in the plant biotechnology. Actin is a fundamental cytoskeletal component that is expressed in nearly every plant cell (McElroy *et al.*, 1991; An *et al.*, 1996). One of the most popular promoters used for transformation is the maize ubiquitin 1 promoter (Christensen *et al.*, 1992). Synthetic SCP1 and UCP3 are strong constitutive promoters in dicot crops (Lu *et al.*, 2000).

Constitutive expression can be problematic when a transgene is overexpressed at the wrong time in biological development in tissues where it is not normally expressed, or at very high levels. It can have unexpected consequences on plant growth and development as well as the environment (Potenza *et al.*, 2004). The development of tissue-specific promoters that are activated precisely when and where they are needed is ideal for developing value-enhanced crops. For example, the strong soybean seed-specific promoter β -conglycinin has been heavily used to improve oil and protein compositions in transgenic soybean seeds as seen in Table 3 (Kinney and Knowlton, 1998; Cahoon *et al.*, 2001; Buhr *et al.*, 2002; Herman *et al.*, 2003; Sato *et al.*, 2004). Expression of transgenic proteins only in the nonedible parts of the plant with the use of tissue-specific promoters may help to ease the public's fears of consuming foods produced from transgenic soybean plants. Potenza *et al.* (2004) listed plant tissue-specific promoters including flower, pollen, pistil, anther, seed, tuber/storage organ, green tissue, fruit, nodule, and root.

In plants, various types of stress, both biotic and abiotic, induce a large number of genes. Pathogens, such as nematodes, can induce promoter activity (Gheyson and Fenoll, 2002). For example, UCP3 and SUP promoters were induced in the SCN nematode feeding sites, whereas SCPI promoter activity was significantly repressed (Hu and Lu, 2006). When a plant is under abiotic stress, genes are mobilized for defense and repair. For example, the promoter for the potato PI II gene also directs low constitutive expression, but was induced to high levels by wounding and methyl jasmonate when transformed into rice (Xu *et al.*, 1993). Many genes induced by abiotic stress contain two distinct cis-acting elements within their promoters. They are a dehydration-responsive element, or DRE (TACCGACAT) (Yamaguchi-Shinozaki and Shinozaki, 1994), and an abscisic acid-responsive element, or ABRE (ACGTGG/TC) (Bonetta and McCourt, 1998). Tissue-specific promoters are still dependent on the plant and the expression pattern of endogenous transactivating factors. Sometimes, a characterized heterologous promoter might act differently when placed in a different genus, species, or even cultivar of a plant. Inducible (and repressible) promoter systems using both endogenous and exogenous components could help to switch transgene expression on or off at precisely the right time.

Most inducible/repressible promoter systems are two-component systems with molecular “on-off” switches that vary in complexity. They include a transcription factor gene that responds to a chemical, binds to a “target” promoter that contains the transcription factor’s cis-acting element. The target promoter is fused to the transgene of interest and the binding of the transcription factor modulates its expression (Potenza *et al.*, 2004). Chemicals that are used to regulate transgene expression include the antibiotic tetracycline (Weinmann *et al.*, 1994; David and Perrot-Rechenmann, 2001), the steroids dexamethasone and estradiol (Martinez *et al.*, 1999; Bruce *et al.*, 2000; Zuo *et al.*, 2000, 2002), copper (Mett *et al.*, 1993), ethanol (Roslan *et al.*, 2001), the inducer of pathogen-related proteins benzothiadiazol (Gorlach *et al.*, 1996), herbicide safeners (De Veylder *et al.*, 1997), and the insecticide methoxyfenozide (Unger *et al.*, 2002). Systems that are suitable for field application are

particularly useful for experimental systems and have potential applications in plant biotechnology.

An ideal inducible gene expression system should have a low basal level of expression, be highly inducible, have a high dynamic range for inducer concentration, involve a rapid response, switch off after the removal of the inducer, and have an inducer of low toxicity and high specificity that is not found in plants (Padidam, 2003). Although the precise time and control of the transgene expression using the inducible promoter system can be achieved, the drawbacks for these inducible systems are the toxicity of the chemical or inducers used, and their tendency to be regulated by the products of their metabolism (Potenza *et al.*, 2004). For product application, the use of environmentally safe chemical inducers is critical. Multiple-inducible systems that can regulate several genes or pathways independently will be of great value to applications in proteomics and systems biology.

3.3.3 RNA interference

As we learned from the virus resistance (Section 2.2.3) and oil modification (Section 2.3.1) sections, RNAi is a useful technology for silencing endogenous genes to achieve desired traits. Hairpin-mediated RNAi has advantages over the antisense and co-suppression or mutation-based reverse genetic methods in terms of silencing stability and efficiency (Kusaba, 2004). RNAi pathways and mechanisms in plants are much clearer now (Wang and Metzlaff, 2005; Meyers *et al.*, 2006), and it will exhibit greater power in developing transgenic traits in soybeans as we move forward. For example, RNAi technology can facilitate broad-spectrum virus resistance (Prins, 2003) and nematode resistance (Hussey and Huang, 2006) with enhanced durability. RNAi in combination with a specific promoter can express transgenes in a more desirable pattern for trait development. In addition, RNAi takes an important role in functional genomics research and facilitates trait gene discovery (Travella *et al.*, 2006).

Although RNAi is in general sequence specific, unintended effects have been reported (Scacheri *et al.*, 2004). Therefore, the gene fragment in the hairpin constructs should be target gene-specific and have no side effects on any other

soybean genes. The soybean gene sequences and bioinformatics tools can be employed to achieve this objective before testing the constructs in soybean tissues.

3.3.4 Gene shuffling

The term “gene shuffling” covers a range of molecular techniques or processes that feature the rapid shuffling and recombination of the DNA sequences that make up a desirable protein function or a desirable protein complex. Gene shuffling improved on this process by using recombination to combine favorable mutations and to remove deleterious mutations from the population of evolving proteins (Stemmer, 1994).

Protein improvement can be achieved through a single or recursive round of shuffling. Most likely, the desired result is obtained from recursive rounds of shuffling in which the best progeny from the previous round of DNA shuffling are used as parents for subsequent rounds. DNA shuffling using single starting genes enabled dramatic improvement in protein properties when compared to previous methodologies of DNA recombination (Lassner and Bedbrook, 2001). The rate of random mutagenesis can be controlled by adjusting reaction conditions or by using different DNA polymerases (Zhu *et al.*, 2005), thereby controlling the amount of new diversity introduced at each generation of protein improvement. DNA shuffling has not been limited to the improvement of single genes, but has also been applied to improving pathways (Lassner and Bedbrook, 2001). Multigene shuffling using recombined natural diversity associated with different starting genes, rather than recombining random mutations associated with single gene shuffling, is an effective approach for direct molecular evolution for plant improvement. The relative success of multigene shuffling attributed to several factors was elucidated by Lassner and Bedbrook (2001). First, natural diversity's proven functionality leads to the recombination of functional sequences allowing the screening of more functional gene variants. Second, multigene shuffling causes sequence block exchanges, yielding chimeras that have greater divergence. Multigene shuffling results in the sampling of a much greater area of sequence space.

Several examples including GUS as a reporter gene, carotenoid biosynthesis pathway to produce antioxidants, triazine detoxification for herbicide tolerance, and *Bt* genes for insect resistance, demonstrated the effectiveness of the gene shuffling for plant improvement (Lassner and Bedbrook, 2001). Recently, a new mechanism for detoxifying glyphosate *in planta*, deactivation by acetylation, was developed (Castle *et al.*, 2004). The GAT gene derived from a naturally occurring soil bacterium (*Bacillus licheniformis*) was shuffled for 7–11 rounds to optimize both the enzyme's acetylation efficiency and its specificity before incorporating the gene into plants including soybean (Castle *et al.*, 2004; Siehl *et al.*, 2005).

The technology allows plant molecular biologists to improve genes and pathways in a manner that mimics the breeding procedures used by plant breeders. As in plant breeding, desirable characteristics from otherwise undesirable or uncharacterized parents can be bred into progeny through backcrossing for several generations. Backcrossing, a tool used by plant breeders to introduce novel traits into high quality breeding lines, can also be used in molecular breeding projects to remove unwanted and unnecessary mutations. Gene shuffling is becoming a highly effective tool for creation of desirable input or output traits for soybean improvement.

3.3.5 Transformation efficiency and plastid transformation

Transformation efficiency is the outstanding bottleneck of soybean biotechnology. As other legumes, soybean has been regarded as recalcitrant to transformation. Only a few specific genotypes can be regenerated *in vitro*, however, cultivated varieties are rarely amenable to regeneration, with an even lower transformation frequency (Somers *et al.*, 2003). A list of the literature on the regeneration and transformation of soybean globally is compiled at the web site: <http://www.cropsoil.uga.edu/soy-engineering/index.html>. Since the first transgenic soybean plants were produced via *Agrobacterium* (Hinchee *et al.*, 1988) and particle gun bombardment (McCabe *et al.*, 1988), the transformation systems have been improved (Zhang *et al.*, 1999; Xing *et al.*, 2000; Clemente and Klein, 2004; Olhoft

et al., 2004). Although soybean transformation efficiencies are consistently greater than 5% in Jack (Soper *et al.*, 2003), the frequency is quite low compared to corn (20–60%). Clemente and Klein (2004) discussed the current issues and strategies for improving soybean transformation efficiency. As described in Section 2.1, a number of factors impact the transformation efficiency including tissue type, culture medium, genotype, wounding, and temperature. Combination of particle bombardment and *Agrobacterium* methods is worth further investigation. Any factors that can increase proliferation can enhance the transformation rate. Gene transfer directly into meristematic cells by particle bombardment or *Agrobacterium* is encouraging since it skips the tissue culture steps. Optimizations of the particle bombardment conditions and identification of more efficient *Agrobacterium* strains will likely enhance the transformation productivity.

Plastid (or chloroplast) transformation was first successfully achieved in 1990 (Svab *et al.*, 1990) and is routine in tobacco (Svab and Maliga, 1993; Staub and Maliga, 1995b). It has been demonstrated in a limited range of other species (Daniell *et al.*, 2005). Particle bombardment is most often used for chloroplast transformation, although tobacco protoplasts can incorporate foreign DNA into their plastids (Golds *et al.*, 1993). Chloroplast transformation offers certain advantages, including the ability to precisely deliver genes into predetermined locations in the plastid genome by homologous recombination, high levels of transgene expression due to the presence of multiple copies of the plastid genome in each cell, consistent expression between independent transgenic events, and the lack of transgene transmission through pollen. Multiple genes can be inserted into plastids as polycistrons (Staub and Maliga, 1995a, b; Quesada-Vargas *et al.*, 2005); which could facilitate efforts to engineer particular biochemical pathways. Certain traits, such as the expression of proteins that control insects or genes conferring herbicide tolerance, are good candidates for plastid-based expression. Other genes that require precise tissue specific or temporal expression are probably not suited for insertion into the chloroplast since specific promoters and control elements that can confer controlled expression are not available.

Dufourmantel *et al.* (2004) reported the first demonstration of plastid transformation in soybean. These researchers delivered a gene that detoxifies spectinomycin into embryogenic cultures by particle bombardment. The transformation cassette was similar to that used for tobacco transformation, employing a strong plastid promoter (Prn) from the 16S ribosomal RNA gene from tobacco chloroplast (Maliga, 2004). Two days after bombardment, the embryogenic tissue was placed on solid medium containing spectinomycin and subsequently subcultured about every 2 weeks to fresh medium. Transgenic events appeared as green, embryogenic calli from the bleached, spectinomycin-sensitive tissue. About one transgenic event was recovered from every two plates of bombarded tissue. The selection scheme produced tissue that was fully “transplastomic” meaning that all copies of the plastid genome harbored the transgene. This group went on to demonstrate that the plastid genome stably maintained the transgene for at least six generations and that application of spectinomycin was not necessary for stable inheritance (Dufourmantel *et al.*, 2006). They also showed that *Bt* genes can be incorporated into the plastid genome of soybean, leading to high level expression (Dufourmantel *et al.*, 2005). Expression of *Bt* genes in crops by the nuclear machinery requires synthetic and codon modification to improve translation, protect the mRNA from degradation, and prevent early translation termination. In contrast, the native *Bt* genes can be expressed in plastids from bacterial coding segments. High copy numbers of plastid genomes (up to 10000 per single cell) with highly active inter- and intramolecular exchange would allow for the propagation of the transgene throughout the plastid genome pool very quickly (Goulding *et al.*, 1996), and result in high transgene expression. Field-level tolerance to glyphosate was obtained by expression of prokaryotic *EPSPS* genes in tobacco plastids; the protein levels were as high as 5% of total soluble protein, which is much higher than the expression level from transgenes in the nucleus (Ye *et al.*, 2001). Recently, Dufourmantel *et al.* (2007) introduced a bacterial gene coding for 4-hydroxyphenylpyruvate dioxygenase (HPPD) into the plastid genome of soybean. This enzyme catalyzes a step in the pathway leading to

plastoquinone and vitamin E and herbicides such as sulcotrione and isoxaflutole (IFT). Expression of HPPD from the plastid genome led to high levels of expression (5% of total soluble protein) and tolerance to IFT. Nuclear transformants carrying a chloroplast targeted *HPPD* gene under control of the strong *rbcS* promoter displayed herbicide damage after treatment with IFT, while the chloroplast transformants did not show damage.

Finally, a majority of cultivated crop plants inherit their plastid genomes uniparentally through a strict maternal inheritance (Maliga, 2004). Transferring transgenes in plastids under the control of plastid promoters can reduce or eliminate the transgenic pollen drift that could cause gene flow.

Expression of transgenes in the plastid genome for containment and high-level expression of recombinant proteins for pharmaceutical and industrial applications are desirable. Soybean represents a promising target for plastid transformation. The nature of the tissue used for gene transfer into the nuclear genome provides an ideal target for chloroplast transformation. DNA is typically delivered to this target by microprojectile bombardment using the gene gun, which is the preferred means. However, plastid transformation is routine only in tobacco, the soybean plastid transformation remains a challenge.

3.3.6 Marker-free products

Selectable marker genes based on antibiotic or herbicide resistance are widely used for identification of transformed tissues and plants. However, marker genes may be undesirable in the final products due to their potential horizontal transfer and other unexpected effects (Rommens, 2006). The presence of resistance genes against antibiotics in food products might theoretically lead to the spread of these resistances via intestinal bacteria in human populations, although there is no evidence supporting this proposition (Puchta, 2003).

There are several strategies to generate marker-free transgenic plants. (1) Without using marker genes, the isopentenyl transferase gene from the T-DNA of *Agrobacterium* was successfully used for the selection of transformants (Ebinuma *et al.*, 1997). Positive selection can be used to identify

the transformants by PCR assay or enzymatic assay if the transgene encodes an enzyme. (2) Eliminate marker genes by co-transformation, the desired gene and the marker gene can be supplied on two T-DNAs within the same binary vector (Komari *et al.*, 1996; Lu *et al.*, 2001) or on two binary vectors within the same *Agrobacterium* (Daley *et al.*, 1998) or with two different *Agrobacterium* strains (Komari *et al.*, 1996). However, the limitation of this strategy is that nonlinked transgene loci have to be separated by crossing, which is time consuming. (3) Excise the marker gene using site-specific recombination, a kanamycin gene placed between two *lox* sites was excised from the tobacco genome by the expression of Cre recombinase (Dale and Ow, 1991). This could be applied with other two site-specific recombination systems: FLP/frt (Luo *et al.*, 2000) and R-Rs (Sugita *et al.*, 2000). The recombinases, Cre, Flp and R, are not host-specific factors and can function in plants. A chemical-inducible site-specific DNA excision system has been developed in *Arabidopsis*. In the system, Cre expression was tightly controlled by the estradiol inducible XVE transactivator (Ow, 2001, 2002; Zuo *et al.*, 2001). Srivastava and Ow (2004) proposed the use of two site-specific recombination systems—one for integrating the DNA and the second for removing DNA sequences that are not needed after DNA transfer.

Transgenic plants without marker genes could win regulatory approval and public acceptance with less concern of food safety and environmental impact. The future transgenic soybean plants should adopt the marker-free technology in order to eliminate undesired sequences from the final commercial products.

3.3.7 Site-specific integration

Site-specific integration technology would help place gene(s) or trait(s) at a known and characterized site(s) in elite genotypes to yield predictable function as a hybrid product. Such targeted gene expression may also be necessary to satisfy regulations regarding food safety, such as potential allergenicity effects resulting in increased health risk to consumers.

Site-specific integration using flippase recombinase (FLP)/FLP recombinase target (FRT)

from yeast (Sadowski, 1995) and Cre/lox system (Sauer and Henderson, 1988; Ow, 2002) required two types of transgenic events—a target event (permanent insertion site, i.e., FLP recombinase that stimulates chromosomal recombination and binds to FRT sites) and a donor event (transgene donor of gene of interest). FRT sites will present in both target and donor events for exchange or excision of DNA sequences between FRT sites within two events via pollination. Target sites are fully characterized for characteristics like copy number, flanking sequence, chromosome location, and position before trait insertion.

This strategy will significantly reduce transgenic event production efforts compared to random insertion of transgenes. Furthermore, this technology could help to advance molecular gene stacking for multiple traits.

3.3.8 Transgene-stacking technology

The next generation of transgenic soybean products will have stacked traits, such as stacking two *Bt* genes or herbicide resistance genes for more effective insect or weed management. Therefore, it is very important to develop efficient gene-stacking technology. Transgene stacking can be achieved by sexual crossing, co-transformation, or retransformation as described by Eckert *et al.* (2006).

Crossing two transgenic lines with different transgenes can be easily achieved by conventional breeding with MAS technology. Co-transformation of two or more transgene constructs is possible using particle bombardment (Eckert *et al.*, 2006; Kinney, 2006). One or more transgenes can be expressed in a same expression cassette. Retransformation of a transgenic line with a new construct can be carried out with the same method as the initial transformation. However, this method faces other challenges. Retransformation requires new or different selection markers, and the frequency of events that exhibit proper expression for all of the transgenes is low. Site-specific integration technology takes an important role in developing an optimized gene-stacking method.

Kinney (2006) successfully co-expressed five genes and established a novel fatty acid synthetic pathway in transgenic soybeans. These results

demonstrate that gene-stacking technology has advanced and it is possible to develop transgenic soybeans with multiple improved traits. Transgenes can also be stacked with native genes by conventional breeding. Narayanan *et al.* (2004) successfully developed broad-spectrum bacterial blight resistance in rice by combining MAS technology and transgenic engineering. They transformed the *Xa21* transgene into rice and combined this transgene with other R genes by MAS technology. This is an example of how conventional breeding and the transgenic approach can synergistically advance product development.

3.4 Intellectual Property Rights, Industrial Perspectives, Biotechnology Acceptance, and Economic Consequences

Plant biotechnology holds a great deal of promise to enhance our lives and protect our planet. With the world population expected to reach nearly nine billion by 2050, biotechnology offers new potential for sustainable living, healthy eating, and battling diseases while reducing our footprint on the planet.

The broad field of biotechnology presents important opportunities that should be explored and developed to identify those safe and commercially viable applications that bring significant benefits to the society. These opportunities arise in areas including food, materials, energy generation, polymers, sensors, and electronics. Benefits may include lower cost, higher quality products and reduced reliance on fossil fuels, along with other environmental benefits.

3.4.1 Intellectual property rights (IPR) in agricultural biotechnology

At present, the majority of plant biotech investments occur in developed countries, and about 70% of that investment is from the private sector. Several developing countries currently have access to transgenic products that are spillover from developed countries, particularly from the United States (Acquaye and Traxler, 2005). As the pace of scientific discovery in agricultural biotechnology has accelerated over the past few decades, the use of

patents and other IPR to protect these discoveries has increased tremendously.

IPR refer to inventors' exclusive rights provided by various laws including patent law, copyright law, trademark law, trade secret law, industrial design law, and potentially others. These rights allow their owners to exclude competitors from "making, using, offering for sale, or selling" an invention for a limited period of time. The plant variety protection (PVP) for plants was first available in 1970, but for hybrids beginning only in 1994. Conversely, patent protection for plants first became available in 1985, and firms used both patents and PVP system for some years. Although cost of utility patents is higher, PVP allows farmers' reuse of seed (although not an issue for F₁ hybrids) as well as open breeding access. In recent years patents have clearly become the form of choice. Pioneer Hi-Bred Inc., A DuPont Company holds more patents and PVP certificates than any other company (Lesser, 2005).

The investment in an innovation, such as herbicide tolerance through plant biotechnology, is often large including higher regulatory costs. However, an incentive for private-sector innovation in plant biotechnology for crop improvement is that the return of the investment is expected to be higher since the marginal cost of producing additional improved seed is very small (Acquaye and Traxler, 2005). The IPR has provided innovating firms with some monopoly power to set a price higher than marginal cost in the seed market (Falck-Zepeda *et al.*, 2000).

The high transaction cost of tracking down and licensing the multiple patents required to be able to commercialize the products is a concern. For example, DeKalb's patent claiming transgenic Basta-resistant corn delayed Holden's launch of Basta-resistant corn for at least a year (Pray and Naseem, 2005). Excessive patenting upstream could result in increased transaction costs for downstream innovators, slowing research activities. The patent holders could extract licensing fees or royalty payments from downstream innovators. Therefore, it will increase costs for downstream innovators to divert scarce research resources from product development to "reinventing the wheel" in order to get around existing roadblocks (Pray and Naseem, 2005). After the first regulatory release for planting a genetically engineered crop was granted in 1983, plant biotechnology research

increased very rapidly in the late 1980s and 1990s in response to the technological opportunities offered by the breakthroughs of cellular, molecular, and genomic biology, and stronger IP rights. A relatively small number of firms own key IPRs for the development of agricultural products through plant biotechnology.

To avoid higher costs of licensing and royalty payments and to facilitate successful commercialization of new complex biotechnology products, industry responded with aggressive unprecedented consolidations of the agricultural plant biotechnology and seed industries in the late 1990s so that enabling intellectual properties were owned by the same company. After the industrial consolidation, just six big firms—US firms Dow, DuPont, and Monsanto and European firms BASF, Bayer, and Syngenta, controlled over 40% of the private-sector agricultural biotechnology patents issued in the United States through 2000. DuPont and Monsanto hold the largest corporate portfolios, with 13% and 14% of the industry's count of US patents, respectively, followed by Syngenta with 7%, Bayer with 4%, and Dow with 3% (Graff *et al.*, 2003). The six big firms also account for over 80% of transgenic crop field trials for regulatory release in the United States. It is a clear indication that the research being done has become quite concentrated. Subsidiary firms contributed 70% of the patent stocks of the "Big Six", although parent firms contributed better patents by one measure of patent quality (King and Schimmelpfennig, 2005).

There are some concerns of IPRs owned largely by few private firms. Farmers are concerned that the monopoly power of agricultural biotechnologies may set a higher premium price for the improved seeds. Meanwhile, too many patents and too much concentration in the Big Six may slow down research for new biotechnology development. The IPRs could raise the bar for new entry of agrobiotech business. Japanese companies have been forced out of this arena because of difficulties reaching a reasonable deal for the key promoters, markers, and transformation technology with Monsanto and others (Pray and Naseem, 2005).

The IPRs in plant transformation, as an example, illustrated the relationship between the public and private sectors and the history and impact of the IPRs on plant biotechnology.

Most plant transformation technology, including soybean transformation, came out of research conducted at public-sector labs. The major private biotech firms also contributed to the development of these techniques and now own (or have exclusive licenses to) all of the major technologies (Pray and Naseem, 2005). Monsanto, the Max Planck Institute, University of Leiden (owned by Mogen/Zeneca now Syngenta) and The Washington University group applied for patents on *Agrobacterium*-mediated transformation methods in 1983, but they were not issued until almost 17 years after the application due to interference proceedings in the United States. The Max Planck Institute in Europe eventually won the patent. Japan Tobacco holds the patent on using *Agrobacterium* to transform monocots, Cornell holds the patent on the Helium Biorad particle that was exclusively licensed to DuPont, and the patent for the electric discharge particle gun is held by Agracetus (now owned by Monsanto).

Lawsuits can easily cost millions of dollars to litigate. In 2002, Syngenta claimed that Monsanto and Delta and Pineland infringed their patents on *Agrobacterium* transformation of dicots such as cotton and soybeans, and required them to pay royalties on their *Bt* and herbicide dicot crops or to stop selling them (Syngenta, 2002). Monsanto then launched countersuits, leading this to become a long, expensive battle that was not settled for several years. The difficulty of getting access to the whole package of plant transformation technology appears to have kept some firms from entering the plant biotechnology business. On the other side, the net impacts of patents have stimulated research on these tools that has led to major increases in their technical efficiency. The cost of plant transformation has also dropped dramatically—although possibly much more for the patent holders than the licensees (Pray and Naseem, 2005).

Internationally, the public sector has generated 24% of the IP in agricultural biotechnology. The largest public-sector patent holders are the University of California and the USDA, with 1.7 and 1.2%, respectively (Graff *et al.*, 2003). Public-sector technology transfer has several major goals: bringing the benefits of public research and development to potential users, finding innovative ways to fulfill agency missions in an era of relatively scarce resources, influencing the direction of

technology development, and enhancing research funds through licensing revenues (Rubenstein and Heisey, 2005).

Public funding for research emphasizes the discovery of a wide spectrum of previously unknown gene functions and private research focuses on application-driven research in a more narrow range of established product lines (Graff *et al.*, 2003). We shall see the continued trend of cooperation between public and private sectors in working toward the development of IPR.

3.4.2 Industrial perspectives

Transgenic product development through plant biotechnology research is not a single activity but a sequence of activities that covers the continuum from product concept, gene discovery, transformation, greenhouse and field evaluation, transgenic event and line selection, breeding into commercial varieties with transgenic trait, field production, marketing, and post marketing to regulatory approvals and biotechnology acceptance. The process of producing a transgenic variety uses public knowledge, proprietary knowledge, technology protected by intellectual property, money, and other inputs. Scientists in a research program start with basic knowledge about plants, pests, the environment, and market needs. To develop transgenic plants, they insert promising genes and promoters into available plant lines using plant transformation technology. Once the plants have been transformed, they are first tested in greenhouses. The varieties that survive this round of testing are then tested in fields. Before firms or research institutes can conduct field trials, they must obtain permission from USDA-APHIS. Under the authority of the Federal Plant Pest Act, APHIS regulations provide procedures for obtaining a permit or providing notification prior to introducing a regulated article 1 in the United States. Firms or research institutes that are conducting research to develop genetically modified organisms (GMOs) must apply to APHIS for a permit or notification 2 to conduct field tests (also known as a “release into the environment” permit).

To obtain legal approval to use a new transgene in commercialization, the firm must apply to the USDA for deregulated status. If the USDA

grants the application, the new plant is no longer a regulated article and can be treated as a nontransgenic plant for commercialization purposes. Meanwhile, the scientific community will continue to weigh the risks and benefits of plant biotechnologies, realizing that there may never be enough evidence to ensure zero risk. Only data will reveal tolerable levels of environmental risks on a case-by-case basis (Gewin, 2003).

Herbicide resistant soybean developed through plant biotechnology demonstrates the benefits that can be obtained in soybean production. More emerging plant technologies will be applied to further improvement of soybean to meet needs in the food, feed, industrial use, and energy businesses. Despite all the opportunities, transgenic soybeans face numerous challenges. Every transgenic product is subject to extensive regulatory scrutiny and will face many requirements of field research and data for biosafety assessment. From gene discovery to the launch of a transgenic product, regulatory approvals can reach \$44–88 million US dollars over 8–10 years (Monsanto data; Dill, 2005). Given the commitment necessary to commercialize these products, timely technology introductions are the keys to acceptance by the commodity industry and public. Sometimes, it is difficult for developers to earn sufficient returns on research investment for many biotech traits that could be beneficial for producers, processors, and consumers.

At least six commercial milestones have been identified that should be met before launching a product. The milestones include: (1) regulatory approvals in the United States, Canada, and Japan, (2) approvals or marketing arrangements in place in major export markets, (3) grain handling, sampling, and detection methods implemented, (4) comprehensive stewardship programs in place, (5) quality varieties that meet end user need, and (6) buyers identified (Dill, 2005).

To accelerate the development of transgenic products, Bradford *et al.* (2005) proposed that the regulatory emphasis should focus on the phenotype of the transgenic products as the conventional breeding products. This can save a lot of efforts in characterizing the transgene in the transgenic crops. They suggested elimination of event-specific regulation. With a deeper understanding of the transgenic products, the expensive and time-consuming regulatory process

may be simplified while still acquiring the key risk assessments.

3.4.3 The challenge of global biotechnology acceptance

Although more than one billion acres of transgenic crops, including glyphosate-resistant soybeans, have been planted globally since 1996 (James, 2006), global biotechnology acceptance is a challenge. To improve the regulatory system for food and environmental safety, the questions relating to environmental monitoring, traceability, and labeling for risk assessment can be addressed. Since 1998, the European Union has imposed a moratorium (9433/1/99, 90/220/EEC) on new approvals for the release of GMOs. The risk assessments include evaluations of both direct and indirect, as well as short- and long-term effects, plus the uncertainty at various levels (2001/18/EC; Tencalla, 2006). One in two Europeans, polled in the latest Eurobarometer survey published in June 2006, believes biotechnology will improve quality of life. Most Europeans are in favor of medical (red) and industrial (white) biotechnology, but they are still mostly skeptical about agricultural (green) biotechnologies (Gaskell *et al.*, 2006).

Regulations covering the traceability and labeling of GMOs and the traceability of food and feed products produced from GMOs came into force in April 2004 in the EU. According to these, foods produced from biotechnology products must be labeled. The threshold for labeling measurable incorporation of GM material is 0.9% in any ingredient in the food product (Madsen and Sandoe, 2005). Since 2003, new rules and guidelines are in place to address these risk assessment questions, but the system is still moving forward very slowly. The United States, Canada, and Argentina launched an official complaint to the World Trade Organization (WTO) against the EU regarding the technical barrier for the agricultural commodity in May 2003. Such unresolved issues caused a deeper economic analysis of some trade-related consequences of GM crop adoption (Sobolevsky *et al.*, 2005).

An identity preservation system for agricultural commodity could be used to ensure that GM

and non-GM products are segregated along the production, marketing, processing, and distribution chain in countries requiring product stream segregation. However, US farmers growing conventional soybeans could spend \$19.8 per metric tons, or 11% of the price received (Sobolevsky *et al.*, 2005), if required to segregate GM products. When growing transgenic crops that require identity preservation, the high cost of segregation may negate most of the welfare gain and make the earnings almost equal (Sobolevsky *et al.*, 2005). For international trade, the soybean imports alone in China could total about 13.98 million US dollars in 2005, and for some regions in Brazil, the costs could be close to 9% of a shipments product value (www.agritrade.org).

In addition to the political and trade impacts on the global acceptance of plant biotechnology, public concern is also an important factor for biotechnology acceptance. The public seems to have an agenda that goes beyond risks to human health and the environment. A major concern here is that GM crops, including herbicide- and insect-resistance traits, are primarily useful to the farmer and only indirectly useful to citizens. Concerns about socio-economic effects (industrial patents, monopolization, and globalization) and moral issues (sustainable agriculture through organic farming, freedom of customer choice, and unnaturalness of biotechnology derived products) seem to add to the opposition (Madsen and Sandoe, 2005; Gaskell *et al.*, 2006).

Public perception about risks can be explained by the social psychology model (Slovic, 1987). Three factors are the primary determinants of perceived risks: dread risk (defined as lack of control, dread, catastrophic, fatal consequences), unknown risks (defined as hazards judged to be undetectable, unknown, delayed harmful consequences), and the number of people exposed to the risks. The risks from RRTM crops are associated with two technologies—DNA technology and pesticides. According to the above model of social psychology, both of these technologies are viewed as high on the risk scale from a lay point of view. Customers cannot control the technology because biotech products are visually undetectable. Many people are concerned that GMOs could have unknown irreversible long-term effects on humans and biodiversity, however, there is no evidence supporting the fears thus far.

Trust is created rather slowly, but may be destroyed in an instant, and negative events are far more likely to have a powerful effect on trust than positive events. Distrust tends to perpetuate distrust (Madsen and Sandoe, 2005). The first introduction of Flavr Savr delayed-ripening GM tomato in United Kingdom in 1996 was largely successful. However, a multiple wave of food scandals (Mad Cow Disease, *E. coli* 157, etc.) generated a context of distrust toward policy makers, industry, and scientists. This unease may translate into the generalized “zero risk” approach toward new innovations, which is experienced today in some EU countries with regard the plant biotechnology (Tencalla, 2006).

The potential for rejection of products derived from biotechnology in the EU and other countries has caused growers, millers, grain processors, and others to hesitate in the adoption of plant biotechnology. However, no scientific advisory panel in any country has confirmed any concern as to the safety of biotechnology-derived products that have been evaluated (Dill, 2005). It is unfortunate that political, trade, and economic issues rather than scientific data can negatively influence plant biotechnology acceptance in the EU and the rest of the world.

Developing countries accounted for 40% of the global biotech crop area in 2006, equivalent to 40.9 million hectares (James, 2007). However, the common challenges in the developing countries are establishing biosafety laws and regulations, building capacity to evaluate and approve the release and import of transgenic products, and managing the IPR. In developing countries, the public sector's scientific contributions occurred far upstream, or were missing altogether. Paraguay is a striking example. In 2005, it had the sixth largest GMO area in the world, yet it has no biotechnology research capacity. It seems likely that while in the short term technology can be accessed without these capacities, future use of GMOs will be limited (Traxler, 2006).

Two international fora are also involved in biosafety, the Cartagena Protocol on Biosafety (BSP) (<http://www.biodiv.org/biosafety/default.aspx>) and Codex Alimentarius (<http://www.codexalimentarius.net/web/index.en.jsp>). Codex, which establishes international quality and food safety standards, is currently having discussions on transgenic traceability and labeling. The

BSP, which establishes requirements for the movement of “living modified organisms” (LMOs) between countries, is in the process of setting forth documentation requirements for commodity shipments of LMOs for food and feed purposes. The United States is not a party of the BSP. Requirements relating to documentation of the presence of LMOs in shipments of products in international trade provide an additional cost and challenge for US exporters.

The scientific and technical success of biotechnology products is the first step toward commercialization and benefits to all human beings. More work toward a broader approach, taking into account the politics, economics, society, and international trade issues, is needed for the acceptance of plant biotechnology as a cutting-edge innovation for plant improvement. Some of these factors are nonscientific in nature, and cannot be addressed from a scientific/technical perspective alone (Madsen and Sandoe, 2005).

There are many stakeholders of plant biotechnology products. Engagement of all the stakeholders in addressing the value judgments, uncertainties, and limitations involved in the risk assessment is likely to be the best way of avoiding public distrust (Madsen and Sandoe, 2005). Public education on scientific risk assessment must be presented in a way that is easy to understand. Industrial stakeholders will need to work together with biotechnology stakeholders to help the general public understand the impact of additional regulatory proposals and the safety procedures and national regulatory systems that are already in place.

Industry in plant biotechnology must take scientific, societal, ethical, environmental, and cultural impacts into consideration when designing and commercializing products. In 1999, DuPont formed an independent and prestigious panel of individuals from around the world “to guide our actions, help us create positions on important issues, and guide and challenge us in the development, testing, and commercialization of new products based on biotechnology.” The Bioethics Guiding Principles were established in 2003 to show their commitment to a comprehensive stewardship approach to biotechnology and the use of this science to help build a sustainable future (http://www2.dupont.com/Biotechnology/en_US). The use of biotechnology

still has room to grow in developing countries, particularly Africa, and building the capacity for the market in these countries is largely dependent on major players in the industry.

The latest European survey on biotechnology showed that 52% of the 25 000 people polled believed biotechnology will improve their quality of life, which indicates that the optimism about biotechnology has increased since 1999 after a period of decline. The survey also shows a growing level of trust in university and industry scientists at 73% and 64%, respectively (Gaskell *et al.*, 2006). With improved biotechnology products for the consumer’s benefits, a trusted regulatory system, solid science-based benefits and risk assessment, and a responsible product stewardship from premarket to postmarket, the benefits of plant biotechnology will reach more people in the world in the future.

3.4.4 Economic impacts of transgenic soybean

The annual economic value of soybean production in the United States is in the range of 12.6–18 billion US dollars during 2001–2005 (Table 4) (www.nass.usda.gov). Yield is vitally important for soybean producers. Soybean prices have trended downward since 1980. Although prices have been declining, per-acre income from soybeans has increased due to yield improvements and farmer programs that have helped increase net farm income (Soper *et al.*, 2003).

Although soybean yields and production in the United States have increased steadily, the United States share of world soybean production has been

Table 4 US soybean production and economic value (USDA-NASS)^(a)

	2005	2004	2003	2002
Acreage (Million acres)	72.1	75.2	73.4	74.0
Yield (Bushels/acre)	43.3	42.2	39.0	38.0
Price (US \$/bushel)	5.5	5.7	7.3	5.5
Value (Billion US \$)	16.9	17.9	18.0	15.3

^(a) Source: USDA-NASS

declining. In 1991, US soybean production was approximately 2 billion bushels, which was 50% of the world's production. In 2002, production climbed to nearly 3 billion bushels but only accounted for 43% of the world's production. Brazil has massive growth potential in agriculture due to the availability of virgin lands in its vast interior. Under the right economic circumstances, much more of that land could be converted for soybean production. Brazilian soybean producers have proven to be remarkably competitive in terms of relative production costs, and Brazil's average soybean yield has exceeded the United States average in some years (Ash *et al.*, 2006).

Global soybean trade is predicted to rise nearly 4% annually to 97 million metric tons in 2013–2014, compared to 70 million metric tons in 2004–2005 (www.ers.usda.gov). China is the world's largest importer of soybean, with the current United States share of that market at 40%. Increased world soybean production will come mainly from Brazil, because of its available land for cultivation. Ash *et al.* (2006) showed that soybean exports from South America have exceeded US exports since 2002/03, including 2004/05 despite a record US harvest and export volume. Brazil and Argentina have each surpassed the United States in soybean meal and soybean oil exports. However, once Brazil's planting areas are completely opened; there will remain little arable land to develop for global soybean production. Therefore, higher yields will be needed to feed a growing world population. The movement to an enriched diet globally, in the developing world in particular, is putting more pressure on the soybean meal market.

Glyphosate-resistant RR soybeans became available to US growers in 1996, and within 10 years, 89% and 98% of the soybeans grown in the United States and Argentina, respectively, were RR varieties. The economic benefits of the diffusion of transgenic soybean have been widely shared among farmers, industry, and consumers even though delivery has been through the private sector. Transgenic soybean has had a favorable environmental impact by facilitating reduced pesticide use and the adoption of conservation tillage (Traxler, 2006).

Yields of RR soybeans are not significantly different from yields of conventional soybeans in either the United States or Argentina. The

farm level benefits of RR soybeans are generated primarily through reduced herbicide, tillage, and management costs. Many farmers switched to low-till or even no-till cultivation practices after the adoption of RR soybeans. Machinery and labor costs are also lower due to the reduced time needed for harvesting (Qaim and Traxler, 2005). Analysis shows that there have been substantial net economic benefits at the farm level amounting to a cumulative total of \$27 billion (Brookes and Barfoot, 2005).

Farmers, seed firms, and consumers in Argentina and the United States had significant welfare gains that increased as RR soybean adoption increased. In 2001, RR soybeans created more than 1.2 billion dollars, or 4% of the value of the world soybean crop, in economic benefits at the global level. The largest share of these overall benefits went to soybean consumers, who gained \$652 million (53% of total benefits) due to lower prices. Soybean producers received net benefits of \$158 million (13%), and biotechnology and seed firms received \$421 million (34%) as technology revenue (Traxler, 2004, 2006). In 2004, the \$4.14 billion additional farm income generated by RR soybeans is equivalent to adding 9.5% to the value of the crop in the GM-growing countries, or adding the equivalent of 6.7% to the \$62 billion value of the global soybean crop. These economic benefits should, however, be placed within the context of a significant increase in the level of soybean production in the main GM-adopting countries. Since 1996, the soybean area and production in the leading soybean-producing countries such as the United States, Brazil, and Argentina increased by 56% and 66%, respectively (Brookes and Barfoot, 2005).

Even though all of the transgenic RR soybeans used in Brazil and Argentina are the result of technology spillovers from US private firms, the benefits have been widely distributed among the private firms, farmers, and final consumers. In 2001, farmers in Argentina were receiving a surplus of more than \$300 million compared to the US farmers who received a surplus of \$145 million. Although the RR area in the United States is larger than in Argentina, net producer surplus has been larger in Argentina since 1999 because the share of adopting farmers in Argentina exceeds the share in the United States. In Argentina, total variable cost of production is about 8% ($\$21 \text{ ha}^{-1}$) lower

for RR soybeans than for a conventional crop because of weak intellectual property protection and the widespread use of farm saved and black market seeds. Farmers in developing countries have much to gain when they are given access to suitable foreign technologies (Traxler, 2006). Evidence from China (Pray *et al.*, 2002), Argentina (Qaim and Traxler, 2005), Mexico (Traxler *et al.*, 2003), and Romania (Brookes, 2005) suggests that small farmers have had no more difficulty than larger farmers in adopting the new technologies. This suggests that the monopoly position engendered by intellectual property protection does not automatically lead to excessive industry profits.

RR soybean has clear environmental benefits. According to the international classification of pesticides, glyphosate belongs to toxicity class U, "active ingredients unlikely to present acute hazard in normal use." Adoption of RR soybeans led to an increase in the use of a relatively harmless herbicide and a significant reduction in the use of more hazardous herbicides (Traxler, 2006). Since 1996, the use of pesticides was reduced by 172 million kilograms (a 6% reduction), and the overall environmental footprint from GM crops was reduced by 14%. The volume of herbicide use on GM soybeans decreased by 41 million kilograms since 1996 (a 4% reduction), and the overall environmental footprint decreased by 19% (Brookes and Barfoot, 2005). The introduction of the glyphosate-resistant technology has caused a significant reduction in the price of all major herbicides for soybean. It has been estimated that these price reductions have saved soybean growers between \$216 and \$307 million per year in weed control costs (Gianessi, 2005).

Meanwhile, 53% of US soybean growers reported making fewer tillage passes through their field with an average reduction of 1.8 tillage per hectares. RR soybeans have enabled growers to eliminate \$385 million per year worth of tillage in their fields (Gianessi, 2005). No-till helps to preserve the soil texture and reduces the risk of wind and water erosion, with concomitant positive environmental effects. Overall, the number of machinery hours is reduced by 20%, and fuel savings are almost 10 l ha⁻¹ (Traxler, 2006). The adoption of no-tillage farming systems reduces cultivation fuel usage by 36.6 l ha⁻¹ compared with

traditional conventional tillage and 16.7 l ha⁻¹ compared with the average of chisel plough/disk tillage. In turn, this results in reductions of carbon dioxide emissions of 98.8 kg ha⁻¹ and 45.0 kg ha⁻¹, respectively (Brookes and Barfoot, 2005). Furthermore, the use of no-till and reduced-till farming systems utilize less plowing, increasing the amount of organic carbon stored or sequestered in the soil in the form of crop residue. This carbon sequestration reduces carbon dioxide emissions to the environment. The additional soil carbon resulting from reduced tillage with GM crops accounted for a reduction in 9.4 billion kilograms of carbon dioxide emissions in 2004. This is equivalent to removing nearly 4.7 million cars from the roads for a year (equal to 19% of all registered cars in the United Kingdom) (Brookes and Barfoot, 2005).

The new wave of value-enhanced crops or quality traits using plant biotechnology includes those plant varieties that have one or more modified output characteristic, adding end user value to the commodity. These output traits have the potential to add momentum to the agricultural biotechnology industry and to enhance productivity worldwide. The high oleic soybeans have been introduced commercially in the United States, US biosafety field trial data indicate that product quality traits accounted for 18% of all US trials in 2005 (Traxler, 2006).

As illustrated by the RR and high oleic soybeans, biotechnology and molecular farming have exhibited their great power in advancing the development of novel soybean products. Transgenic crops such as RR soybeans significantly benefit growers and permit more efficient use of renewable resources such as land, water, and soil nutrients. Furthermore, transgenic crops such as high oleic soybeans significantly benefit human life by providing more nutritious foods, desirable feeds, and valuable industrial products. With increased awareness of the benefits and wider distribution of existing and new transgenic soybeans, soybean scientists will further contribute to the public acceptance of plant GMOs. New transgenic soybean products with stacking traits can further benefit human life, environment preservation, and global economic advancement. Once biotechnology has gained widespread acceptance, the miracle soybean will fully express its beauty.

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Oilseed Brassicas

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Taxonomically, *Brassica* is a diverse genus within the Cruciferae family containing over 100 species, some of them including several major oilseed species such as *B. napus*, *B. rapa* (syn. *B. campestris*), and *B. juncea*. Other two lesser-known oilseed species are *B. carinata* and *B. nigra*. *B. napus*, also known as rapeseed, is the most widely cultivated oilseed in many parts of the world followed by *B. juncea*, which is known as brown mustard or Indian mustard. *B. rapa* is grown on a very limited area in countries like Canada, Finland, China, and India. The word “rape” in rapeseed comes from the Latin word “rapum” meaning turnip. Turnip, rutabaga, cabbage, cauliflower, brussel sprout, mustard, and many other well-known vegetables are relatives of the three major oilseed Brassicas. The term “rapeseed and mustard” includes these three major oil species (*B. napus*, *B. juncea*, and *B. rapa*).

The cultivated oilseed species *B. juncea*, *B. napus*, and *B. carinata* originated in nature from interspecific crossing between the diploid progenitors followed by chromosome doubling of the interspecific hybrids as hypothesized by U (1935) and shown in Figure 1. The relationship between the diploid species and tetraploid species presented in Figure 1 is also called as the U-Triangle.

Brassica crops are among the oldest cultivated plants known to humans with written records dating back to 1500 BC (Prakash, 1980). Archaeological evidence of their importance dating back to 5000 BC has been reported (Yan, 1990). Ancient Sanskrit writings dating back to 2000 BC describe *B. juncea* and *B. rapa*. *B. napus* is thought to have originated in Europe especially in Mediterranean areas where *B. rapa* and *Brassica oleracea* overlapped (McNaughton, 1976). *B. juncea* originated in the Middle East (Prakash, 1980), where its diploid progenitors overlapped. *B. rapa*, which is diverse and widely populated in many parts of Europe and Asia, is thought to have originated in Europe.

1.2 Botany and Reproductive Systems

Flowers in the Cruciferae family have four bifurcated petals with varying degree of incision and deep yellow to pale yellow or cream color. There are six stamens in the flower. The anthers are lower than the stigma at bud stage, however, prior to flower opening, the filaments elongate and push anthers above or at the same level of stigma. Different degrees of self-pollination and outcrossing occur in different species of *Brassica*. For example, *B. rapa* and *B. oleracea* are largely cross-pollinated species due to self-incompatibility (SI); however, *B. napus* is largely a self-pollinated

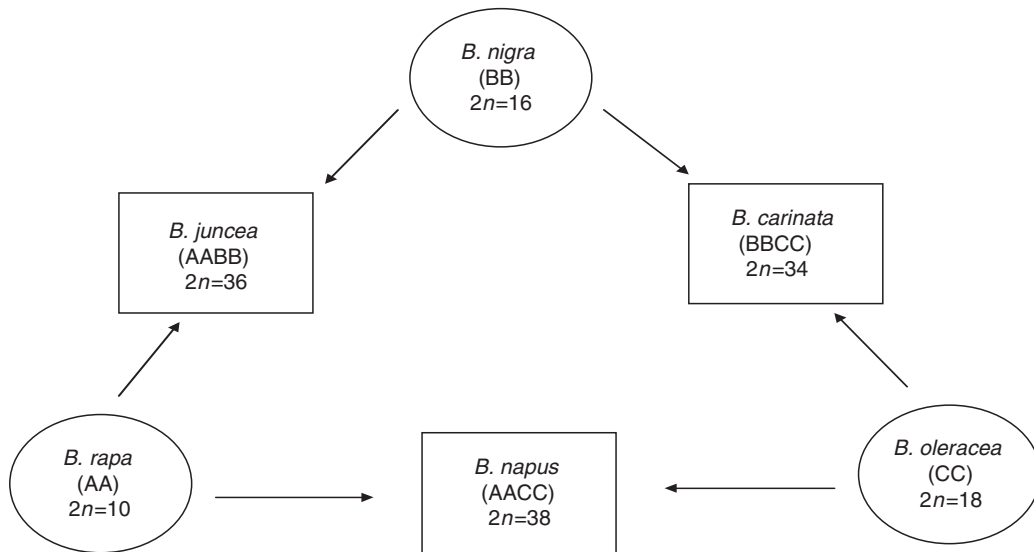


Figure 1 Genetic relationships in different *Brassica* species

species. Due to bright yellow open flowers, insects, especially honey bees can easily be attracted; therefore, there is a varying degree of outcrossing in *B. napus* depending on insect activities and wind. *B. juncea* is also a mostly self-pollinated species similar to *B. napus*. Yellow sarson, which belongs to *B. rapa*, is largely self-pollinated although *B. rapa* as a species is known to be self-incompatible and cross-pollinated.

1.3 Area, Production, Utilization, and Economic Importance

Rapeseed and mustard is the world's third largest oilseed crop after soybean and oil palm. In 2005–2006, the world produced 391 million metric tons of oilseeds, of which rapeseed accounted for 46.4 million metric tons. Total world cultivation of rapeseed and mustard grew steadily by two-and-a-half-fold from about 10 million ha in 1980 to slightly more than 25 million ha in 2005 (Figure 2a). However, in the same time period production increased almost fourfold from 10 million tons to 47 million tons. This increase in productivity has been the result of adoption of improved production technologies and adoption of improved, high yielding, disease and pest resistant varieties. The world's major rapeseed,

mustard, and canola producing countries in 2005 are listed in Table 1.

1.3.1 Transition from rapeseed to canola

Canada played a key role in transition from rapeseed to canola. Canola was an unknown term to many people around the world prior to the 1980s. Canola is not a botanical term but rather a generic term, which describes specific oil and meal quality standards for rapeseed. Canola refers to the seed of *Brassica* species (*B. napus*, *B. juncea*, and *B. rapa*) that contains less than 2% erucic acid in oil, and less than 30 $\mu\text{mole g}^{-1}$ of aliphatic glucosinolates in oil-free meal. Canadian plant breeders, Baldur Stephenson of the University of Manitoba, and Keith Downey of Agriculture Canada, played a key role in developing canola, transforming rapeseed to a major oilseed crop through improvements in its oil and meal quality.

Although in some parts of world, rapeseed and mustard oil has been used in human diets for thousands of years, its use in North America and industrialized world was limited until recently. *B. napus* has been grown in parts of Europe for many centuries and its oil was used in lamps as well as for cooking. Rapeseed and mustard oil is still being used extensively for cooking in India

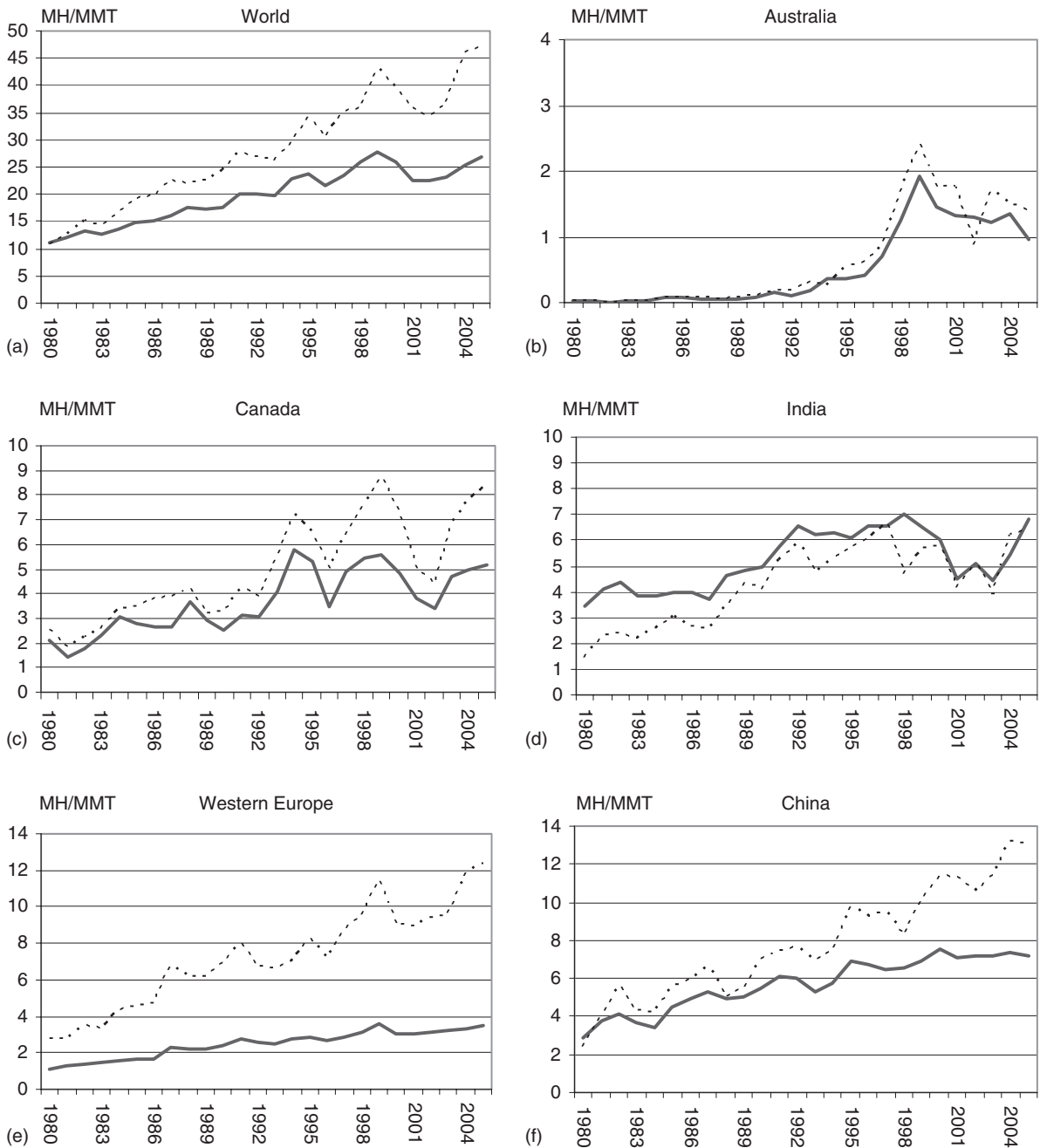


Figure 2 Twenty five years of canola/rapeseed harvested area in millions of hectares (MH) represented by solid line and production of grain in millions of metric tons (MMT) represented by dotted line in (a) World, (b) Australia, (c) Canada, (d) India, (e) Western Europe, and (f) China

Table 1 World's major rapeseed mustard and canola producing countries in 2005^(a)

Country	Area or production (million ha)	Production (million metric tones)	Yield ha ⁻¹ (metric tones)
China	7.22	13.05	1.81
India	6.80	6.40	0.94
Canada	5.15	8.45	1.64
Western Europe	3.52	12.32	3.50
Eastern Europe	1.74	3.47	1.99
Australia	0.96	1.41	1.46
World	25.79	47.16	1.76

^(a)Source: FAOSTAT

and China. When steam powered engines were invented, it was found that rapeseed oil was a better lubricant than other oils, because it would cling to steam washed metal surfaces better than other lubricants. For this reason, its use in marine engines was widespread prior to and during World War II.

During the Second World War, there was an acute shortage of rapeseed oil for the allied navy vessels because most of the supply came from Asian and European sources. The allied navy decided to source rapeseed oil from a safer place—Canada. Prior to the war, rapeseed was grown in Canada experimentally at various research farms. These experiments showed that it could be grown very well in Canada. An immigrant farmer from Poland, who settled in Saskatchewan, introduced *B. campestris* (now known as *B. rapa*) in his garden. He sold it to his neighbors and friends when rapeseed cultivation started to increase in Canada due to war demand. Because of its Polish origin, this species later was known as “Polish” rapeseed. Several tons of seeds of rapeseed (*B. napus*) originating from Argentina was purchased by some US companies and later introduced into Canada. Therefore, *B. napus* was later known as Argentine rapeseed. During World War II, cultivation of rapeseed peaked at about 18 000 ha. After the war, cultivation dropped dramatically to a few hundred hectares. Other oils were in plentiful supply; so there was no demand for rapeseed oil. Since rapeseed was very well suited for Western Canadian conditions, the National Research Council and some oil processors were interested in developing rapeseed oil for edible uses.

Rapeseed oil has a high proportion of eicosenoic and erucic acids in its oil, which are not essential to human growth. Also, erucic acid is poorly digested in human intestine, therefore, nutritionally rapeseed oil had little value as compared to other oils. During the mid-1960s, feeding experiments were conducted with high erucic acid rapeseed (HEAR) oil as a source of fat. In some of these experiments, it was discovered that certain experimental animals developed lesions in their heart tissues after prolonged feeding with high erucic acid feed. Although, the effect of a high erucic diet on human and experimental animals has remained controversial, Canadian plant breeder Baldur Stefansson at the University of Manitoba and Keith Downey at the Agriculture Canada, Saskatoon, started developing low erucic acid rape varieties. The first low erucic acid rapeseed variety Oro was released in 1968 from the University of Manitoba program. During the mid-1960s, rapeseed production increased to about 400 000 ha. In 1967, the Rapeseed Association of Canada was founded.

After oil extraction, leftover rapeseed meal contains protein and sulfur-containing substances called glucosinolates. Glucosinolates, when broken down by an enzyme called myrosinase, produce isothiocyanates, thiocyanates, and toxic nitriles. These substances are toxic to nonruminant animals, and they give bitter taste and reduce palatability to the meal. The rapeseed meal when fed to brown egg-laying hens caused a fishy smell in eggs. Therefore, reduction of total glucosinolates was the only practical solution to these problems. During the late 1960s and early 1970s, breeding efforts to develop low glucosinolate-containing rapeseed were started in Canada. The first low erucic acid and low glucosinolate variety, Tower, was released in 1974. It was demonstrated that the oil and meal produced from this variety was superior to rapeseed oil and meal. Other low erucic acid and low glucosinolate rapeseed varieties followed. During this time, the double low (low erucic acid and low glucosinolates) rapeseed varieties were gaining popularity in Canada. In 1978, the term “Canola” was trademarked by the Western Canadian Oilseed Crushers’ Association (now the Canadian Oilseed Processors Association—COPA) to differentiate the superior low erucic acid and low glucosinolate varieties and their products from the older rapeseed varieties.

During 1979, 3.4 million ha of canola was seeded in Western Canada. People around the world noticed the developments in Canada. Japan started to import large quantities of canola grain for its domestic use. In 1980, the canola trademark was transferred to the Canola Council of Canada. Canola revolution had already begun in Canada.

1.3.2 Canola and rapeseed production in major growing countries

1.3.2.1 Canada

Rapeseed, when introduced in Canada in early forties had less than 18 000 ha of cultivation. During the sixties, after significant breeding efforts toward the development of high-quality canola varieties, area of production increased steadily. Rapeseed became a very important crop in Western Canada. In 1970, Canada cultivated 1.64 million ha of rapeseed, and produced 1.64 million tons of rapeseed averaging about a ton per hectare. In 2005, the cultivation increased to 5.15 million ha, producing 8.45 million tons of canola and averaging about 1.64 t ha⁻¹ (Figure 2c). The average yield has increased during the last 10 years due to the introduction of high-yielding canola varieties resistant to disease and various herbicides.

Canada was the first country in the world to introduce herbicide tolerant *B. napus* varieties in mid-1980s. Herbicide tolerant canola makes weed management easy. Herbicide tolerant canola can be sprayed with the herbicide for which the tolerance is carried by the variety. All other weeds will be killed while plants of the herbicide tolerant (HT) variety will survive. A wild *B. campestris* mutant tolerant to atrazine herbicide was found in natural weed population (Beversdorf *et al.*, 1980). It was later discovered that this trait was inherited through cytoplasm and was introduced in commercial canola cultivars. The first atrazine tolerant canola variety OAC Triton was released in 1984. Because of negative impact of this cytoplasmic mutation on yield, vigor, and oil content, Triazine tolerant canola varieties were grown on limited acres before the development of other modes of herbicide tolerance. Later, three modes of herbicide tolerant canola (*B. napus*)

varieties: Glyphosate tolerant—called Roundup resistant, Glufosinate tolerant—called Liberty resistant, and Imidazolonone tolerant also known as Pursut Smart, Odyssey Smart, and later known as Clearfield canola were registered in 1995. Originally, sources for the first two modes of herbicide tolerance (Glyphosate and Glufosinate) were developed through genetic engineering while the third one (Imidazolonone) was developed through microspore mutagenesis. First time in 1996, the herbicide tolerant varieties were grown on limited acres in Western Canada, but within a short period of time, the herbicide tolerant canola varieties were widely adopted by Canadian farmers. By 2005, more than 90% of Canadian canola crop was seeded with one of the three types of the herbicide tolerant canola.

Up until 1995, Canadian canola included two species, *B. napus* and *B. rapa*, with roughly 70:30 ratio. *B. rapa*, being frost tolerant and earlier maturing than *B. napus* has been suitable for cooler areas of prairies, especially in Northern Alberta (Peace River area), Central Alberta in the foothills of Rocky Mountains, and Northern Saskatchewan where frost-free days are limited. Traditionally, *B. napus* has been higher yielding than *B. rapa*. With the introduction of herbicide tolerance technologies and heavy breeding effort in *B. napus*, yield gap between *B. napus* and *B. rapa* widened during the later part of nineties. Also, mid-early maturing high-yielding herbicide tolerant *B. napus* varieties and hybrids became lot more profitable than conventional *B. rapa* varieties, which caused severe drop in *B. rapa* acreages to the point that it is hardly grown in Western Canada.

The Canadian canola varieties, mostly spring type, are planted in late April and early to mid-May and are harvested in fall (late August to early October) depending on location and season. In 1985, canola oil was granted GRAS (Generally Recognized As Safe) status by American Food and Drug Administration. This development paved the way for increased interest in canola oil by the US food manufacturers and oil processors. Also, more and more reports were coming out from around the world about the nutritional value of canola oil. With the increased import of Canadian canola grain and oil, interest in canola cultivation began to increase in the United States where

winter canola varieties were tried in Kentucky, Ohio, Idaho, and Washington states and spring canola varieties were tried in Georgia, Alabama, North Dakota, Minnesota, and Montana. Due to several production related issues and disease and pest problems, canola production in the United States has remained restricted to 0.4–0.6 million ha only in Northern Plains (North Dakota, Minnesota, Montana, etc.)—where many of the Canadian bred spring varieties are suitable for cultivation.

1.3.2.2 Europe

The principal canola growing countries in Western Europe are France, Germany, the United Kingdom, Denmark, Sweden, and Finland. In Europe, the winter form of rapeseed is grown, which is planted in fall and harvested in late spring to early summer the following year. A very small proportion of spring rape is also planted in the United Kingdom, Sweden, Denmark, and Finland. Eastern and central European countries where rapeseed is planted are Poland, Czechia, Slovakia, Hungary, Russia, and Ukraine. Table 2 lists major European countries for 2005 where rapeseed/canola is produced.

The switchover from rapeseed to canola in Western Europe was completed in the mid-

eighties; a decade behind Canada, because of the fact that the breeding cycle in winter rape is much longer than spring canola. Although, the area of cultivation in European countries is less than in Canada, the total production is more than Canada (Figure 2e). Winter type crops generally yield higher than spring type crops and winter canola is no exception to that rule. As shown in Table 1, the average winter canola yield in Western Europe is 3.5 t ha⁻¹ compared to 1.64 t ha⁻¹ in Canada. Herbicide tolerance technology has not been introduced in Europe, mostly due to anti-GM (genetically modified) sentiment and regulatory hurdles.

1.3.2.3 Australia

The spring form of canola is planted in Australia during the fall (mid-April to early May) and harvested anywhere from mid-October (Western Australia) to early December (New South Wales). Canola was not a major oilseed crop until the mid-nineties. During the seventies, Canadian spring canola varieties were introduced in Australia and were not successful because of a virulent form of blackleg (*Leptosphaeria maculans*). Substantial improvement in disease resistance was achieved during the mid-1980s to early nineties and the acreage expanded along with the world canola demand in the nineties (Table 1; Figure 2b). Although, herbicide tolerant canola, mostly triazine and imidazolonone tolerant, have been in cultivation in Australia, the transgenic form of herbicide tolerant canola is not commercialized yet due to state moratoria on transgenic canola crop.

1.3.2.4 China and India

China and India are the two countries in Asia with a huge area planted under rapeseed and mustard. In China, most rapeseed varieties are *B. napus* type and are having semi-winter habit. China has already started to switch from rapeseed to canola type in recent years and has also made tremendous progress in yield and disease improvement. Rapeseed yield per hectare is high in China due to favorable rapeseed growing conditions (Table 1; Figure 2f) and also due to introduction of production technologies and

Table 2 Europe's rapeseed and canola producing countries in 2005^(a)

Country	Area (million ha)	Production (million metric tons)	Yield (Mt ha ⁻¹)
Denmark	0.112	0.342	3.06
Finland	0.077	0.106	1.37
France	1.226	4.486	3.66
Germany	1.343	5.052	3.76
Norway	0.007	0.011	1.69
Sweden	0.082	0.198	2.41
United Kingdom	0.593	1.902	3.21
Hungary	0.121	0.284	2.34
Poland	0.544	1.434	2.63
Romania	0.089	0.154	1.73
Russia	0.260	0.273	1.05
Slovakia	0.107	0.250	2.33
Ukraine	0.197	0.280	1.42

^(a)Source: FAOSTAT

development of high-yielding, disease-resistant varieties.

In India, a huge proportion of acres are planted to spring form of *B. juncea*. Traditionally, a huge area is planted under low rainfall, dry land where moisture stress compounded by disease stress reduces yield. Although, low erucic acid and low glucosinolates (canola quality) traits are available in *B. juncea*, transition to canola quality in Indian *juncea* crop has been very slow. Also, yield has been very low (Table 1; Figure 2d).

1.4 Traditional Breeding and Genetic Improvements

The breeding objectives fall into three major categories: (a) improvement in yield and agronomic traits, (b) improvement in oil and meal quality, and (c) improvement in disease and insect resistance. Overall, these improvements have increased productivity of oilseed *Brassica* crops around the world.

1.4.1 Improvement in yield and agronomic traits

Canola/rapeseed has undergone intense genetic improvements during the last 20 years because of tremendous investments in biotechnological research in public and private organizations as well as commercial plant breeding activities. *B. napus* has been the most amenable species when it comes to tissue culture response, transformation response, embryogenesis, and application of other DNA-based technologies.

Prior to the introduction of hybrid technologies in rapeseed and canola, pedigree selection has been widely used in developing open-pollinated rapeseed and canola cultivars. One of the main reasons why there was tremendous amount of private investments went in canola research from mid-eighties to mid-nineties was due to the fact that there was a possibility of developing commercial hybrids. Published reports indicated that there was tremendous heterosis available in rapeseed. The seeding rate was reasonably low so that farmers could purchase necessary quantity of hybrid seed and seed multiplication ratio was quite favorable for commercial increase

of seed. Also, there were several pollination control systems, which could be fully developed and used to produce commercial quantities of hybrid seed. Major seed companies saw the opportunities of creating value by combining hybrid performance with traits such as herbicide tolerance, disease and insect resistance, etc. During the last 20 years, hybrid production technologies were developed in *B. napus* and *B. juncea* and were applied in commercializing hybrid canola and rapeseed in Canada, Europe, China, Australia, and India.

In Europe, first winter canola commercial hybrid based on MSL system (Male Sterility Lembke) was registered in 1995 (Frauen and Paulmann, 1999). The MSL system is based on spontaneous mutant selected in the NPZ nursery in 1982. The female line based on MSL system produces pollen under certain environmental conditions making it possible to increase female seed for commercial hybrid production. All other *B. napus* lines restore the MSL female. The transfer of recessive male sterility nuclear mutant from one female line to other line is challenging for MSL system. The hybrids using other male sterility system have been commercialized in Europe since then.

In China, cytoplasmic male sterility (CMS) system called *Polima* was discovered (Fu, 1981). Since then, several other pollination control systems have been reported in China. Between 1992 and 2002, Fu's group released five *Polima cms* based oilseed rape hybrids. According to the Ministry of Agriculture of China, two of these (Huaza No. 3 and Huaza No. 4) were among the top 10 most widely cultivated rape varieties in China between 1999 and 2002. Another successful rapeseed hybrid Quinyou No 2 was widely grown in China which used Shann 2A as a male sterile line.

In Canada, two commercial hybrids, each using different pollination control system, *polima cms* and SI, were released in late 1980s and early 1990s. None of these hybrids was able to capture significant acres due to seed production limitations and only marginal performance advantage compared to the open-pollinated varieties. The *Polima* CMS system broke down and female line produced small amount of pollen under high temperature, therefore, seed production was challenging.

Plant Genetic Systems, Inc. developed a novel *B. napus* oilseed rape hybridization system through genetic engineering. The development of the MS1 and RF1 lines was based on recombinant DNA technology by the introduction of bacterial genes into the *B. napus* variety “Drakkar”. Nuclear male sterility of the first parental line results from the localized production of a ribonuclease (*barnase*) in a specific anther cell layer and at a specific stage in anther development. Fertility restoration in the hybrid line was obtained through insertion, in the second parental line of a gene coding for *barstar*, a specific inhibitor of the enzyme *barnase*. A gene conferring tolerance to the herbicide glufosinate ammonium was inserted in both lines, coding for phosphinothricin acetyltransferase, an enzyme that inactivates glufosinate ammonium through acetylation. Bayer Crop Science acquired PGS system through their acquisition of AgriEvo and commercialized high-performing Glufosinate (Liberty) resistant canola hybrids such as InVigor 5020, InVigor 5030, and InVigor 5070. These canola hybrids set new standards of hybrid performance in Canada and gained increased popularity due to their performance advantage.

INRA in France worked on OGU CMS system in canola and licensed to major companies in early nineties. OGU cytoplasm came from *Raphanus* and was discovered by Ogura (1968). Restorer gene for this sterility was transferred from radish to *B. napus* through interspecific crossing and backcrossing followed by translocation of radish chromosome fragment into *B. napus* genome. The resulting restorer line ended up being higher in glucosinolates. Further crosses and testcrosses revealed that the restorer gene was tightly linked to the gene responsible for high glucosinolates, which made it impossible to develop canola

quality commercial hybrids through OGU CMS system. Various groups, including INRA, spent tremendous effort in breaking an apparent linkage between the restorer gene and high glucosinolate content, and ultimately developed restorer lines, which could be used in commercial canola hybrid development. Pioneer Hi-Bred International, Inc. successfully developed low glucosinolate restorer source and commercialized the glyphosate (roundup resistant—RR) hybrid 45H21 in 2002, which was widely grown in Western Canada. Several Roundup resistant hybrids based on the NPZ-Lembke system have also been commercialized in Canada. By 2004, imidazolonone tolerant canola hybrids were also commercialized from Pioneer Hi-Bred and Advanta Seeds. Western Canadian farmers had wide variety of choices of hybrids with different herbicide tolerance. Although on an average yield basis, the first canola hybrids commercialized in the early and mid-1990s did not outperform open-pollinated varieties by a huge margin, the performance advantage of hybrids was enough in high-moisture environments that farmers realized the benefits of hybrid vigor. Farmers in southern Manitoba and parts of Alberta, therefore, started to adopt canola hybrids. After 2001, newer canola hybrids started to show a tremendous performance advantages over open-pollinated varieties. Results of 2006 Prairie wide Canola Variety Trial (PCVT), co-ordinated by Canola Council of Canada to evaluate commercial canola varieties and hybrids over 32 locations in western Canada, clearly demonstrated superiority of hybrids over open-pollinated varieties (Table 3).

Using the INRA-OGU CMS system canola hybrids have also been commercialized recently in Australia. In India, different male sterility systems are reported (Labana *et al.*, 1992) in *B. juncea*.

Table 3 Average yield expressed as % of control—46A65 for three sets of entries in 2006 Prairie wide Canola Variety Trials^(a)

Type of entries	Number of entries	Range of performance yield % of 46A65	Average performance yield % of 46A65
Hybrid	26	102–129	117
Open-pollinated	19	89–108	101
Synthetics	4	108–116	112

^(a)Source: Canola Council of Canada

To date, large-scale commercialization of Indian mustard (*B. juncea*) hybrids has not occurred in India. As hybrid canola breeding evolves, the diversity of male and female pools becomes very important and introgression of genetic variation from materials of different geographic origin becomes necessary to improve hybrid performance (Btruille *et al.*, 1999; Quijada *et al.*, 2004; Udall *et al.*, 2004).

Early maturing rapeseed and canola cultivars are important when the growing season is short due to weather conditions. In Canada, there are areas in Alberta and Saskatchewan, where frost-free days are very limited. Ten years ago, farmers in these areas used to grow short season *B. rapa* varieties, which matured before frost hit the crop. With the widespread *B. napus* cultivation in Canada, short season early maturing *B. napus* varieties are important in these areas. In Australia, especially western Australia and parts of New South Wales and Victoria under low rainfall conditions, early maturing canola is extremely important. Also, in India, early maturing short season varieties are extremely important in low rainfall areas. In central China, early maturing rapeseed is extremely important for crop rotation with rice and rapeseed. The crop must finish pod filling and be ready for harvest before it becomes too dry otherwise yield would be severely affected in late maturing crop. Usually, early maturing varieties are lower yielding. Degenhart and Kondra (1984) concluded that it was possible to develop early maturing high-yielding canola varieties. Earlier maturity has been one of the important objectives of canola breeders and early maturing *B. napus* varieties such as 44A89, Hudson, 44A04, Peace, 43A56, InVigor 5108, etc. in Canada; *B. napus* varieties such as Monty, Karoo, Drum, 44C73, 44C11, etc. in Australia; and *B. juncea* varieties such as GM-1, Agrni in India were commercially released.

Short plant height and good lodging resistance have been other two important objectives for most canola and rapeseed breeding programs and especially in Europe where winter rapeseed plants can grow very tall up to 2 m in height. Farmers routinely apply growth regulators to reduce plant height of winter rape in Europe. A winter rapeseed dwarf mutant gene called *RREIZH* (*Bzh*) was discovered at INRA in France and molecular markers were developed for this mutant (Foisset

et al., 1995; Barret *et al.*, 1998). This mutant has been utilized in development of commercial semi-dwarf hybrids such as Lutin, PR45D01, PR45D03, Belcanto, Saturnin, and Bambin in Europe. In China, another dwarf mutation called *ndf1* was discovered and inheritance of this mutant was worked out (Wang *et al.*, 2004). Although, short plant height is desirable in Western Canada, Australia, and India, plants do not grow as tall as winter rape in Europe; therefore, semi-dwarf hybrids are not urgently required. Shorter plants tend to stand well and do not lodge making swathing and harvesting operation easier. Lodging resistance is highly influenced by environment and requires recording of observations at many locations in order to characterize a variety.

Canola plants (*B. napus*) shatter and lose seeds if the pods are fully ripened and completely dry. To avoid shattering losses, farmers swath canola and mustard crop when crop is physiologically matured and still has some green pods. It is commonly known that *B. napus* is more prone to shattering than *B. juncea* and *B. rapa*. Although, shattering resistance is very important, it is difficult to screen this trait on large scale in breeding nurseries. Kadkol (1985) studied inheritance of shattering resistance in *B. rapa* and *B. napus*. Later, sequence characterized amplified region (SCAR) markers were developed for shatter resistance (Mongkolporn *et al.*, 1999).

In many parts of the world, rapeseed, mustard, and canola are grown in nonirrigated land relying totally on rainfed conditions. Sometimes during the growing periods, moisture and heat stress are encountered causing significant yield losses. Many times in Western Canada, spring canola crop goes through without rain for prolonged period. Canola plants are very vulnerable during flowering and may not recover from stress after the dry spell is finished. The drought stress reduces yield and oil content and can affect other quality traits. Canola breeders have always been indirectly selecting for consistent performance by testing plant material in varying environmental conditions. It is a well-known fact that *B. juncea* is drought and heat tolerant and can be used in transferring drought and heat tolerance to *B. napus*, however, screening for drought tolerance is complex and would require setting up of a special nursery or screening for other traits associated with drought (Niknam and Turner, 1999). *Sinapis alba* is considered a

drought tolerant species as well, which can be used in transferring the trait to canola.

1.4.2 Improvement in oil and meal quality

Canola grain has about 40–45% oil and 20–25% protein on a whole seed basis at 8.5% moisture. Canola breeders have been trying to increase oil and meal protein contents in canola over several decades. Oil content is highly influenced by environment, especially, availability of moisture and temperature during grain filling. In Canada, the harvested canola crop (Canada #1) over the years has seen slow and steady improvements in oil content (Figure 3) in spite of adverse weather conditions during 2002 and 2003. The improvements in oil contents in Canada would have been even greater if there were incentives for farmers to grow high-oil varieties. Canadian farmers are not paid for how much oil their canola crop produces. In Australia and some European countries the farmers are paid premiums above the defined base oil level. With the recent interest in bio-based fuel, canola oil has gained lot of attention from nonfood users. Increasing oil content in canola, therefore, became a very important goal.

Grami *et al.* (1977) observed high heritability for oil content and reported—0.8 correlation

coefficient value between oil and protein contents. Zhao *et al.* (2005) in a doubled haploid (DH) population derived from Chinese \times European winter rape cross, observed eight quantitative trait loci (QTL) with additive effect, and nine pairs of additive \times additive interactions explained 80% of the genetic variation for oil content. Unpublished research indicates that there is mid-parent and high-parent heterosis for high-oil content. Also, hybrids tend to finish and mature well compared to open-pollinated varieties resulting in higher oil than open-pollinated checks. Recently, the high-oil-producing hybrid 46P50, having 2.5% higher oil than the commercial hybrid check 45H21, has been released for commercial cultivation in Canada.

Canola oil possesses several good nutritional properties in addition to having low erucic acid. It is known to have the lowest total saturated fatty acid content (7.0% in total) among all vegetable oils. Clinically, it is proven that increased intake of fatty foods, especially high in total saturated fatty acids, increases the chance of blocked arteries in adult men and older women. Such blockage, medically known as atherosclerosis, can cause heart attack if not checked and treated. Canola oil, with the lowest total saturated fatty acids, is highly recommended by medical professionals to the high-risk patients and also to the general public. Canola oil is high (60–65%) in monounsaturated fatty acids (oleic acid), which is known to reduce

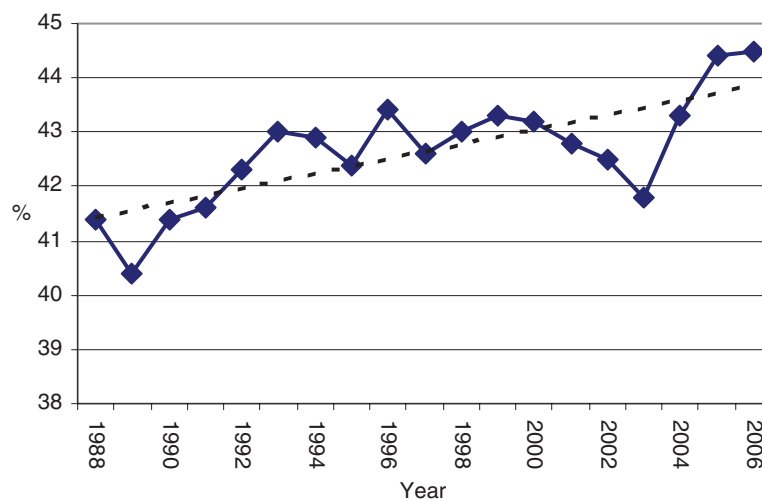


Figure 3 Oil contents (%) in Canadian canola crop (Canada #1) from 1988 to 2006 expressed at 8.5% moisture [Source: Canadian Grant Commission]

serum cholesterol and low-density lipoprotein (LDL) cholesterol level while not affecting high-density lipoprotein (HDL) cholesterol. The LDL cholesterol is known to be positively correlated with increased risk of heart disease. Canola oil has about 20–22% linoleic acid and about 10–12% linolenic acid. Both of these fatty acids are polyunsaturated fatty acids. Linoleic acid is considered an essential fatty acid because the human body can not synthesize it. Therefore, it must be consumed in the diet. Linolenic acid is converted into ω -3 fatty acids, which are also known to reduce the risk of heart diseases and increase the health of the nervous system.

Since canola oil is slightly higher in linolenic acid, reduction of linolenic acid without changing linoleic acid has been one of the objectives to improve oil quality. Low linolenic acid in canola oil makes canola oil stable, improving its frying properties, and also reduces or eliminates hydrogenation while processing canola oil to margarine. The use of chemical mutagens was applied to induce mutants those resulted in lines with low linolenic acid up to 5% (Rakow, 1973). Later these mutants were used in developing a commercial canola variety at the University of Manitoba, which had less than 3% linolenic acid (Scarth *et al.*, 1988). Jourden *et al.* (1996a) concluded that low linolenic acid in Stellar was controlled by two genes with additive effect. In order to facilitate selection of desired genotypes for low linolenic acid, molecular markers have been developed (Jourden *et al.*, 1996b; Barret, 1999). During the process of hydrogenation trans-fatty acids are produced. Recently, trans-fat has gained a lot of negative attention due to its association with cancer and heart disease. Also, new labeling laws in the United States require all food manufacturers to show the total amount of trans-fat and saturated fat on the labels of all packaged foods. Because of these developments, canola oil with low linolenic acid has been in high demand. Currently in Canada, Dow Agro Sciences has commercialized several NexeraTM high oleic and low linolenic acid canola varieties having Imidazolone tolerance. Cargill has commercialized VictoryTM series of low linolenic acid, open-pollinated canola varieties and hybrids with Roundup resistance. Commercial varieties of *B. napus* with low total saturates (<5%) are not yet available commercially.

Canola grain oil and protein are negatively correlated; therefore, increase in one would lead to a decrease in the other. However, recently, canola breeders have been able to increase both oil and protein simultaneously. Since canola is an oilseed crop, crushers derive most value from oil. Therefore, canola meal is a by-product after oil is extracted. Unlike soybean meal, which is high in protein, canola meal has about 38–42% protein and about 33% crude fiber. Therefore, it is sold at only 70–75% of the value of soybean meal. Also, due to its deficiency in some amino acids, such as lysine and methionine, it is blended in rations along with soybean meal. One advantage of canola meal over soybean is the higher level of rumen-bypass protein, which makes canola meal a preferred component in dairy rations.

Glucosinolates are sulfur-containing substances in meal, which reduce the nutritional properties of the meal. For 2005 and 2006, the total glucosinolates (whole seed basis at 8.5% moisture) for the Canadian canola crop has been 9 and 10.1 μ mole g^{-1} , respectively. The long-term average for total glucosinolates in the Canadian canola crop has been 11 μ mole g^{-1} making it an ideal source of meal for farm animals. Glucosinolates are also reasonably low in the European and Australian canola crops. China is making a good progress toward reducing total glucosinolates in the rapeseed crop. There are several types of glucosinolates, each under the control of different gene or set of genes. The genetics and inheritance of glucosinolates is documented in past publications (Kondra and Stefansson, 1970; Magrath *et al.*, 1994; Mithen *et al.*, 1995; Parkin *et al.*, 1994; Toroser *et al.*, 1995; Sodhi *et al.*, 2002). Rakow and Raney (2003) demonstrated that it was possible to develop *B. napus* lines having zero aliphatic glucosinolates and a total glucosinolates of about 1.0 μ mole g^{-1} . This would further increase the feed value of rapeseed meal.

B. napus seeds are inherently dark brown in color. Yellow seed color was transferred into *B. napus* through interspecific hybridization and tested for agronomic performance (Rashid *et al.*, 1994; Rashid and Rakow, 1995; Rakow *et al.*, 1999; Relf-Eckstein *et al.*, 2003). Due to lower fiber contents, yellow-seeded *B. napus* has high oil. Meal from yellow-seeded canola has higher protein and lower fiber contents (Simbaya *et al.*, 1995).

Somers *et al.* (2001) developed random amplified polymorphic DNA (RAPD) markers for yellow seed *B. napus*. Liu *et al.* (2005) postulated in their study that seed color was controlled by three pairs of genes—a dominant yellow-seeded gene (*Y*) having an epistatic effect on the two independent dominant black-seeded genes (*B* and *C*), thereby inhibiting the biosynthesis of seed coat pigments. It is difficult to develop yellow-seeded canola hybrids because the genes responsible for this trait have to be transferred into both male and female inbreds, and the source pools from which inbreds are developed. If there was a single dominant gene available for the yellow seed trait, yellow-seeded hybrid development would be very simple.

In Canada, the canola crop is ready to swath from mid-August to mid-September. The crop is swathed at physiological maturity when the seeds in the bottom one-third of the pods on main raceme start changing color from green to dark brown. At this stage, seeds within pods of other branches are still green. As the swath lies in the field, chlorophyll within the seed is broken down and seed changes color from green to dark brown. If there is a frost, that causes plant tissue to die immediately, causing interruption in breakdown of chlorophyll. This causes premature death of seeds leaving them high in chlorophyll which ends up in the oil following crushing. The process of chlorophyll removal is an additional, expensive step that is unnecessary if the seeds are uniformly matured and have lowest possible green seed. In

Canada, at the point of delivery, a simple test verifies green seed number in the grain. If the number of green seed is higher than 2%, the crop is downgraded to Canada #2 instead of Canada #1 and farmer would receive lower value for the grain. This adds another breeding objective for the Canadian canola breeders: breeding for low chlorophyll and low green seed. Usually, later maturing varieties are more prone to higher green seed if not managed properly. At the same time there is variation among varieties because certain varieties consistently produce crops with low green seed content.

1.4.3 Improvement in disease resistance and insect resistance

Rapeseed and mustard have several important diseases and pests. The most common and widely occurring diseases and insects of canola and rapeseed in different countries are listed in Table 4. In Canada, Europe, and Australia, blackleg, caused by *L. maculans*, also known as stem canker or *Phoma* is the most important disease of canola. Currently, many of the commercial Canadian canola cultivars are rated “R” (resistant) for the most prevalent isolate of blackleg. The Australian isolates are the most virulent and have been reported to be present in a very small area in western Canada. There is tremendous genetic variation present in *B. napus* as far as

Table 4 List of most important rapeseed-mustard and canola diseases and insects in different countries

Country	Disease	Insects/pests
Canada	Blackleg (<i>Phoma lingam</i>) Sclerotinia (<i>Sclerotinia sclerotiorum</i>) Fusarium wilt (<i>Fusarium oxysporum</i> and <i>Fusarium avenaceum</i>)	Cabbage flea beetle (<i>Phyllotreta</i> spp.) Bertha armyworm (<i>Mamestra configurata</i>) Diamondback moth (<i>Plutella xylostella</i>)
China	Sclerotinia (<i>Sclerotinia sclerotiorum</i>) Turnip mosaic virus	Aphids (<i>Myzus persicae</i> , <i>Lipaphis erysimi</i> , <i>Brevicoryne brassicae</i>) Weevil (<i>Ceutorhynchus asper</i>) Diamondback moth (<i>Plutella xylostella</i>)
India	Alternaria (<i>Alternaria brassicae</i>) White rust (<i>Albugo candida</i>) Downy mildew (<i>Perenospora parasitica</i>)	Aphid (<i>Lipaphis erysimi</i>) Mustard saw fly (<i>Athalia proxima</i>) Painted bug (<i>Bagrada cruciferarum</i>)
Europe	Blackleg (<i>Phoma lingam</i>) Sclerotinia (<i>Sclerotinia sclerotiorum</i>) Light leaf spot (<i>Pyrenopeziza brassicae</i>)	Pollen beetle (<i>Meligethes aeneus</i>) Seedpod weevil (<i>Ceutorhynchus assimilis</i>) Cabbage stem flea beetle (<i>Psylliodes chrysocephala</i>)
Australia	Blackleg (<i>Phoma lingam</i>) Sclerotinia (<i>Sclerotinia sclerotiorum</i>) Damping off (<i>Rhizoctonia</i> spp., <i>Pythium</i> spp., <i>Fusarium</i> spp.)	Earth mites (<i>Halotydeus destructor</i> , <i>Penthaeus</i> spp.) Lucerne flea (<i>Sminthurus viridis</i>) False wireworm (<i>Isopteron punctatissimus</i> , <i>Adelium</i> spp.)

resistance is concerned. Also, *B. juncea* and *B. nigra* are mostly tolerant to blackleg, making them good sources of resistance. Plieske *et al.* (1998) concluded that the B genome provided strong, monogenic dominant resistance to blackleg. *Phoma* resistance is classified into two categories: (a) seedling resistance or cotyledon resistance, which is controlled by a few major genes and (b) stem resistance or adult plant resistance, which is controlled by many genes. The identification of genes responsible for race-specific resistance (cotyledon resistance) was reported (Rouxel *et al.*, 2003). Resistance caused by a major gene (seedling resistance) is not durable and can break down when used in varieties grown on a large scale. In Australia, resistance to blackleg caused by a single dominant gene was introduced in commercial cultivars in 2001, but it broke down within a short period of time. Adult plant resistance is complex and governed by several genes (Zhu and Rimmer, 2003). In a breeding program, it is desirable to utilize adult plant resistance since it is durable.

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* is another important disease of canola that affects canola cultivation in major markets. In China, continuous and widespread presence of *Sclerotinia* in an environment conducive to disease development has forced breeding programs to develop tolerant varieties. In *B. napus*, there has been very little genetic variation available for resistance to *Sclerotinia*, however, over the years, through methodical screening and well-managed breeding, rapeseed varieties with improved resistance have been developed in China (Wang *et al.*, 2003a). *Sclerotinia* fungus also attacks other two important oilseeds, soybeans and sunflower. The screening for disease is very tedious and inheritance is very complex. Zhao and Meng (2004) identified six QTLs associated with this disease resistance of which three were associated with leaf resistance explaining 40.7% variation while the other three were associated with mature plant resistance explaining 49% of phenotypic variation.

In India, *Alternaria* blight caused by *Alternaria brassicae* is a devastating disease attacking the *B. juncea* crop in many parts of the country. It is reported that there is resistance available in other related species (Siemens, 2002). Klewer *et al.* (2003) reported transfer of *Alternaria* resistance from *S. alba* and other related species into *B. napus*. Although, there are very well-established screening techniques and natural conditions available, no

commercial *B. juncea* variety is available, which carries a high degree of tolerance to this disease. *Alternaria* blight is not a serious canola disease in Canada, Europe, and Australia.

Fusarium wilt is not a major disease in Canada. It was first reported in 2002 in some of the Canadian commercial canola varieties. It is easy to screen breeding material if the screening nursery is in the right environment. Most of the current commercial canola varieties in Canada are resistant to this disease.

Downy mildew caused by *Peronospora parasitica* is another important disease in *B. juncea* in India. This disease is not a serious problem of canola in Canada, Europe, and Australia. Resistance sources are available in *B. napus* and *B. juncea*. White rust is caused by *Albugo candida* and it is more prevalent in *B. rapa* and *B. juncea*. It is not a serious disease in Canada. Currently, all commercial *B. napus* varieties in Canada are resistant to white rust.

Flea beetle, *Phyllotreta cruciferae* (Goeze), is a serious insect of canola in western Canada. The damage is usually severe at cotyledon stage and at seedling stage. Feeding damage can result in complete death of young seedlings, severely reduced growth, and delayed crop establishment resulting in lower seed yield. Mechanism of resistance to flea beetle in *S. alba* has been reported (Bodynaryk and Lamb, 1991). Elliott and Rakow (1999) observed that flea beetle damage was least in *B. carinata* and *S. alba* lines and it was greatest in *B. juncea* and *B. rapa* lines. There is not complete *B. napus* resistance source available for breeding.

The cabbage seedpod weevil (*Ceutorhynchus obstrictus*) was introduced to North America from Europe about 70 years ago. It was first found infesting canola in southern Alberta in 1995 and since then, the weevil has spread to central Alberta and southwestern Saskatchewan. Canola, brown mustard, cole crops (e.g., cabbage, broccoli, cauliflower) and cruciferous weeds (e.g., wild mustard, flixweed, stinkweed) are all hosts to this insect. Cabbage pod weevil, also known as seedpod weevil, is a very destructive insect and causes a lot of damage to the crop in warmer conditions. *S. alba* (yellow mustard) is known to carry resistance against this insect. Dosdall and Kott (2006) successfully transferred resistance from *S. alba* into *B. napus* lines.

There are three aphid species that attack rapeseed mustard: (1) Indian mustard aphid (*Lipaphis*

erysimi), (2) cabbage aphid (*Brevicoryne brassicae*), and (3) green peach aphid (*Myzus persicae*). Of these, mustard aphid is a very devastating insect of rapeseed and mustard in India. Cabbage aphid and green peach aphids sometimes infest canola in Australia. Sekhon and Ahman (1992) described source of resistance in *B. juncea*, *B. napus*, and other species against mustard aphid. Also morphological, anatomical, and biochemical characteristics of resistant *Brassica* plants are described in detail. Kanrar *et al.* (2002) reported the development of a transgenic Indian mustard (*B. juncea*) line with resistance to Indian mustard aphid. Gowers and Christy (1999) were able to transfer aphid resistance from *B. oleracea* to *B. napus*. Aphid infestation is not a serious problem in canola in Canada.

Diamondback moth (*Plutella xylostella*) is another canola insect in Western Canada and feeds on all *Brassica* plants. Although, the diamondback moth is found throughout Western Canada, its damage varies from year to year. In their study, Andrahennadi and Gillott (1998) found that some of the varieties of *B. juncea* showed resistance to diamondback moth.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional breeding can work for any trait as long as there is genetic variation available and it is possible to evaluate and select the trait in question. Usually, the traits for which there is little genetic variation initially could be improved slowly once the selection pressure is applied. The development of *Sclerotinia* tolerant rapeseed varieties is a good example, as there was very low level of tolerance available initially; however, after continuous breeding and selection for resistance, the level of tolerance increased. There are various methods of plant breeding that could be applied to increase the frequencies of desired alleles in breeding populations. As the frequencies of desired alleles increase, the level of expression for that trait would automatically improve.

For many traits in *B. napus* and *B. juncea*, there is sufficient genetic variation and it is possible to improve the traits through well-managed breeding, therefore, a transgenic approach does not make economic sense. Also, one has to remember that a transgenic approach is tedious that requires a

lot of developmental work, isolating, and cloning gene and then transforming plants. There is tremendous work involved in identifying the most desirable event once the plants are transformed and several events are developed. Data generation for regulatory approval is also very costly and time consuming, therefore, a transgenic approach can not be used for every trait. The trait has to have high economic value, very low or nonexistent genetic variation, and low possibility of genetic improvement through conventional plant breeding.

Table 5 lists all the important traits in spring and winter form of *B. napus* and *B. juncea* as they are the major oilseed crops. Some of these traits may not apply to *B. juncea*. Only the economically important diseases and insects are listed in the table, although there are many more diseases and insects that attack oilseed *Brassica*. The traits for herbicide resistance are listed in this table, but these are already developed and are incorporated into commercial material with very high success rate. There are traits with reasonably good genetic variation, yet the rate of genetic progress can be slow. This is because the trait is either difficult to screen and score or expensive to evaluate and has high environmental influence with low heritability. Yield is a good example of this as it requires multiple locations of replicated testing over several years in order to identify suitable genotypes making this an expensive trait to evaluate. Also, breeding priority has to be taken into consideration. The trait may have decent genetic variation and it would be easy to make genetic progress, but if the breeding priority is low, the trait would not be listed as one of the breeding objectives. White rust screening of *B. napus* genotypes in Canada fits this example. Transgenic approach has been reported for many traits in the literature, but its application and commercialization would always be determined by economic and socio-political aspects, which would be out of control of scientists.

2. DEVELOPMENT OF TRANSGENIC OILSEED BRASSICAS

The topics covered in this section have been extensively reviewed (Poulsen, 1996; Earle and Knauf, 1999; Palmer and Keller, 2002; Cardoza

Table 5 List of important *B. napus* and *B. juncea* traits and their priority for transgenic work

Trait	Genetic variation ^(a)	Feasibility of conventional breeding	Breeding priority	Rate of possible genetic progress	Need for transgenic approach
Yield	7	Yes	High	Low	
Pod numbers per plant	7	Yes	Medium	Moderate	
Seed size	5	Yes	Medium	Low	
Short height	5	Yes	Medium	Moderate	
Early maturity	5	Yes	Medium	Low	
Drought stress tolerance	2	Yes	Medium	Very low	Yes
Cold tolerance	2	Yes	Low	Very low	Yes
Shattering resistance	1	Yes	Medium	Very low	Yes
Oil content	6	Yes	High	High	Yes
Protein content	5	Yes	Low	Moderate	Yes
Reduced glucosinolates	3	Yes	Low	Low	
Reduced total saturates	1	Yes	Medium	Very low	Yes
Low green seed	4	Yes	High	Low	
Low linolenic acid	3	Yes	Low	Moderate	
High oleic and low linolenic acids	2	Yes	Low	Moderate	
Yellow seed coat	1	Yes	Low	Very low	Yes
Herbicide tolerance	1	Yes	High	Very high	Yes ^(b)
Pollination control system	2	Yes	High	Very high	Yes ^(b)
Blackleg resistance	7	Yes	High	High	
Sclerotinia resistance	3	Yes	High	Low	Yes
Fusarium wilt resistance	8	Yes	High	Very high	
Alternaria resistance	1	Yes	High	Very low	Yes
White rust resistance	7	Yes	Low	Very high	
Downy mildew resistance	4	Yes	Medium	Low	
Light leaf spot resistance	3	Yes	Medium	Low	
Damping-off resistance	3	Yes	Low	Low	
Flea beetle resistance	1	Yes	High	Very low	Yes
Diamondback moth resistance	2	Yes	High	Low	Yes
Pod weevil resistance	1	Yes	High	Very low	Yes
Aphid resistance	3	Yes	High	Low	
Pollen beetle resistance	2	Yes	High	Very low	Yes

^(a) 1 = extremely low or exists in related species, 2 = very low, 3 = low, 4 = medium low, 5 = medium, 6 = medium high, 7 = high, 8 very high, 9 = extremely high

^(b) Already commercially exploited

and Stewart, 2004; Pua and Lim, 2004), thus readers are also referred to these articles.

2.1 Transgenic Traits in Oilseed Brassicas

Genes isolated from microbes, animals, plants, and in particular the model plant *Arabidopsis thaliana*, have been used as a source of candidate genes for transforming oilseed Brassicas. The promoter used to drive the selectable marker or the genes of interest has been, in most cases, the 35S promoter from the tobacco mosaic virus, but spatial and

temporal expression of a target gene is usually driven by tissue or developmental phase-specific promoters. The use of the 35S promoter, when placed next to a tissue-specific promoter, can be problematic, because the strong promoter can “read through” and lead to “leaky” expression of the target gene that is intended to be tissue specifically expressed (Jagannath *et al.*, 2001). This problem can be alleviated by replacing the 35S promoter with a weaker one or by insertion of a spacer DNA as an insulator. The nos terminator from *Agrobacterium* has been widely used as a transcriptional terminator.

2.1.1 Herbicide tolerance

This subject is comprehensively reviewed by Warwick and Miki (2004) and readers are referred to this article. Herbicide resistance was among the first transgenic traits to be developed and commercialized in canola and other crops. This can be largely attributed to the relatively simple genetic manipulation (single gene control), the existing background knowledge of the physiological and biochemical characteristics of herbicide actions, the relatively more advanced tissue culture and plant regeneration technologies in Brassicas, and the enormous importance of weed control in crop production. Herbicide resistant transgenic canola commenced commercial production in 1995, and has since been rapidly and widely adopted by growers in Canada. There are four classes of herbicides for which transgenic canola has been obtained: glufosinate ammonia resistance conferred by the *bar* (DeBlock *et al.*, 1989) and *pat* genes (Oelck *et al.*, 1991); glyphosate (Roundup) resistance conferred by a mutant allele of the gene encoding 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Parker *et al.*, 1991); resistance to chlorsulfuron and related herbicides conferred by a mutant allele of the gene for acetolactate synthase (ALS) (Miki *et al.*, 1990); and resistance to bromoxynil by a nitrilase enzyme gene (Freyssinet *et al.*, 1996). Glyphosate (N-[phosphonomethyl]glycine) is the active ingredient of the commercial herbicide Roundup. It acts as an inhibitor of EPSP synthase, which functions in the aromatic amino acids biosynthesis pathway located in the chloroplast. The pathway is also present in bacteria and fungi but not in mammals or birds (Monsanto, 2002). Glyphosate resistant (Roundup Ready[®]) canola was obtained by transformation of *B. napus* cv. Westar with two genes in a single transfer DNA (T-DNA) vector via *Agrobacterium*-mediated transformation, *cp4 epsps* and *goxv 247* (Monsanto, 2002). The *cp4 epsps* gene encodes a bacterial form of EPSP synthase from *Agrobacterium* strain CP4, which is naturally tolerant to glyphosate. The chloroplast transit peptide sequence of the *Arabidopsis* EPSP synthase gene was fused to the gene to ensure transport into the chloroplast and processing of the bacterial enzyme. The second gene, *goxv 247* from *Ochrobacterium anthropi* strain LBAA, encodes a form of glyphosate oxidase (GOX),

which catalyzes the breakdown of glyphosate into the nontoxic products glyoxylic acid and aminomethylphosphonic acid (AMPA). The *goxv 247* gene was fused to the chloroplast transit peptide sequence from the *Arabidopsis* ribulose biphosphate carboxylase (*rbc*) small subunit gene, *SSUIA-CTP1*. Both chimeric genes were driven by the 35S promoter and terminated with the E9 pea *rbc* small subunit gene (*rbcSA*) 3' region. Thus, two mechanisms of resistance are working in these commercial lines: an alternate EPSP synthase with reduced herbicide binding and a novel enzyme for degradation of the herbicide. The line was first given regulatory approval in 1995 in Canada. Subsequently, the trait was introduced into *B. rapa* varieties via conventional crossing (CFIA 2002).

Phosphinothricin (PPT; marketed as glufosinate-ammonium, Basta, Ignite, Liberty, Finale) is an analogue of L-glutamic acid and a strong inhibitor of glutamine synthetase (GS). GS is the only enzyme in plants that can catalyze the assimilation of ammonia into glutamic acid, which is a donor for almost all nitrogenous compounds in plants. Inhibition of GS by PPT may lead to the accumulation of ammonia and subsequently cell death. Two genes, *pat* and *bar*, were used for generating transgenic canola. The *pat* gene encodes PPT N-acetyltransferase from *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988). The acetyl transferase acetylate PPT detoxifies it. The homolog of the gene from *Streptomyces hygroscopicus* is called *bar* (Thompson *et al.*, 1987). They share approximately 87% identity at nucleotide level and require some codon optimization for expression in plants. The *pat* gene driven by a strong constitutive promoter was introduced into *B. napus* cv. Topas by *Agrobacterium*-mediated transformation of microspore-derived embryos (Oelck *et al.*, 1991). The transgenes was later crossed into other *B. napus* and *B. rapa* varieties for field evaluation (Kumar *et al.*, 1998) and commercial production (CFIA 2002). The same construct was also introduced directly into *B. napus* cv. Excel (CFIA 2002). These lines have been approved for use in human oil consumption and livestock meal in Canada, the United States, Japan, and Australia (Agbios, 2002). The *bar* gene was first introduced into *B. napus* cv. Drakkar (De Block *et al.*, 1989) and subsequently introduced

into a number of cultivars. It has also been approved in Canada, the United States, and Japan (Agbios, 2002).

The oxynil herbicides, bromoxynil and ioxynil, inhibit photosystem II electron transport in plants. The *bnx* gene encoding the bromoxynil nitrilase, which catalyzes the conversion of bromoxynil into 3,5-dibromo-4-hydroxybenzoic acid, was isolated from *Klebsiella pneumoniae* ssp. *Ozaenae* (Stalker and McBride, 1987). The gene under the control of the 35S promoter was introduced into *B. napus* cv. Westar by *Agrobacterium*-mediated transformation and high level of resistance was observed (Freysinet *et al.*, 1996). The transgenic line has been approved for commercial production in Canada and Japan (Agbios, 2002; CFIA 2002).

At least four classes of herbicides, i.e., sulfonylureas, imidazolonones, triazolopyrimidines, and pyrimidinyl thiobenzoates, target ALS or acetohydroxy acid synthase (AHAS), a regulatory enzyme in the branched-chain amino acid biosynthesis pathway in chloroplasts (Singh and Shaner, 1995). The enzyme appears to be encoded by a small gene family (Rutledge *et al.*, 1991). A variety of mutant ALS alleles have been identified and characterized, which confer resistance to a specific herbicide class or to multiple classes (Guttieri *et al.*, 1996). In *B. napus*, a mutant AHAS3 gene, *ahas3R*, was cloned, which has a single mutation responsible for multiple herbicide resistance (Hattori *et al.*, 1995). A mutant AHAS3 gene, *csr1-1*, has been introduced into canola, *B. napus* cvs. Profit (Miki *et al.*, 1990) and Westar (Blackshaw *et al.*, 1994), under control of either the 35S promoter or its native promoter. The *csr1-1* gene was isolated from *A. thaliana* known to confer increased resistance to the sulfonylurea herbicide chlorsulfuron (Haughn *et al.*, 1988). Resistance to herbicides was demonstrated in greenhouse and field tests, but these transgenic canola lines have not been commercialized.

2.1.2 Insect resistance

The topic has been reviewed by many authors, including Estruch *et al.* (1997), Jouanin *et al.* (1998), Schuler *et al.* (1998), Hilder and Boulter (1999), Bruenn (2000), and Earle *et al.* (2004). Two main types of transgenic insect resistant oilseed

Brassicas have been developed to date: those expressing *Bacillus thuringiensis* (*Bt*) genes and those expressing proteinase inhibitor (PI) genes. None of the transgenic lines developed have been approved for commercial production.

A wide range of insects can feed on Brassicas, targeting their roots, stems, leaves, and reproductive parts. At least seven orders of insects have species that feed on Brassicas (Earle *et al.*, 2004). Among these, diamondback moth (*P. xylostella*) may be the most devastating pest of Brassicas worldwide. Control strategies need to take into account the various feeding mechanisms displayed by different insects. Genes encoding insecticidal crystal proteins (ICPs) from the soil bacterium *Bt* have been most commonly used in most transgenic studies of insect resistance. One of the attractive features of *Bt* for insect control is its specificity. Different bacterial strains produce different ICPs, each of which has specificity for insects in different orders. This offers the possibility to control certain types of insects without harming others. Another major feature of *Bt* is that there are many ICPs that have unique modes of action or target sites in insects. This offers the potential to control some pests that have developed resistance to other insecticides.

Bt genes have been introduced into oilseed Brassicas (Stewart *et al.*, 1996; Li *et al.*, 1999; Halfhill *et al.*, 2001; Kuvshinov *et al.*, 2001; Winterer and Bergelson, 2001; Wang *et al.*, 2005b). The synthetic *cry1Ac* gene with codon optimized for better expression in plants was used for most studies. In some cases, only one *Bt* gene is transformed; while in some other cases a reporter such as the β -glucuronidase (GUS; Kuvshinov *et al.*, 2001) or the green fluorescent protein (GFP) (Halfhill *et al.*, 2001) was co-transformed. The GFP marker may be useful for selection of transformants, bypassing the need for antibiotics or herbicides, and may also be useful in monitoring the movement of the linked *Bt* transgene beyond the original transgenic crop (Halfhill *et al.*, 2001; Stewart, 2006). In a couple of studies, transgenic *B. napus* plants containing a *Bt* gene and another gene for enhanced resistance were generated. Winterer and Bergelson (2001) crossed *B. napus* cv. Westar carrying either a *cry1Ac* gene or potato PI (*P12*) gene and recovered homozygous lines carrying both the genes. Wang *et al.* (2005b) introduced *aroA-M12*, an EPSPS

gene, and a *Bt*slm recombinant gene encoding *Bt* toxin gene, into *B. napus* cv. Xiangyou No. 15. The 35S promoter was used to drive the *Bt* genes in almost all the works, and in only one case the mannopine synthase promoter (also a constitutive promoter) from *Agrobacterium tumefaciens* was used (Winterer and Bergelson, 2001). *Agrobacterium*-mediated transformation of seedling explants was employed in these reports using antibiotics or herbicides for tissue selection.

Significant variations of *Bt* protein expression level is common, as it is for other transgenes, and the development of large number of transgenic lines were necessary for selecting better expressors. The documented *Bt* protein levels range from as low as 0.0005% to a maximum of approximately 0.4% of total soluble protein (TSP) (Stewart *et al.*, 1996). Transgenic plants are tested by bioassays using selected insects on detached leaves and/or on whole plants. The diamondback moth has served as a primary choice of insects because of its agricultural importance and because some populations of these moths have developed resistance to some *Bt* proteins in the field due to exposure to *Bt* sprays. It has been demonstrated that the susceptible moths are well controlled by plants with high expression. In many cases there has been no follow-up to the initial work reported. However, some of the transgenic lines generated have been used in various confined greenhouse and field studies.

PIs are usually found in seeds or other storage tissues, where they can act to disrupt the digestive enzymes used by insects to break down proteins ingested and thus offer the potential to control insects. Different insects have different primary proteinases and are sensitive to different PIs. Therefore understanding the biochemistry of the interaction and choosing the right combination are the keys for successful control. Transgenic *B. napus* plants have been produced with PI genes from different plant sources: *MTI-2* from white mustard (*S. alba*) (DeLeo *et al.*, 2001); *PI-2* from potato (Winterer and Bergelson, 2001); *OCI* from rice (Girard *et al.*, 1998a, b); and *CII* from soybean (Jouanin *et al.*, 1998). The spring cultivar Drakkar was used in all the studies except in one case Westar was used (Winterer and Bergelson, 2001). Only lower levels of PI protein (0.15–0.25% TSP) were expressed in the *MTI-2* lines. These

plants were tested against larvae of various insects and *P. xylostella* was the most sensitive with high mortality and retarded development. No significant impact on other insects was found. Expression of the *PI-2* gene (0.002–0.01% TSP) did not retard the growth rate of *P. xylostella* moth and actually led to a slightly more leaf damage. Even when *Bt* and *PI-2* are co-expressed, insect control was not reduced rather enhanced. Similar negative results were found for the *OCI*, *CII*, as well as the *OCI-CII* double transgene lines. There are a few possible explanations for these results, including the likely mismatch between the insects tested versus the transgenic lines, the possibility of insect adaptation to the PIs, and the paradoxical effects that may be seen when expression of PIs is low (Earle *et al.*, 2004).

Other than *Bts* and PIs, there have been other efforts to control insect pest of *B. napus*. Recently, Wang *et al.* (2005a) generated *B. napus* plants transgenic for two genes, a chitinase gene encoding insect-specific chitinase from *Manduca sexta* and a scorpion insect toxin gene from *Buthus martensii* Karsch. Fifty-seven transgenic lines were obtained and inoculated with diamondback moths larvae using *in vitro* leaf-feeding assay. Significant insecticidal activity was observed and the activity was well correlated with the levels of proteins encoded by the two transgenes estimated by ELISA. These are interesting results, particularly due to the fact that some diamondback moths have developed resistance due to the wide use of *Bt*. Resistant transgenic plants containing new genes or novel combinations of genes may slow down and eventually minimize the development of resistance by insects.

2.1.3 Disease resistance

Oilseed Brassicas are particularly subject to attack by various fungal pathogens, including ascomycetes *A. brassicae*, *A. brassicicola*, *L. maculans*, *Pyrenopeziza brassicae*, *S. sclerotiorum*, and *Verticillium longisporum*, the myxomycete *Plasmodiophora brassicae*, and the oomycete *A. candida* (Dixelius *et al.*, 2004). The loculoascomycete *L. maculans* (Desm.) Ces. et de Not. is the most important pathogen of oilseed Brassicas worldwide, causing stem canker or blackleg disease. For this reason a number of

efforts have been made to control the disease using a transgenic approach. Genes used for these studies include a tomato chitinase gene (Grison *et al.*, 1996), a pathogenesis-related peroxidase of *Stylosanthes humilis* (Kazan *et al.*, 1998), a pea defense gene *DDR206* (Wang *et al.*, 1999, 2001), the R gene *Cf9* from tomato (Hennin *et al.*, 2001a, b, 2002), and a novel antimicrobial peptide (Kazan *et al.*, 2002). Certain degrees of resistance were obtained with these transgenes. Particularly, the transgenic oilseed rape constitutively overexpressing a chimeric chitinase gene gave field tolerance not only to *L. maculans*, but also *P. brassicae*, and *S. sclerotinia*, the causal agents of stem rot and light leaf spot, respectively (Grison *et al.*, 1996). Furthermore, the first resistant gene named *Lm1* effective against *L. maculans* was cloned from *B. nigra* (Wretblad, 2002; Wretblad *et al.*, 2003). Transgenic *B. napus* plants expressing the *Lm1* gene exhibited resistance to PG2, PG3, and PG4 isolates. Stem rot caused by discomycete *S. sclerotiorum* (Lib.) de Bary is another common disease around the world. Transgenic oilseed Brassicas expressing an oxalate oxidase gene derived from *Hordeum vulgare* roots showed enhanced resistance to *Sclerotinia* sp. The enzyme destroys the oxalate, which is required for fungal infection (Thompson *et al.*, 1995). This is significant since no broadly-available, simple genetic source of resistance has yet been identified.

The plant immune response is best studied in the model plant *Arabidopsis* (Chisholm *et al.*, 2006). This model plant has been widely used for cloning and functional studies of defense-related genes and signaling pathways. It is expected that knowledge gained in the *Arabidopsis* system has the potential for application in Brassica crops. However, not all the defense mechanisms are conserved between the two species. For example, the *RPM1* resistance gene from *Arabidopsis* is missing in *B. napus* (Grant *et al.*, 1998). For this reason, international efforts on Brassica genomics have been initiated (www.brassicainfo.org). The genomics resources generated by these efforts have greatly facilitated the direct cloning of genes in Brassica, albeit a tremendously difficult job. Recently, significant progress has been made toward the map-based cloning of *LmRI* locus, which controls seedling resistance to the blackleg fungus *L. maculans* in the *B. napus* cultivar Shiralee (Mayerhofer *et al.*, 2005).

2.1.4 Abiotic stress tolerance

Arabidopsis has been an excellent model plant for studying of abiotic stress responses and biotechnology (Zhang *et al.*, 2004). During the past decade, great progress has been made with this model system in unraveling the abiotic stress signal transduction (salt, cold, and drought stress), and these new insights have provided promising novel approaches to engineer plant tolerance to abiotic stresses. A number of genes have been demonstrated to be effective in conferring stress tolerance when overexpressed or knocked down in transgenic *Arabidopsis* plants. Some of these genes have been shown to be conserved in crop species including *B. napus* (Zhang *et al.*, 2004).

The potential of manipulating ion transporters to improve ion homeostasis is well recognized. For example, several classes of transporters are required in regulating sodium homeostasis under salt stress. The influx of Na^+ is controlled by *AtHKT1*, a low affinity Na^+ transporter (Rus *et al.*, 2001), and other nonselective cation channels, while the efflux is controlled by Salt Overly Sensitive 1 (*SOS1*), a plasma membrane Na^+/H^+ antiporter (Shi *et al.*, 2000). Vacuolar membrane ion transporters, such as the tonoplast Na^+/H^+ antiporter *AtNHX1* (Apse *et al.*, 1999; Gaxiola *et al.*, 2001), also play a vital role in regulating cytoplasm Na^+ homeostasis by sequestering Na^+ ions in the vacuole. It appears that coordination exists between the transporters in the tonoplast and plasma membranes through other components of the *SOS* pathway, albeit the mechanism is not completely understood (Chinnusamy *et al.*, 2006). It is commonly believed that maintaining a low cytosolic Na^+ concentration is essential to achieve salt tolerance and can be achieved by restricting inflow, increasing outflow, or increasing vacuolar sequestration of Na^+ . Thus, theoretically, increasing plasma membrane Na^+ exporters and tonoplast Na^+ importers and/or restricting the amount of Na^+ influx by lowering the amount of plasma membrane Na^+ importers should suffice. Indeed, a number of successes have been documented when these strategies were used (Zhang *et al.*, 2004). For example, increased expression of the *Arabidopsis* tonoplast membrane Na^+/H^+ antiporter, *AtNHX1*, under a strong constitutive promoter was reported to result in salt-tolerant *Arabidopsis* (Apse *et al.*, 1999) and

B. napus (Zhang *et al.*, 2001). The transgenic *B. napus* plants were able to grow, flower, and produce seeds in the presence of 200 mM sodium chloride. Although the transgenic plants grown in high salinity accumulated sodium up to 6% of their dry weight, growth of these plants was only marginally affected by the high salt concentration. Moreover, seed yields and the seed oil quality were not affected.

In contrast to ion homeostasis, a plant's adaptation to cold and drought is to a greater extent under transcriptional control, and some processes are regulated by abscisic acid (ABA), while others are ABA-independent (Shinozaki *et al.*, 2003; Chinnusamy *et al.*, 2004). For these reasons, it is not surprising that transcription factors (TFs) represent one of the best targets for engineering plants to achieve enhanced cold and drought tolerance. Even so, not all TFs involved in the cold and drought signal transduction are suitable targets. The *CBF* genes have been successfully used to engineer abiotic stress tolerance in a number of different species (Zhang *et al.*, 2004). *CBFs* are a class of AP2 TFs that plays a central role in both the ABA-dependent and ABA-independent pathways. Expression of all *CBF* genes in *Arabidopsis* is low under normal growth condition but increases within several minutes after cold or drought stress. In addition, the DNA-binding activity of some *CBFs* can also be modulated by temperature. The orthologous genes of *CBF* have been found in most crop plants examined so far, including *B. napus*. Constitutive overexpression of the *Arabidopsis CBF* genes in canola results in increased freezing tolerance (Jaglo *et al.*, 2001) and drought tolerance (Zhang *et al.*, 2004; Zhang *et al.*, unpublished). In these studies, it was discovered that ectopic overexpression of the *CBF* genes in plants produced, in addition to increased stress tolerance, dark-green, dwarfed plants. To overcome these problems, stress-inducible promoters that have low background expression under normal growth condition have been used in conjunction with the *CBF* genes to achieve increased stress tolerance in tobacco plants without the retarded growth (Lee *et al.*, 2003).

Another biotechnological target for improving plant drought tolerance is the genetic manipulation of the stress response to ABA. ABA modulates stomatal aperture to reduce

transpirational water loss. Under water stress conditions, the endogenous level of ABA increases, which, through a complex signaling cascade, results in stomatal closure (Blatt, 2000). When water relations return to optimal conditions for growth, ABA concentration decreases to reverse the process. Thus, the ability to regulate ABA synthesis and response makes this hormone an excellent target for improving drought tolerance in crop species. A number of genes have been identified, through genetic screens, which either increase or decrease the plant's response to ABA (Finkelstein *et al.*, 2002). Of these, *ERA1* and *ABH1* are of particular interest, as loss-of-function alleles of these genes increase sensitivity of the guard cells to ABA (Cutler *et al.*, 1996; Pei *et al.*, 1998; Hugouvieux *et al.*, 2001). As a consequence, *era1* or *abh1* mutants show reduced wilting during drought stress. In the presence of exogenous ABA, the *era1* leaves display the tightest stomatal aperture closure. *ERA1* encodes the β -subunit of *Arabidopsis* farnesyltransferase (AtFTB), suggesting that a negative regulator of ABA sensitivity must be farnesylated to modulate ABA response in *Arabidopsis* (Cutler *et al.*, 1996). All plant farnesyltransferases identified to date consist of heterodimer of an α - (AtFTA) and β -subunit, each of which belongs to a single-gene family. Loss of function of *ERA1* in *Arabidopsis* results in reduced water loss, but also causes severe pleiotropic phenotypes in growth and development (Bonetta *et al.*, 2000). Interestingly, further transgenic studies demonstrated that downregulation of either AtFTA or AtFTB, by antisense transgene approach, resulted in both increased ABA sensitivity and improved drought stress tolerance in *Arabidopsis* (Wang *et al.*, 2005c). Furthermore, use of a drought-inducible *Arabidopsis rd29A* promoter to drive the antisense expression of AtFTB in canola conferred similar drought protection in this species (Wang *et al.*, 2005c). Transgenic canola plants showed enhanced ABA response during seedling development. In addition, under drought conditions, ABA-hypersensitive plants showed greater reduction in stomatal conductance and leaf transpiration than the parental nontransgenic plants. It was also demonstrated that the downregulation of targeted gene expression driven by this drought-inducible promoter is a conditional, reversible process that is controlled by the available soil water content.

More importantly, results obtained from three consecutive years of field trials indicated that in the field under well-irrigated conditions, these transgenic plants showed similar or greater seed yield than the parental plants. When irrigation was reduced at the peak of flowering, transgenic canola showed significantly greater seed yield than the parental control (Wang *et al.*, 2005c).

2.1.5 Fatty acid modification

Transgenic manipulation of storage lipid biosynthesis represents some of the most successful cases of metabolic engineering in oilseed crops. Research in this field has benefited greatly from the use of *Arabidopsis* as a model system for study of plant lipid biochemistry. Based on genomic information from *Arabidopsis* there are about 600 genes implicated in lipid metabolism, many of which are potential targets of genetic manipulation of storage lipid deposition in seeds. For a detailed description of the metabolic steps and enzymatic components involved in plant fatty acid and glycerolipid synthesis, readers are referred to articles by Ohlrogge and Browse (1995), Ohlrogge and Jaworski (1997), and Weselake (2005). A review by Thelen and Ohlrogge (2002) is recommended for highlights of achievement in seed oil biotechnology in Brassica and other major oilseed crops.

Currently, the Brassica seed oil industry is still primarily focused on edible oil. Improving oil profile for human health has been a predominant theme. Direct downregulation of some of the fatty acid biosynthesis enzymes and introduction of novel fatty acid modification enzymes have resulted in large compositional changes in the fatty acid compositions of seed oil. In order to reduce the need of industrial dehydration that causes the production of trans-unsaturated fatty acids, high stearic oil is preferred for the production of solid fat, such as margarines. Research through antisense the stearyl-acyl-ACP desaturase gene in Brassica resulted in an increase of stearic acid to a level of up to 40% (Knutzon *et al.*, 1992). Since higher monounsaturated fatty acid is required for a desired oil profile, co-suppression of the $\Delta 12$ desaturase has been employed, leading to an oleic level at 89% in seed oil (Stoutjesdijk *et al.*, 2000). Some fatty acids, particularly γ -

linolenic acid (GLA), have documented beneficial effects when used as a nutritional supplement in humans. GLA can be produced from linoleic acid through desaturation mediated by the $\Delta 6$ desaturase. Traditionally, borage has been the main source for GLA. In order to overcome the yield limitation of borage, research has been conducted to introduce a $\Delta 6$ desaturase gene originated from the oleaginous fungus, *Pythium irregulare* into Brassica species, and the transgenic *B. juncea* lines were capable of producing GLA up to 40% of the total fatty acids (Hong *et al.*, 2002). Plant-based production of very long chain polyunsaturated acid (VLCPUFA), particularly eicosapentaenoic acid (EPA, C20: A5, 8, 11, 14, 17) and docosahexaenoic acid (DHA, C22: A4, 7, 10, 13, 16, 19) as a nutraceutical supplements, offers not only economic benefits but also addresses the concern on the ever-decreasing fish stocks from which these fatty acids are primarily obtained. Major enzymatic components required for fatty acid desaturation and elongation to the formation of DHA have now all been identified. Using a series of transformations involving nine different genes, a group at the Bioriginal Food and Science Corporation, achieved an important milestone of producing transgenic *B. juncea* with EPA level of up to 15% of seed oil (Wu *et al.*, 2005).

Large scale and low cost production of specialty oil as renewable industrial feedstocks in Brassica has been a commercial-driven research goal for quite some time (Kinney, 1998). One of the most notable successes of such an endeavor is that of high laurate *B. napus* produced by Calgene. Expression of a 12:0-acyl-ACP thioesters isolated from California bay laurel (*Umbellularia California*) in canola seeds resulted in highly saturated canola oil, known as BTE canola, with lauric content surpassing 50% of the seed oil (Voelker *et al.*, 1996). Subsequent studies revealed that the laurate was found almost exclusively at the sn-1 and sn-3 positions of the glycerol backbone due to an apparent substrate specificity limitation of the lysophosphatidic acyltransferase (LPAAT) in Brassica, which acylates the sn-2 position. To further improve laurate content, a coconuct 12:0-CoA-prefering LPAAT was introduced into the BTE expressing lines, which led to the production of trilaurin, and a level of laurate approaching 67% in seed oil (Knutzon *et al.*, 1999). In a similar study using an acyl-ACP thioesterase gene

from the Mexican shrub *Cuphea hookeriana*, which accumulates up to 75% caprylate (8:0) and caprate (10:0) in its seed oil, transgenic *B. napus* plant accumulating 11 and 27 mol% of these two fatty acids in seed oil have also been reported (Dehesh *et al.*, 1996).

Genetic manipulation of *B. napus* for the purpose of producing the very long chain erucic acid used in plastic film manufacture and the lubricant and emollient industries has sparked research interests for more than a decade. The main goal of transgenic improvement is to increase the level of erucic content, which is currently found around 45% in some of the HEAR cultivars. Two enzymatic steps crucial for the biosynthesis of erucic acid have so far been specifically targeted: the fatty acid elongase FAE1 and the sn-2 fatty acyltransferase LPAAT. FAE1 encodes for a seed-specific condensing enzyme of the fatty acid elongation enzyme complex responding for extending the 18 carbon fatty acid to that of 22. Seed-specific expression of the *Arabidopsis* FAE1 gene in a HEAR germplasm led to increases in the proportions of erucic from 48% to 53% among the best greenhouse grown transgenic lines. Substrate specificity of the sn-2 acyltransferase LPAAT that is incapable of incorporating long chain fatty acids into triacylglycerols is another limiting factor of erucic acid content. When a mutant allele of the yeast LPAAT, *SLC1* was introduced into cultivars of *B. napus* Hero and Reston, the resulting transgenic plants showed increases in both overall proportions and the amounts of erucic acid (Zou *et al.*, 1997; Katavic *et al.*, 2000). Similar strategy has also been applied to *B. juncea*, in which a FAE1 transcript level has resulted in increased erucic content (Kanrar *et al.*, 2006). In addition to transgenic technology, an asymmetric somatic hybridization approach using *B. napus* and *Crambe abyssinica*, an annual cruciferous oil crop with a high content of erucic acid, has also been shown to have potential for improving the amounts of erucic acid in *B. napus* (Wang *et al.*, 2003c).

2.1.6 Modification of other seed composition for improved nutrition

In oilseed Brassicas, the oil-free meal is a useful source of animal feed. There have been a number of efforts using transgenic approaches to improve the

nutritional value of seed meal. Oilseed Brassicas generally contain relatively low levels of essential amino acids such as methionine, lysine, and cysteine. Thus, attempts have been made to modify the protein composition and balance the amino acids proportion (deClercq *et al.*, 1990; Altenbach *et al.*, 1992; Kohno-Murase *et al.*, 1994, 1995; Denis *et al.*, 1995a, b; Falco *et al.*, 1995). Altenbach *et al.* (1992) generated transgenic canola plants expressing the methionine-rich 2S albumin protein precursor gene from Brazil nut, under the control of the phaseolin promoter. The methionine-rich protein was shown to be accumulated to 1.7–4% of the total seed protein and because of this the seed methionine content was increased to 33%. This work also suggests that the Brazil nut precursor protein can be processed and deposited correctly in canola. Using an antisense transgenic approach to downregulate the expression of the 12S seed storage protein cruciferin, Kohno-Murase *et al.* (1995) observed an increase in essential amino acids, methionine, lysine, and cysteine, 8%, 10%, and 32%, respectively. Cruciferin accounts for 60% of total seed proteins in *B. napus* and contains very low levels of the three essential amino acids. The total seed protein and lipid contents were not affected, so it is conceivable that the resources were redirected to enhance the synthesis of otherwise less abundant seed proteins, which may have a more balanced amino acids' composition, resulting in the increase of the content of the three essential amino acids. Falco *et al.* (1995) took a different approach, by manipulating the lysine synthesis pathway, to increase the content of the amino acids in transgenic canola seeds. The two key enzymes in the lysine biosynthesis pathway, aspartokinase (AK) and dihydrodipicolinic acid synthase (DHDPS), are regulated by end product feedback inhibition. To circumvent this normal feedback regulation, lysine feedback-insensitive bacterial DHDPS encoded by the *dapA* gene from *Corynebacterium*, was linked to a chloroplast transit peptide and expressed from a seed-specific promoter in transgenic canola seeds. As a result, more than 100-fold increases in the accumulation of free lysine in the seeds were obtained.

In addition to balanced amino acids composition, efforts have also been made to improve nutritional value of transgenic Brassica oilseeds by overexpression of carotenoids (Shewmaker *et al.*,

1999) and the introduction of phytase (Ponstein *et al.*, 2002). Carotenoids are a group of compounds derived from the five carbon isoprenoid precursor isopentenyl diphosphate, including α - and β -carotene, lycopene, and lutein. These compounds serve a variety of functions in mammals and humans. Amongst them, β -carotene is best known, which serves as a dietary precursor of vitamin A in the body. The carotenoid pathway has been elucidated since the 1960s and complementary DNAs (cDNAs) for most of the major enzymes have been cloned from both plant and microbial sources in the 1980s (Cunningham and Gantt, 1988). The first committed step in carotenoid biosynthesis is the condensation of two geranylgeranyl diphosphate moieties to give phytoene. The gene responsible for this reaction, phytoene synthase, has been cloned from a variety of microbial and plant sources. Phytoene synthase has been considered to be a regulatory point in the pathway. Shewmaker *et al.* (1999) produced *B. napus* transgenic plants overexpressing phytoene synthase gene *crtB* from the bacteria *Erwinia uredovora* in plastid. The phytoene synthase gene fused with the transit peptide of the pea small subunit of ribulose biphosphate carboxylase (rubisco) was under the control of a napin promoter for seed-specific expression, and plastid targeting. The resultant embryos from these transgenic plants were visibly orange and the mature seeds contained up to a 50-fold increase in carotenoids, predominantly α - and β -carotene with a significant amount of phytoene. Other metabolites in the isoprenoid pathway were also examined, and it was found that both tocopherol and chlorophyll were decreased. Unexpectedly, the fatty acid composition was also altered: oleic acid increased while linoleic and linolenic acids decreased.

Seeds are rich in phosphorus, but phosphorus is stored mainly in the form of phytate and it can not be utilized by monogastric animals because they lack the enzyme phytase in the digestive tract for phytate hydrolysis. Therefore, either exogenous phytase or phosphorus needs to be supplemented in the animal feed. This is costly both economically and environmentally, since the phytase production by fermentation is not cost effective, and phosphorus application and the undigested phytate will be released to the environment and cause pollution. Ponstein

et al. (2002) introduced the *Aspergillus niger* gene encoding phytase (*phyA*) into canola *B. napus*. The gene was under the control of the seed-specific cruciferin (*CruA*) promoter for overexpression in seeds. The cruciferin signal peptide was also incorporated into the expression construct to direct the secretion of the enzyme. Phytase expression in the T₁ seeds ranged from 0 U g⁻¹ to 600 U g⁻¹ seed. The transgenic trait has also been shown to be stable for up to four generations under greenhouse and field conditions.

2.1.7 Oilseed as bioreactor

In the past decade, there has been increasing interest and effort in producing recombinant proteins for industrial or biopharmaceutical uses in plants. The major advantages of the plant expression system are: (1) plants are free from potential human pathogen contamination and are thus considered relatively safe; and (2) large scale production in plants is cost effective. Oilseed Brassicas provide an inexpensive source of proteins. Attempts have been made to overexpress recombinant proteins in seeds, particularly in seed oil bodies for easy extraction and purification of the expressed protein. Lee *et al.* (1991) demonstrated that the maize oleosin gene driven by the promoter/terminator of a 2S albumin (napin) gene from Brassica was expressed and correctly targeted to seed oil bodies of transgenic *B. napus*. Soon after, Moloney *et al.* performed a series of studies and established the Brassica seed oil bodies as a vehicle for the production of recombinant proteins (van Rooijen and Moloney, 1995a, b; Parmenter *et al.*, 1995; Liu *et al.*, 1997; Chaudhary *et al.*, 1998; Kuhnelt *et al.*, 2003). First, they demonstrated that a fusion comprising a complete oleosin coding domain and a GUS coding sequence could be expressed specifically in the seeds of *B. napus*, and its product was correctly targeted with approximately 80% of the activity partitioning with oil bodies (van Rooijen and Moloney, 1995a). Recombinant oil bodies can be used to facilitate separation of a recombinant protein from other cellular proteins by flotation centrifugation. The target protein may then be released from the oil bodies by

cleavage with endoprotease and further purified (Kuhnel *et al.*, 2003). The fusion protein is stable in dry seeds for long periods. This demonstrates that foreign proteins other than oleosin can be correctly targeted to seed oil bodies. Further, the domains of oleosin required for correct targeting were mapped and the results indicate that both the N-terminal (1-47 amino acids) and central oleosin domain (48-113 amino acids, which make up a lipophilic core) are important for targeting to the oil body (van Rooijen and Moloney, 1995b). The expression system has been used successfully for the production of the leech anticoagulant protein hirudin (Parmenter *et al.*, 1995) and xylanase, an enzyme encoded by the *xynC* gene of rumen fungus *Neocallimastix patriciarum* (Liu *et al.*, 1997) in *B. napus*. The hirudin has also been expressed in *B. carinata*, an attractive alternative for the production of recombinant proteins (Chaudhary *et al.*, 1998), mainly due to the fact that it is not a crop in most of world, only in a localized area in Ethiopia and neighboring countries, it does not normally cross-pollinate with *B. napus*, thus avoiding transgene flow. All of these proteins retained their native biological activity.

Efforts have also been made to produce commercially useful biodegradable plastics in *B. napus* seeds (reviewed by Hanley *et al.*, 2000). The environmental benefit of such plastics is obvious, and their renewable nature is even more important in the long run. Plants use energy from sun to fix CO₂ and water to synthesize the plastic, which is then degraded back to CO₂ and water after use and disposal. Many bacterial species make polymerized carbon storage products from simple metabolic intermediates under limiting nutrient conditions (Madison and Huisman, 1999). These polymers have plasticlike properties and are collectively known as polyhydroxyalkanoates (PHAs). Many organisms can use PHAs as an energy source by employing secreted de-polymerases to break down the polymer into water-soluble monomers. Many different co-polymers are produced by a variety of organisms, but the homopolymer poly(β -hydroxybutyrate), PHB, is the most common type of PHA. In *R. eutropha* bacteria, three genes are involved in the biosynthesis of PHB, *phbA* encoding β -ketothiolase, *phbB* encoding the NADPH-dependent acetoacetyl-CoA reductase,

and *phbC* encoding PHA synthase. Significant PHB production in leaves of transgenic *Ara-bidopsis* (14% dry weight) was first demonstrated by Nawrath *et al.* (1994). This was achieved by introducing all three bacterial genes into the plastid via the N-terminal addition of the small subunit RUBISCO-targeting peptide. However, *B. napus* oilseed may provide a better system for PHB production because acetyl-CoA, the substrate required in the first step of PHB biosynthesis, is prevalent during fatty acid biosynthesis. For this reason, Houmiel *et al.* (1999) introduced all the three genes into *B. napus* and PHB accumulated in leucoplasts to levels of as high as 7.7% fresh seed weight of mature seeds. The researchers cloned all the three bacterial genes into one binary vector as three modular cassettes, each consisted of the *L. fendleri* oleate 12-hydroxylase promoter, an open reading frame encoding a chloroplast transit peptide fused to the open reading frame of one of the bacterial genes, and the 3' termination region of the *Pisum sativum rbcSE9* gene. The construct was introduced into *B. napus* using *Agrobacterium*-mediated transformation. The group also made the efforts to produce PHBV co-polymer (Slater *et al.*, 1999), which is structurally more flexible than PHB because of reduced crystallinity and is suitable for many commercial applications. The synthesis pathways for PHB and PHBV are essentially identical, differing only in the initial metabolites. Synthesis of PHB is initiated by the condensation of two acetyl-CoA molecules, whereas PHBV synthesis requires the additional condensation of acetyl-CoA with propionyl-CoA. Since the concentration of free propionyl CoA in plants is low, it was necessary to include a fourth gene, *IlvA* from *Escherichia coli*, to redirect intermediates of metabolism to provide a pool for PHV biosynthesis. PHBV co-polymers were accumulated in *B. napus* seeds but only at levels less than 3% of seed weight, which is considerably lower than the calculated economically viable level of 15% polymer in the seed (Houmiel *et al.*, 1999).

2.1.8 Pollination control systems

The topic is comprehensively reviewed by Nasrallah (2004). Heterosis (hybrid vigor) has been observed in canola hybrids (Starmer *et al.*,

1998). The heterotic effect is variable and depends on the parental lines used for hybrid seed production. Both *B. napus* and *B. rapa* oilseed cultivars are largely autogamous and are capable of undergoing both self-pollination and cross-pollination, therefore a pollination control system is required for hybrid seed production. Although SI and CMS exist as pollination control systems for commercial hybrid seed production, these are not always stable and may not be available in the desired germplasm. A number of pollination control systems using transgene approaches have been documented (Perez-Prat and van Lookeren Campagne, 2002). In *B. napus*, male sterility was obtained through expression of the cytotoxic gene *barnase*, a ribonuclease (RNase) gene from *Bacillus amyloliquefaciens*, specifically in the tapetal cells of anthers, thus disrupting normal pollen development (Mariani *et al.*, 1990; Denis *et al.*, 1993). The tapetum-specific expression is achieved by using the TA29 promoter isolated from tobacco. The TA29::*barnase* gene was linked to the herbicide resistance gene *bar*, allowing selection against male-fertile herbicide-sensitive progenies by herbicide treatment shortly after germination. Male sterility was restored by expressing an RNase inhibitor gene *barstar* in the tapetum. Expression of *barstar* in the tapetum has no effect on tapetal development, and plants expressing this protein are male fertile (Mariani *et al.*, 1992). This is a very elegant pollination control system and has been shown to function satisfactorily in the field for the commercial production of hybrids in oilseed rape without adverse effect on the agronomic performance of the crop. A persistent problem in hybrid breeding schemes based on nuclear or CMS is inefficient pollination of male-sterile plants. Male-sterile flowers are less attractive to honey bees than male-fertile flowers because they lack pollen and secrete only one-tenth the amount of nectar produced by male-fertile flowers, and they can also have reduced number or size of nectarines (Pernal and Currie, 1998).

Until recently, attempts to transfer the SI trait into autogamous species by a transgenic approach were not successful (Bi *et al.*, 2000). However, the structural analyses of the Brassica *S* locus regions (Cui *et al.*, 1999; Brugiere *et al.*, 2000), and the identification of the male and female determinants of SI specificity (Schopfer *et al.*,

1999; Cui *et al.*, 2000; Takasaki *et al.*, 2000) have made this approach a feasible strategy for hybrid breeding. Some haplotypes of the Brassica *S* locus is physically short and could be easily transferred as single transgene in one transformation event using the BIBAC vector (Cui *et al.*, 1999, 2000; Brugiere *et al.*, 2000), thus it is possible to transfer the genomic region covering the entire *S* haplotype into a self-fertile elite line of *B. napus*. Similarly, the male and female SI gene pair isolated from one *S* locus haplotype can also be transferred into one plant to introduce the SI trait into elite self-fertile canola lines. The *S* haplotypes may be linked to a selectable marker such as an herbicide resistance gene, allowing for relatively straightforward single-cross hybrid production (Nasrallah, 2004).

2.2 Transformation Strategies

Genetic transformation procedures have been successfully developed for all oilseed Brassicas, but by far more work has been done with *B. napus* than with any other species. Particularly, the spring canola line “Westar” has been widely used in the past. Most of these methods require an efficient plant regeneration system from either single cells or complex tissues.

The most widely used gene transfer technique in oilseed Brassica is *A. tumefaciens*-mediated transformation because it is compatible with a number of *Brassica* species, genotypes, and explants (Dale and Irwin, 1994; Dale and Irwin, 1995; Walden and Wingender, 1995; Damgaard *et al.*, 1997; Poulsen, 1996). The wild type bacterium causes the formation of crown gall tumors on infected plants by inserting DNA containing genes for synthesis of auxin and cytokinin (T-DNA from the bacterial Ti plasmid) into plant chromosomes. Disarming the T-DNA by removing the hormone genes, together with introduction of reporter genes, selectable markers, and genes of interest, has converted *A. tumefaciens* into an excellent system for genetic transformation. Explants are co-cultivated with the bacteria for a brief period (usually several days) and then cultured under conditions that kill the bacteria and allow the growth and regeneration of transformed cells. Various explants have been used for regeneration, e.g., stem internodes (Fry *et al.*, 1987), stem

segments (Pua *et al.*, 1987), cotyledonary petioles (Moloney *et al.*, 1989), hypocotyls segments (Radke *et al.*, 1988; De block *et al.*, 1989; Stewart *et al.*, 1996; Cardoza and Stewart, 2003), microspores (Jones-Villeneuve *et al.*, 1995; Souvire *et al.*, 1996; Fukuoka *et al.*, 1998; Nehlin *et al.*, 2000), and protoplasts (Herve *et al.*, 1993). However, hypocotyls remain the most commonly used explants for transformation and have been used in most transformation experiments. The virulence of *Agrobacterium* can be enhanced by using a phenolic compound called acetosyringone and it is now being used routinely in canola transformation (Halfhill *et al.*, 2001; Cardoza and Stewart, 2003). The transformation efficiency can be improved by preconditioning of the explant on callus inducing media before co-cultivation (Radke *et al.*, 1988; Ovesna *et al.*, 1993; Schroder *et al.*, 1994; Cardoza and Stewart, 2003). The co-cultivation period is also critical and a period of 2 days was found to be optimal (Cardoza and Stewart, 2003).

In planta transformation has been developed and used most extensively in the model plant *A. thaliana* (Clough and Bent, 1998). The method does not require tissue culture and plant regeneration and the procedure is simple. It includes a brief dipping of flowers in *A. tumefaciens* cultures, allowing seeds to set. Subsequently, seeds are harvested and transgenic seedlings selected. Recently, Wang *et al.* (2003b) demonstrated that a similar dipping procedure can be adopted to produce transgenic *B. napus* plants. The published current transformation efficiency is low (up to 0.018%), but once it is optimized the method is expected to have major impact on how transgenic Brassicas are produced. The advantages of *in planta* transformation are obvious, including circumventing the genotype dependence, the labor and facilities needed for tissue culture, and the ease and speed to produce transgenic plants.

Agrobacterium rhizogenes are sometimes an alternative biological vector for gene transfer and relatively high transformation efficiencies have been documented (Boulter *et al.*, 1990; Damgaard and Rasmussen 1991; Spak *et al.*, 1991; Dusbabkova *et al.*, 1993; Zaccomer *et al.*, 1993). Explants incubated with wild-type *A. rhizogenes* can integrate and express T-DNA from its Ri plasmid, leading to the formation of hairy roots. Such roots can serve as a

starting point for recovery of transformed plants, via spontaneous or hormone-induced plant regeneration from transformed hairy root cultures (Christey, 1997). Regenerated plants from such cultures share some general characteristics such as abundant formation of plagiotropic roots, wrinkled leaves, shortened internodes, reduced apical dominance, and later flowering (Tepfer *et al.*, 1989). It was demonstrated that *A. rhizogenes* strains are effective in transforming *Brassica* species by co-transformation of both the Ri plasmid and the binary vector (Boulter *et al.*, 1990; Christey and Sinclair, 1992). Plants regenerated from transgenic hairy roots show a relatively wide range of Ri phenotypes due to different expression levels of the *rol* loci of the Ri plasmid, but some normal plants carrying the gene of interest can later be obtained in progeny since the two T-DNAs may be integrated into different chromosomes and segregate (Boulter *et al.*, 1990).

Particle bombardment (biolistics, gene gun) is a widely used alternative method for direct DNA delivery for species that are not readily amenable to *Agrobacterium* infection. It has been successfully used in the generation of transgenic plants from cultured and isolated Brassica microspores (Chen and Beversdof, 1994; Fukuoka *et al.*, 1998). The method is attractive for its high efficiency compared to that of *Agrobacterium*, but there is a tendency for multiple copy insertions and potential instability of the transgenics.

Microinjection is another possible way for direct transfer of DNA. There has been only one successful report using this method. Transgenic plants were obtained when DNA was injected into the nucleus of 12-cell microspore-derived embryos (Neuhaus *et al.*, 1987); but not when uninucleate microspores were used as targets (Jones-Villeneuve *et al.*, 1995). The method, however, is potentially useful because of the precision of DNA delivery and the ability to select the targets for injection.

All the transformation methods described above for oilseed Brassicas are used for targeting the transgenes into the plant nuclear genome. This means the transgenes could be transmitted to the progeny and related weedy species through pollination, an environmental concern. Recently, a method has been developed for chloroplast transformation in *B. napus*, where the transgenes were integrated into the chloroplastic rather than

nuclear genome (Hou *et al.*, 2003). The authors inserted genes for antibiotics resistance gene and insect tolerance into a chloroplast transformation vector and introduced into cotyledon petioles by bombardment. Transgenic plants were subsequently obtained, which demonstrated insect tolerance, as expected. The plastid transformation offers at least two advantages over the nuclear transformation. First, the chloroplast traits are maternally inherited and can not be transmitted to genetically related species by pollination and thus preventing the transgene flow to related species. Second, high copy numbers of transgenes means increased transgene expression.

2.3 Selection of Transformed Tissue

For most transformation methods, only a small number of cells are infected with the introduced DNA and the majority of the cell populations do not contain the gene of interest. To give these transformed cells a selective advantage for growth and organ differentiation, it is necessary to screen them at an early stage after infection. To this end, some selectable markers are incorporated into the construct along with the genes of interest. Antibiotic resistance genes are commonly used, such as neomycin phosphotransferase (NPTII) gene from Tn5, which confers resistance to kanamycin and other aminoglycoside antibiotics neomycin, gentamycin, and also paromomycin. Another effective selection marker for Brassica transformation is *hygromycin phosphotransferase (hpt)* from *E. coli* providing resistance to hygromycin (van den Elzen *et al.*, 1985). Aminoglycoside-3-adenyltransferase (*aadA*), the gene responsible for resistance to spectinomycin and streptomycin, is also a suitable selection marker for *B. napus* (Schroder *et al.*, 1994). The herbicide-resistance gene *bar*, which confers resistance to both bialaphos- and D+L-phosphinothricin is frequently used as selectable marker (de Block *et al.*, 1989). Among the selection markers used in oilseed Brassicas, kanamycin is the predominant marker. There is, however, evidence that it limits regeneration in brassicas (Thomzik and Hain, 1990; Gupta *et al.*, 1993).

A positive selection employing the enzyme phosphomannose isomerase, which allows transformed cells to use mannose as carbon source, has been applied successfully to Brassica oilseed

transformation (Bojsen *et al.*, 1994). The selection is based on the fact that most plants are unable to metabolize mannose or mannose-6-phosphate and introduction of the phosphomannose isomerase gene that converts mannose-6-phosphate into fructose-6-phosphate facilitates the growth and selection of transformed cells and plants.

To address the concerns over the use of these marker genes, primarily from an ecological perspective, such as the possible transfer of antibiotic and herbicide resistance genes from transgenic plants to pathogenic bacteria or related weedy species, strategies to produce marker-free transgenic crops have been developed in the recent years, based on genetic segregation and site-specific DNA deletion systems (Puchta, 2003). Marker-free *B. napus* transgenic plants have been generated by using co-transformation (Daley *et al.*, 1998). Plants were transformed with a single *A. tumefaciens* strain containing two binary plasmids, each of which carried either NPTII or the reporter GUS gene. Approximately 50% of the primary transgenic plants were shown to express both the transgenes. Progeny expressing only one of the transgenes were observed in about 50% of the co-transformed lines, indicating that the genes were inserted at different loci.

2.4 Regeneration of Whole Plants

Plant regeneration occurs mainly through organogenesis for most explants but somatic embryogenesis occurs in a number of cases (Pua, 1990; Graves *et al.*, 1991). There are a number of factors that affect the efficiency of organogenesis, such as source and age of the explants, genotype, ethylene inhibitors, and media constituents. Although organogenesis at high frequency can be achieved from various explants, hypocotyl segments remain the most desirable explants for tissue culture and have been used for most *Brassica* species. A survey of 70 articles on transgenic oilseed Brassicas conducted by Pua and Lim (2004) shows that 85% employed hypocotyls and cotyledons from less than 2-week-old seedlings for the study of transformation, while only 15% used stem segments from the older plants. In seedlings, the majority of studies (72%) used explants of less than 1-week-old. The survey also shows the preference of hypocotyls (82%) over cotyledonary explants

(18%). Most researchers have found that explants excised from 3–4-day-old seedlings gave optimal regeneration rates.

Regeneration of Brassicas is highly genotype dependent. Ono *et al.* (1994) surveyed 100 *B. napus* cultivars for their ability of shoot regeneration from cotyledonary explants. A huge variation was found, ranging from 0% to as high as 99%, under the optimal explant age and plant growth regulators. Phogat *et al.* (2000) also found that the *B. napus* cultivar GSL-1 has better regeneration efficiency than Westar. Thus, genotype dependency is a limiting factor in Brassica tissue culture and regeneration and consequently transformation efficiency, which can only be overcome by *in planta* transformation strategy (Wang *et al.*, 2003b).

Ethylene inhibitors, such as aminoethoxyvinylglycine or silver nitrate, supplemented to the medium has been shown to be beneficial to regeneration of *B. campestris* (Radke *et al.*, 1992; Kuvshinov *et al.*, 1999), *B. napus* (Halfhill *et al.*, 2001; Cardoza and Stewart, 2003), and *B. juncea* (Pua and Lee, 1995; Mehra *et al.*, 2000; Prasad *et al.*, 2000). The inclusion of ethylene inhibitors is intended to inhibit the synthesis and action of ethylene that has long been found to be accumulated in cultured plant cells and tissues (La Rue and Gamborg, 1971), and cause abnormal plant growth and development (Lentini *et al.*, 1988) and also possibly affect shoot organogenesis (Pua and Gong, 2004). In addition, there are possibly many other media supplements that might increase regeneration efficiencies. For example, methylglyoxal-bis-(guanylhdyrazone), an inhibitor of spermidine biosynthesis, was reported in two studies to significantly increase regeneration frequencies (Sethi *et al.*, 1990; O'Neill *et al.*, 1996).

Somatic embryogenesis in Brassica crops is relatively lagging compared to other plant species, likely due to the advanced organogenesis techniques developed in Brassica. However, there have been a few reports on somatic embryos obtained from hypocotyls (Kohlenbach *et al.*, 1982), protoplast-derived colonies (Kranz, 1988), and immature cotyledons (Turgut *et al.*, 1998) in *B. napus*. Somatic embryogenesis has also been reported in rapid-cycling *B. napus* hypocotyl explants using MS basal medium with low pH (3.5–5) (Koh and Loh, 2000).

2.5 Testing of Transgenic Lines

Putative transgenic plants obtained after *in vitro* selection and plant regeneration need to be assayed to confirm whether the introduced gene(s) is stably integrated into the host genome and efficiently expressed. There are several standard methods commonly used to verify transgene integration and expression. Putative transgenic plants can be assayed for a selectable marker enzyme system, such as neomycin phosphotransferase or phosphinothricin acetyl transferase activity in the extracts of transgenic tissues (Reiss *et al.*, 1984; McDonnell *et al.*, 1987; Radke *et al.*, 1988). The presence of these enzymes can also be detected using antibody-based assays such ELISA and immunoblot analysis (Girich *et al.*, 1995). The *E. coli* β -glucuronidase (*uidA*) gene is very often included in the transgene construct and used as a reporter of the transformation event. The expressed β -glucuronidase activity, GUS, can be easily assayed qualitatively by histological staining or quantitatively by spectrophotometric measurement of the activity in plant extracts (Jefferson *et al.*, 1986, 1987). It is also used in promoter analysis for examining the gene expression pattern. Since early 1990s, polymerase chain reaction (PCR) has become a quick way to amplify the integrated transgenes. However, the well-established DNA blot and RNA blot analyses remain as definitive assays for transgene integration, copy number, and expression level.

Once confirmed transformants are identified, the transgene inheritance stability and segregation can be analyzed in the selfed progeny of primary transformants. The segregation data not only demonstrate whether the transgene is inheritable and efficiently expressed, but is also a good estimation of the copy number of active transgene insertion loci. The stability of gene expression in transgenics is a fundamental issue for commercial acceptance. Gene silencing and inactivation occur frequently in transgenics and in some instances may not be evident for several generations (Kumpatla *et al.*, 1997). This phenomenon exists in transgenic oilseeds but not to the extent observed in other species; there are very few reports for *Brassica* species (Blakemore *et al.*, 1995; Jones *et al.*, 1995; Metz *et al.*, 1997; Conner *et al.*, 1997). It is generally accepted that transgene

inactivation is related to gene copy number and the sequence similarity between the transgenes and endogenous genes (Finnegan and McElroy, 1996). Indeed, by using a *SRK* allele that has less sequence identity and a weaker native promoter; Takasaki *et al.* (2000) were able to circumvent the co-suppression problem and successfully expressed the *S*-locus receptor kinase gene *SRK* in *B. rapa*. There are other strategies reportedly helpful to minimize transgene silencing (Kasschau and Carrington, 1998; Vain *et al.*, 1999), but their efficacy in *Brassica* species remains to be proved.

The concept of “substantial equivalence” is currently used by all jurisdictions as a first step in evaluating whether a transgenic crop is safe for production and consumption (Kuiper *et al.*, 2001, 2002). That means that transgenic plants must have no negative attributes and be the same as its nontransgenic progenitor except for the engineered trait. Therefore, the transgenic plants need to be thoroughly analyzed, before commercial release, for pleiotropic effects that are unrelated to the intended primary trait of interest. Such unintended pleiotropic effects have been documented for transgenic oilseed Brassicas. Knutzon *et al.* (1992) developed a transgenic rapeseed line with enhanced levels of saturated fatty acid, stearic acid, in its seed oil. Later analysis revealed that the stearic acid was not only present in the storage oil as expected but also “leaked” into the membrane lipids of the developing seeds. The tiny amount of leaked stearic acid is expected to reduce membrane fluidity and lead to functional defects. Indeed, the stearate-rich seeds tended to have relatively poor germination rates (Thompson and Li, 1997). In another example, changes in chemical composition of plant tissues as a result of unrelated genetic manipulation were observed. Abidi *et al.* (1999) analyzed oil derived from different lines of transgenic canola for phospholipids, tocopherols, and phytosterols. Besides the changes in phospholipids, they also found variations in the concentration of tocopherols and phytosterol composition. These unintended pleiotropic effects were all found by analyses of certain limited target traits.

Approaches to monitor/assess the global changes caused by the insertion of transgenes have been developed in the model plant *Arabidopsis*. Similar strategies could soon be potentially

applied to transgenic oilseed crops, given the international efforts devoted to the Brassica genomics (www.brassica.info) and the fact that they belong to the same plant family and are thus genetically closely related. Recently, El-Ouakfaoui and Miki (2005) conducted a microarray analysis to search for transcriptome changes in *Arabidopsis* with strong expression of transgenes (*nptII* and *uiaA*) regulated by constitutive promoters using the *ATH1 Arabidopsis* GeneChip from Affymetrix. Their data implies very little functional disturbance to the genomes of transgenic plants by the insertion of simple T-DNA constructs, i.e., transgenic plants generated with simple T-DNA constructs containing common marker genes are fundamentally equivalent to nontransgenic plants. Similarly and conceivably, metabolomics (Keurentjes *et al.*, 2006), proteomics (Peck, 2005), and ionomics (Salt, 2004) technologies developed in *Arabidopsis* could also applied to assess the issue “substantial equivalence” of transgenic crops. However, substantial equivalence is a starting point in the safety evaluation, rather than an endpoint of the assessment, which may include immunological, toxicological, and biochemical testing (Kuiper *et al.*, 2001, 2002).

2.6 Regulatory Measures

Regulations of transgenic plants vary greatly from country to country.

2.7 Canada

In Canada, all four herbicide-resistant types of *B. napus* are regulated identically as “plants with novel traits (PNTs).” The Canadian Food Inspection Agency (CFIA) defines PNTs as “plant varieties/genotypes that are not considered substantially equivalent, in terms of their specific use and safety both for environment and for human health, to plants of the same species in Canada, having regard to weediness potential, gene flow, plant pest potential, impact on nontarget organisms, impact in biodiversity, antinutritional factors, and nutritional composition. PNTs may be produced by conventional breeding, mutagenesis, or more commonly by recombinant DNA techniques” (www.inspection.gc.ca/english/sci/biotech/reg).

2.8 United States

The use of crops produced via biotechnology is regulated by three US government agencies. Their responsibilities are complementary, and in some cases overlapping. The USDA Animal and Plant Health Inspection Service (APHIS) regulates field testing of biotechnology-derived plant varieties (www.aphis.usda.gov). The US Environmental Protection Agency (EPA) is the lead agency for evaluating crop plants with pesticidal traits developed via biotechnology. The US Food and Drug Administration (FDA) Center for Veterinary Medicine is the primary agency regulating the safety of livestock feeds developed via biotechnology. The federal government of the United States of America has a co-ordinated, risk-based system to ensure new biotechnology products are safe for the environment and human and animal health (<http://usbiotechreg.nbio.gov>). Established as a formal policy in 1986, the Coordinated Framework for Regulation of Biotechnology describes the Federal system for evaluating products developed using modern biotechnology. The Coordinated Framework is based upon health and safety laws developed to address specific product classes. A searchable database is available (http://usbiotechreg.nbio.gov/Search_pub.asp) that covers genetically engineered crop plants intended for food or feed that have completed all recommended or required reviews for food, feed, or planting use in the United States. Included in the database are a number of canola transgenic lines that were engineered for a variety of traits, such as herbicide tolerance, phytate degradation, altered fatty acid and oils, and male fertility.

2.9 Australia

There are currently no commercial transgenic canola crops grown in Australia. The Office of Gene Technology Regulator (OGTR) oversees gene technology research in Australia (www.ogtr.gov.au). OGTR has set out guidelines and advice on how GM organisms can be handled. An Institutional Biosafety Committee (IBC) must be set up for each transgenic type. The IBC ensures that OGTR guidelines and advice are followed for that particular type. The 1998 requirements for field trials in Australia were that the trial must be

400 m from any other Brassica crop, the trial area including the buffer and a 50 m area surrounding the trial must be kept free of Brassica and related weeds, a 15 m canola buffer is to be planted around the trial as a pollen trap, and canola can not be sown on the trial area for three seasons after the trial.

3. FUTURE ROADMAP

The advent of genomics opened many new doors for the biotechnology improvement of Brassica crops. The volume of expressed sequence tag (EST) collections in the public domain (GenBank) has been increasing drastically over the last few years. In parallel, proteomics and metabolomics data sets are being generated from various stage of development. A multinational consortium of *B. rapa* genome sequencing is also currently underway, and the full genome sequence information is expected to be available in the coming years. Such resources will serve as a foundation for an integrated understanding of genome composition, gene expression profile, post-transcriptional regulation of gene products, and interaction of the metabolic network, all of which are important for biotechnology improvement of plant performance and crop productivity.

An emerging trend in Brassica biotechnology is the appreciation of complementarities between traditional breeding efforts employing classic genetics and molecular biology approaches predominated by target gene identification. Many of the agronomic traits are governed by genetic components that exert quantitative control. Aided by the availability of breeding populations developed through DH technology first established several decades ago (Keller *et al.*, 1975, 1977), genetic linkage analyses aimed at fine mapping of QTL in Brassica have been plentiful (Liu *et al.*, 2006). Identification of specific genes corresponding to the QTLs, however, remains a major obstacle. The last few years have witnessed many successes in the combined application of genomic tools with genetic linkage analysis to identify QTLs in *Arabidopsis* (Keurentjes *et al.*, 2006). Similar research is now expanding to crop species. Research in Brassicaceae has the unique advantage of extensive genome synteny between

Brassica crops and *Arabidopsis* (Parkin *et al.*, 2005). In practice, what this would mean is that many of the QTLs relevant to agronomic traits can be aligned to specific regions in the *Arabidopsis* genome within which a guided and focused search can be conducted to identify the corresponding genes. Furthermore, the voluminous EST database enables the development of comprehensive gene chips in Brassica, which will be available from several sources in the near future. This would allow mapping of hundreds of expression QTL (eQTL) and integrated mining of combined genetic linkage and expression experiments (Clarke and Zhu, 2006).

Improvement of seed yield remains a top priority in Brassica biotechnology. Such a need is further accentuated by the potential of seed oil as a renewable resource for biodiesel. While biofuel can only contribute to the overall energy scheme as a supplement to the petroleum industry in the foreseeable future, the superior efficiency of seed oil in energy conversion (Hill *et al.*, 2006), and its unique property as lubricant in enhancing engine fuel efficiency, can not be ignored. Nonetheless, in comparison to cellulose, considered a major bioenergy solution, a constraining factor of seed oil based biofuel is the limited biomass crops produce. With extensive efforts on study of QTL genes concerning yield, delineation of primary genomic control points that can serve as genetic modification targets will pave the road for future endeavors in this direction. Enhancing oil content through modifying lipid metabolism offers a matching opportunity to seed yield, and there have been reported successes under green house conditions in this regard (Jacko *et al.*, 2001). TFs that control seed oil deposition in *Arabidopsis* have also been identified (Cernac and Benning, 2004). Utility of transgenic alteration of such genes is expected to enhance oil yield in Brassica crops. Despite of these progresses, there is still a pressing need for extensive field trial experiments to assess the potential of these technologies under variable environmental conditions. Recently, a major breakthrough in understanding carbon flux control in developing seed of Brassica revealed a new pathway that employs Rubisco without the Calvin cycle to increase the efficiency of carbon use during the formation of seed oil (Schwender *et al.*, 2004). Better understanding of this pathway, known as the "Rubisco bypass", promises to

provide insight into the regulation of storage lipid biosynthesis and has obvious ramifications for improving seed oil content.

A variety of metabolites possessing either positive or negative health effects exist in Brassica. As a major source of vegetable and edible oil for human consumption, Brassica crops have been the subjects of extensive efforts in biotechnology improvement of nutritional value. Carotenoids and vitamin E contents in oil have been the major targets for quite some time (Shewmaker *et al.*, 1999; Ravanello *et al.*, 2003; Kumar *et al.*, 2005). Such a trend will likely to continue. Phytosterol, a significant component in seed oil with well-established effects in lowering the risk of cholesterol and cardiovascular associated diseases (Ostlund, 2002), has attracted considerable research efforts. In recent years, Brassica seed meal as a source of animal feed has also been explored. Phytate, glucosinolate, flavonoids, anthocyanins, and proanthocyanidins metabolism are all being targeted for improvement of meal quality. Studies in a broad spectrum of genes encoding enzymes of various biochemical pathways have generated mounting evidence suggesting that metabolic regulation is often distributed in, not one, but multiple steps of metabolic pathways. Thus, increasingly, transgenic efforts are gearing toward introduction of multiple genes, commonly referred to as "stacking" strategy, in order to achieve maximum effects in metabolic pathway engineering. Such a strategy generally requires several individual gene expression cassettes, each one of them would demand a separated promoter to drive a tissue-specific gene expression. Since identical promoters introduced into the same genome contour usually lead to co-suppression during the subsequent generations of the transgenic plants, identifying promoters differing in sequence content but similar in tissue specificity are desirable for the purpose of performing multiple gene transformation.

A research area crucially important for the agricultural production of Brassica crops worldwide is to improve crop efficiency in soil nutrient utilization, particularly with regard to nitrogen (N) and phosphorus (P) (Raghothama and Karthikeyan, 2005). N and P are predominant nutrient factors limiting plant productivity. Applications of N and P fertilizers bring major economic burdens to farmers in developing countries, and modern

agricultural reliance on fertilizer is also believed to have had negative impact to the environment, particularly on ground water quality and aquatic ecosystems. Unlike other major crops, Brassica species do not establish symbiotic interactions with microrhiza. Therefore, improvement of nutrient utilization would have to be focused solely on the plants. There has been major progress in understanding plant responses at the metabolic and gene expression level (Wu *et al.*, 2003). Various advancements in transgenic improvement of P acquisition have also been reported. But there is a need for continued search for novel approaches of genetic engineering for not only nutrient uptake but also efficient utilization.

Release of transgenic Brassica crops has been scrutinized intensively as it should be for a technology that has such a broad relevance to our daily life. The debate continues. A contentious issue particularly concerning transgenic Brassica crops is the separation of food crops from the ones destined for industrial usage (Miller, 2003). As outlined in other sections of this chapter, *Brassica* species are increasingly viewed as an amendable and productive host for producing industrial feedstocks. Indeed, high erucic Brassica cultivars generated through traditional breeding efforts have been cultivated in the Canadian prairies for quite some time (Scarth *et al.*, 1991). However, any real or perceived risk of gene flow from transgenic industrial crops to canola industry as a whole. Fortunately, in addition to *B. napus*, *B. juncea*, *B. carinata*, and *B. rapa* that are traditional crops, there are also other species such as *C. abyssinica* and *Camelina sativa*, which are genetically and metabolically closely related to oilseed crops. Within this context, it can not be overemphasized that future efforts are in urgent need to develop these species into platform hosts for genetic engineering of industrial crops.

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Sunflower

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1. INTRODUCTION

Cultivated sunflower (*Helianthus annuus* L.) is the only significant agricultural commodity with a North American center of origin. There are two primary types of cultivated sunflower: oilseed sunflower and confectionary sunflower. Oilseed sunflower represents the largest cultivated area of the two and boasts seed oil content in excess of 40%. Most of the seed from this type is crushed to extract its valuable oil that has uses in both food and industrial applications. Oilseed sunflower is also sold as birdseed and is used to some extent to augment feed rations in the poultry, dairy, and beef industry.

Oilseed sunflower is one of the most important oilseed crops in the world and is the preferred source of oil for domestic consumption and cooking in much of central and eastern Europe. Sunflower oil is stable at high temperatures, has a neutral taste, contains less than 11% total saturated fat and does not contain any trans fat. A recent study conducted at Harvard University indicates that consuming sunflower oil in the diet helps to reduce the level of “bad” cholesterol (low-density lipoprotein, LDL) and increases the level of “good” cholesterol (high-density lipoprotein, HDL) while reducing total cholesterol levels in the body (<http://www.sunflowernsa.com>). These characteristics make sunflower oil the preferred oil for health conscious consumers. In an effort to improve the quality and healthfulness of its

products, Frito Lay, the largest salted snack manufacturer in the world, recently announced that by 2007 only sunflower oil will be used in the manufacture of Lays and Ruffles brand potato chips as well as a host of other core snack offerings (<http://www.sunflowernsa.com>).

Confectionary sunflower produces large seeds with low oil content (less than 20%) that are sold as a salted snack in the shell and consumed like peanuts or de-hulled and used in bread or other baking and snack applications. Salted confectionary sunflower seeds have grown in popularity in the United States as celebrity baseball players help to associate the snack with the popular sport. In the last several years, salted confectionary sunflower seeds have become as common at baseball games as hot dogs and cracker jacks.

The inexpensive production of biodiesel from renewable plant vegetable oils such as sunflower oil has been achieved. The demand for biodiesel is increasing rapidly. Furthermore, sunflower can produce high quality rubber in its leaves and stems. The various usages of sunflower products in food, feed, and industry are stimulating the development of sunflower molecular breeding in combination with conventional and transgenic breeding methods. This chapter describes the history of sunflower as an oil seed crop, summarizes the achievements of conventional and transgenic breeding, and discusses the impact of new technologies on sunflower product development.

1.1 History, Origin, and Distribution

Sunflowers of the genus *Helianthus* comprise 49 species native to the Americas that are widely distributed from Mexico to Canada and all of which are found in the United States (Seiler and Rieseberg, 1997). Sunflower has a long history as an important domesticated plant in North America. Much evidence suggests that monocephalic (single headed) sunflower biotypes were cultivated by prehistoric native North American people and may have been domesticated even before corn (*Zea mays*) (Seiler and Rieseberg, 1997). Carbon-14 dating has provided evidence that sunflower was cultivated in the Missouri–Mississippi basin over 2800 years ago (Lees, 1965). After Europeans colonized North America there were many references to the cultivation of sunflower by native people. Heiser (1951a, b) has excellently reviewed this subject.

It is assumed that domesticated sunflower was introduced into Europe by Spanish explorers to the new world. The earliest record is of sunflower seed from New Mexico obtained by a Spanish expedition in 1510 (Zukovsky, 1950). The herbalist Dodoneus made the first published description of sunflower in 1568 describing single-headed forms in Spain that appeared much like the modern cultivars of today. Sunflower spread from Spain into Italy and France where it was used as an ornamental plant in gardens. By the late 16th century it was growing in gardens in Belgium, the Netherlands, Switzerland, Germany, and England (Heiser, 1951a, b; Semelcczi-Kovacs, 1975). As sunflower became widely grown as a garden ornamental in Europe, people began eating the seeds. The custom of eating seeds drove the selection of biotypes with large single heads and large seeds, and promoted the development of landrace cultivars during the late 16th and early 17th centuries.

The first record of extracting oil from sunflower seeds is an English patent (No. 408) that was granted to Arthur Bunyan in 1716. The patent references the use of oil in industrial applications such as the manufacture of paint and wool products. Peter the Great is credited with introducing sunflower into Russia in the 18th century (Zukovsky, 1950) with seed brought from the Netherlands (Semelcczi-Kovacs, 1975). Initially, it was cultivated as an ornamental but by 1779,

sunflower was being cultivated for oil extraction (Gundaev, 1971). By 1830, the extraction of sunflower oil began on a commercial scale in Russia (Atkinson *et al.*, 1919; Quesenberry *et al.*, 1921; Clydesdale and Hart, 1948).

By 1854, cultivation of sunflower for oil had expanded significantly and in the region of Voronzeth, 84 small mills were producing some 2000 tons of oil per year. By 1880, the crop had introduced into Ukraine and in Russia, there were 150 000 ha of sunflower in production (Semelcczi-Kovacs, 1975). By 1915, the area under production in Russia had grown to 0.9 million ha and consisted of two principal varieties: a thin hulled oil type containing between 20% and 30% oil and a large-seeded, thick-hulled variety with low oil content that was grown for direct human consumption (Severin, 1935).

Russian peasants began selecting local cultivars in home gardens with earlier maturities than the original introductions that required long growing seasons and were restricted to warmer environments (Gundaev, 1971). By 1925, breeding programs had commenced at several agricultural institutes, the most important being the institute at Krasnodar where V.S. Pustovit succeeded in raising yield and oil content such that by 1965, he was testing varieties with 55% oil. In other European countries, the adoption of sunflower as a major oilseed paralleled that of Russia. By 1900, sunflower was the primary oilseed crop in Hungary and was being exported to western Europe and the United States (Semelcczi-Kovacs, 1975). France was exporting oil cake to Britain in the middle 1800s. In Romania there were over 320 000 ha of production by 1948 and in Bulgaria, sunflower production had grown to 156 000 ha by 1936 (Semelcczi-Kovacs, 1975). In the Ukraine, production currently exceeds 2 million ha.

Sunflower was reintroduced to North America late in the 19th century with high-yielding Russian cultivars. Two seed companies were offering the variety “Mammoth Russian” by 1880 (Heiser, 1976). It is likely that Russian immigrants brought their favorite cultivars to North America even before this time. While sunflower was an important oilseed in Europe, it was first cultivated in North America as a high-yielding silage crop that was comparable in nutritive value to corn (Wiley, 1901). In the early 1900s, the states with the largest

sunflower production were Missouri, Illinois, and California (Hensley, 1924; Sievers, 1940).

Between 1930 and 1940, the Canadian government requested the Canadian Department of Agriculture to examine sunflower as a potential oilseed crop to reduce Canada's dependency on foreign sources of edible oil (Putt, 1997). Tall, late maturing silage cultivars like Mammoth Russian were not adapted to Canada's short growing season and earlier cultivars that were amenable to mechanical harvest were needed if the crop were to be viable (Putt, 1997). In 1936, about 400 single sunflower heads that were representative of the cultivars that had been selected for earlier maturity and grown by Mennonites in home gardens were collected from the gardens of three farmers in the community of Rosthern (Putt, 1997). In addition to the Mennonite material, about 20 inbred lines from the Central Experiment Farm in Ottawa and 20 cultivars from Russia were planted in progeny rows to form the first major sunflower-breeding nursery in Canada (Putt, 1997).

Inbreeding and selection for desirable agronomic characteristics such as reduced plant height, early maturity, stalk strength, and high seed yield commenced in 1937 and it was observed that many of the Russian cultivars were uniform. One of these cultivars, "S-490" was of short stature, matured early, and produced small seeds with high oil content. It was increased and licensed as the cultivar "Sunrise" in 1942. At the same time the edible oil supply in Canada was critically restricted because of World War II (Putt, 1997).

After 1943, Mennonite farmers who occupied the Red River valley of Southern Manitoba province grew the cultivar "Sunrise" as a cash crop that was more valuable than oats (*Avena sativa*) and barley (*Hordeum vulgare* L.) and was profitable as corn and wheat (*Triticum aestivum*). By 1949, over 24 000 ha were in production in Manitoba and about 8000 ha were being grown in the Southern Red River Valley in the states of Minnesota and North Dakota in the United States (Putt, 1997).

Putt (1940) recognized that cross-pollination occurred at a frequency up to 78% between cultivars. This observation led to the exploitation of heterosis in sunflower hybrids that outyielded inbred parents by up to 250% and outyielded open-pollinated cultivars by up to 60%. One hybrid called "Advance" was released to the public and was produced by crossing a highly

self-incompatible female line descended from Mennonite cultivars with Sunrise as the male. Unfortunately, when the hybrid was produced on a commercial scale, only about 45% of the seed was a true hybrid thus the full heterotic potential could not be realized (Putt, 1997).

Two significant events occurred that would have a lasting impact on the sunflower industry in North America. The first was the introduction of the early maturing cultivar "Perdovic" that had similar yield but higher oil content than "Advance" and other partial hybrids that relied on natural outcrossing and self-incompatibility for seed production (Putt, 1997). The second event was the discovery of cytoplasmic male sterility and fertility restoration genes by the French breeder Leclercq in 1968 (Enns *et al.*, 1970; Kinman, 1970).

With the advent of a cytoplasmic male sterility and fertility restoration system, commercial production of 100% hybrid seed was possible. This greatly facilitated breeding and allowed producers to realize the full potential of heterosis, dramatically increasing yields. Today, sunflower is grown on every continent where crops are cultivated and is the second largest hybrid crop in area after corn.

1.2 Botanical Description

1.2.1 Taxonomy

Helianthus is a member of the subtribe *Helianthinae* of the family *Asteraceae* (Robinson *et al.*, 1981; Seiler and Rieseberg, 1997). Within *Helianthinae*, *Helianthus* is a member of the *Viguiera* assemblage, which comprises *Viguiera*, a large genus of approximately 160 species, as well as a number of smaller, related genera including *Helianthus*, *Helianthopsis*, *Heliomeris*, *Iostephane*, *Pappobolus*, *Phoebanthus*, *Simsia*, and *Tithonia* (Schilling and Jansen, 1989; Seiler and Rieseberg, 1997). This group of genera are characterized by a base chromosome number of $x = 17$ (Robinson *et al.*, 1981) with an estimated genome size of 2871–3189 Mbp (mega base pair) (Arumuganathan and Earle, 1991), the similar nature of the pappus and pales, production of similar floral chalcone/aurone compounds (Crawford and Stuessy, 1981), and the presence of numerous chloroplast deoxyribonucleic acid

(cpDNA) restriction site mutations (Seiler and Rieseberg, 1997).

The genus *Helianthus* has undergone considerable reorganization since Linnaeus originally described nine species in the genus in 1753 (Seiler and Rieseberg, 1997). Herein is presented a modified version of classification of Schilling and Heiser's that was presented by Seiler and Rieseberg in 1997. Seiler and Rieseberg make three minor modifications to the earlier work by Schilling and Heiser: (1) the sectional name *Atrorubens* is given taxonomic priority replacing the section *Divaricati* E.; (2) *Helianthus exilis* A. Gray is recognized as a species instead of an ecotype of *H. bolanderi* A. Gray; (3) the species name *H. pauciflorus* Nutt. was given priority over *H. rigidus* (Cass.) Desf. (Table 1).

1.2.2 Distribution

There are 49 sunflower species of the genus *Helianthus* and all are native to the Americas (Seiler and Rieseberg, 1997). Some species are rare and endangered and restricted to unique environments while others are widespread throughout North America. The genus *Helianthus* is represented by both perennial and annual species with remarkable diversity and found in a wide range of habitats from newly disturbed areas to tall grass prairie and even climax forests (Seiler and Rieseberg, 1997). For a detailed review of species-specific distribution in North America, see *Sunflower Species of the United States* (Rogers et al., 1982).

Several species have been domesticated as ornamentals including the annual species *H. annuus*, *Helianthus argophyllus*, *Helianthus debilis* Nutt., and the perennial species *Helianthus decapetalus* L., *Helianthus maximiliani* Schrad., and *Helianthus salicifolius* among others (Seiler and Rieseberg, 1997). Two wild sunflower species have the distinction of being the only important food plants that were domesticated during prehistoric times in what is now the United States of America; *H. annuus* L. which gave rise to modern cultivated oilseed and confectionary hybrids and *Helianthus tuberosus* L., a perennial species that is cultivated for its edible tuber (Seiler and Rieseberg, 1997).

Charles Heiser at the Indiana University accomplished early work with the *Helianthus* genome involving collection and interpretation of

Table 1 Infrageneric classification of *Helianthus*^(a)

Section	Series	Species
<i>Helianthus</i>	—	<i>H. annuus</i> L. ^(b) <i>H. anomalous</i> Blake ^(b) <i>H. argophyllus</i> T. and G. ^(b) <i>H. bolanderi</i> A. Gray ^(b) <i>H. debilis</i> Nutt. ^(b) <i>H. deserticola</i> Heiser ^(b) <i>H. exilis</i> A. Gray ^(b) <i>H. neglectus</i> Heiser ^(b) <i>H. niveus</i> (Benth.) Brandege ^(b) <i>H. paradoxus</i> Heiser ^(b) <i>H. peteolaris</i> Nutt. ^(b) <i>H. praecox</i> Engelm. and A. Gray ^(b) <i>H. agrestes</i> Pollard ^(b)
<i>Agrestes</i>	—	
<i>Ciliares</i>	<i>Ciliares</i>	<i>H. arizonensis</i> R. Jackson <i>H. ciliares</i> DC <i>H. laciniatus</i> A. Gray <i>H. cusickii</i> A. Gray <i>H. gracilentus</i> A. Gray <i>H. pumilus</i> Nutt.
<i>Ciliares</i>	<i>Pumili</i>	
<i>Atrorubens</i>	<i>Corona-solis</i>	<i>H. californicus</i> DC. <i>H. decapetalus</i> L. <i>H. divaricatus</i> L. <i>H. eggertii</i> Small <i>H. giganteus</i> L. <i>H. grosseserratus</i> Martens <i>H. hirsutus</i> Ralf. <i>H. maximiliani</i> Schrader <i>H. mollis</i> Lam. <i>H. nuttallii</i> T. and G. <i>H. resinosus</i> Small <i>H. salicifolius</i> Dietr. <i>H. schweinitzii</i> T. and G. <i>H. strumosus</i> L. <i>H. tuberosus</i> L.
<i>Atrorubens</i>	<i>Microcephali</i>	<i>H. glauophyllus</i> Smith <i>H. laevigatus</i> T. and G. <i>H. microcephalus</i> T. and G. <i>H. smithii</i> Heiser
<i>Atrorubens</i>	<i>Atrorubens</i>	<i>H. atrorubens</i> L. <i>H. occidentalis</i> Riddell <i>H. pauciflorus</i> Nutt. <i>H. silphoides</i> Nutt.
<i>Atrorubens</i>	<i>Angustifolii</i>	<i>H. angustifolius</i> L. <i>H. carnosus</i> Small <i>H. floridanus</i> A. Gray ex Chapman <i>H. heterophyllus</i> Nutt. <i>H. longifolius</i> Pursh. <i>H. radula</i> (Pursh) T. and G. <i>H. simulans</i> E. E. Wats.

^(a)Reproduced from Seiler and Rieseberg (1997) and with permission from the National Sunflower Association

^(b)Annual species, all other species are perennial

morphological and cytological data in a series of classic studies from the 1940s to the 1960s (Heiser, 1947, 1949, 1951a, b, 1954, 1965; Seiler and Rieseberg, 1997). Heiser demonstrated the natural occurrence of several interspecific hybrid derivatives in the genus and postulated that natural hybridization and subsequent interspecific gene exchange via natural introgression led to increased morphological and genetic variation in some species that allowed an expansion of their range and may even have led to the formation of introgressive races tending toward *H. debilis*, *H. argophyllus*, *Helianthus Petiolaris*, and perhaps *Helianthus bolanderi* (Seiler and Rieseberg, 1997).

More recently, molecular genetic studies by Rieseberg and others have largely confirmed several of Heiser's hypotheses regarding the important role of introgressive hybridization in the evolution of the *Helianthus* genus (Rieseberg *et al.*, 1990, 1991; Dorado *et al.*, 1992; Seiler and Rieseberg, 1997). For more detailed review of this subject, see Seiler and Rieseberg (1997), Jan (1997), and Jan *et al.* (1998).

1.3 Economic Importance

1.3.1 Planted area

Sunflower is currently grown in North and South America, Europe, Africa, Asia, and Australia. It is the second most important hybrid crop in the world after corn and provides the primary source of vegetable oil in many countries, especially eastern Europe. Table 2, reproduced with the permission from the National Sunflower Association in the United States, describes cultivated hectares and utilization by region from 2000 to 2005. From the table, it is evident that most sunflower seed is consumed domestically and the European Union is the principal net importer of sunflower seeds.

1.3.2 Uses

Sunflower is grown for several uses including oil for cooking and baking, oil for the manufacture

Table 2 Sunflower seed world supply and utilization^(a)

	2000/01	2001/02	2002/03	2003/04	2004/05	2005/06
Area harvested						(forecast)
(1000 ha)	19 540	18 485	19 892	22 918	21 262	22 791
Yield (mt ha⁻¹)	1.18	1.18	1.2	1.17	1.23	1.29
Seed production						
Argentina	2950	3720	3340	2990	3650	3800
Eastern Europe	1657	1861	1648	2295	2270	1950
European Union	3333	3030	3718	4078	4133	3765
China	1954	1750	1946	1820	1750	1850
Russia/Ukraine	7368	4936	7194	9348	8001	10450
United States	1608	1551	1112	1209	930	1824
India	730	870	1060	1160	1300	1250
Turkey	630	530	830	560	640	790
Other	2880	3551	3108	3467	3505	3665
Total	23 110	21 799	23 956	26 927	26 179	29 344
Seed import						
Mexico	23	10	104	38	11	23
European Union	1999	1155	1007	1473	763	1000
Other	704	467	812	1249	813	801
Total	2726	1632	1923	2760	1587	1824
Oilseed crushed	21 116	18 514	21 149	23 442	23 115	25 510
Seed export						
Argentina	94	342	232	44	97	121
United States	153	176	122	136	116	225
Russia/Ukraine	1768	100	524	1271	73	560
Other	711	1084	1112	1277	1257	957
Total	2726	1702	1990	2728	1543	1863

^(a)Reproduced with permission from the National Sunflower Association

of vitamin E supplements, seeds for snack and baking applications, birdseed, and in some cases as a ration component for the poultry, dairy, and beef industries (Dorrell and Vick, 1997; Lofgren, 1997; Park *et al.*, 1997). Sunflower is also widely used as an ornamental plant in gardens all over the world and is used to beautify public by ways such as railways in Japan. In limited instances, sunflower is also grown as a forage crop. Native Americans used sunflower both as a source of food and as a source of pigment in prehistoric times.

Sunflower oil is produced from oilseed sunflower varieties. Sunflower oil is light in taste and appearance and contains more vitamin E than any other vegetable oil. The oil consists primarily of monounsaturated and polyunsaturated fats with low levels of total saturated fats and no trans fat. Sunflower oil is a liquid at room temperature. It is the preferred choice for cooking in most households in central and eastern Europe but is virtually unknown to consumers in the United States where other oils like canola, corn, and soybean have dominated supermarket shelves for years. Most supermarkets in the United States currently do not offer bottled sunflower oil for sale to consumers.

There are three primary oilseed sunflower classes that are differentiated by their fatty acid profiles: linoleic, mid-oleic, and high oleic sunflower (Dorrell and Vick, 1997). Linoleic sunflower is the traditional and most widely cultivated form of the oilseed types. Its oil is predominantly composed of linoleic acid, which is an ω -6 fatty acid. Linoleic acid is one of two essential fatty acids required for human health that must be consumed in food. Linoleic sunflower oil is composed of about 65% polyunsaturated linoleic acid, 21% monounsaturated oleic acid, and about 11% total saturates (palmitic and stearic fatty acids). Linoleic sunflower oil is the preferred cooking oil in much of central and eastern Europe. Mid-oleic sunflower oil is characterized by oleic acid content between 55% and 75% with the balance comprised of linoleic acid and saturated fatty acids. Mid-oleic sunflower contains less than 10% saturated fat, which is lower than linoleic sunflower oil. Mid-oleic sunflower oil is stable at high frying temperatures without the need for partial hydrogenation, a process that is used to stabilize other oils like soybean oil. The hydrogenation process results in the conversion of

some fatty acids to trans fat, which has recently been found to increase the risk of heart disease in humans. The US Food and Drug Administration (FDA) has stated that no level of trans fat in the diet is safe and recent US legislation requires all food manufacturers to list the amount of trans fat present on food products in the United States.

In the United States, mid oleic sunflower is the preferred oil type for commercial frying applications due to its relatively high oxidative stability, health benefits, and neutral taste profile. Crossing a linoleic inbred line with a high oleic inbred line produces a mid oleic sunflower hybrid. The resulting progeny and harvested seed exceed the 55% oleic acid threshold that defines the mid oleic category. Mid oleic sunflower has been given the trademark NuSunTM by the National Sunflower Association in the United States. NuSunTM sunflower oil has recently been adopted by Frito Lay, the largest salted snack manufacturer in the world, as the sole source of oil utilized in Ruffles, Lays, and several other branded chips and snacks including the Lay's "Natural" line. Like all sunflower oil, NuSunTM contains no trans fat, has significantly lower levels of total saturated fats than conventional hydrogenated oils, and provides desirable frying stability and better taste attributes than alternatives. NuSunTM oil has only been available to manufacturers since 1998 when commercial volumes became available for the first time following efforts by the National Sunflower Association to promote commodity-priced sunflower oil that was stable at frying temperatures and low in saturated fats. Before then, only linoleic and high oleic varieties were grown in the United States (Dorrell and Vick, 1997).

High oleic sunflower boasts an oleic acid content exceeding 80%. High oleic sunflower oil is used both in food and industrial applications including spray coatings for cereals, crackers, and dried fruits and use in nondairy creamers. In Europe, high oleic sunflower oil is blended with linoleic sunflower oil to produce targeted oil profiles for niche market applications or is used directly for specialty uses including the manufacture of baby foods and hydraulic fluids. In the United States, high oleic sunflower oil is used in food and industrial applications (Dorrell and Vick, 1997).

Confectionary sunflower, characterized by its large, thickly hulled seeds are sold as salted

snacks in the shell or are de-hulled for use in baking and snack applications. In China, salted confectionary sunflower is a popular snack and can be found in markets across the country. In the United States, salted confectionary sunflower seeds have grown in popularity and are sold at most major league baseball parks. They are available as traditional salted seeds and recently there has been an explosion of flavored offerings including cool ranch, pickle, and hot chili flavors.

1.4 Traditional Breeding

1.4.1 Breeding objectives

As with other commodity crops, the primary objective for sunflower breeders is to increase the overall yield and agronomic performance of newly developed confectionary, high oleic, linoleic, and NuSunTM hybrids (Fick *et al.*, 1974). To accomplish these goals, breeders need to address pathogens, pests, and environmental constraints that have the potential to drastically reduce yield where sunflowers are grown. Chief among these constraints are diseases including *Sclerotinia* (*Sclerotinia sclerotiorum*), downy mildew (*Plasmopara halstedii*), rust (*Puccinia helianthi*), *Phomopsis* (*Phomopsis helianthi*), *Verticillium* wilt (*Verticillium dahliae*), Charcoal rot (*Macrophomina phaseolina*), and head rot (*Rhizopus* spp.).

Other breeding considerations include increasing resistance to insect pests such as moths (*Homoeosoma nebulellum*, *Homoeosoma electellum*, *Cochylis hospes*, and *Suleima helianthana*), seed weevils (*Smicronyx sordidus*, *Smicronyx fulvus*), sunflower midge (*Contarinia schulzi*), sunflower beetle (*Zygogramma exclamationis*), and wireworms (*Pterohelaeus altenatus*) among others. Breeders also strive to increase inbred yields to lower the cost of hybrid production and develop hybrids with shorter plant height and a range of maturities adapted to a wide range of growing environment. Stalk strength, lodging resistance, drought resistance, and salt tolerance are other breeding targets.

Breeders in some markets including Turkey, Spain, Romania, and the Ukraine must develop genetic resistance to the rapidly evolving parasitic plant broomrape (*Orabanche cummana*) that can completely destroy farmers' fields if resistant

hybrids are not utilized. Recently, breeders have incorporated resistance to imidazolinone (IMI) and Sulfonyleurea herbicides into sunflower hybrids to improve weed control options for farmers. The chemical company BASF labeled the herbicide "Beyond" for use on Imazimox-tolerant hybrids in the United States in 2003. DuPont labeled the Express[®] SX herbicide for use on sunflower hybrids with tolerance to tribenuron-methyl in Romania, Hungary, and Bulgaria in 2005 and will label Express for use on resistant hybrids in the United States in the spring of 2007. Pioneer Hi-Bred International, Inc. was the first seed company to market Express-tolerant sunflower and other companies are expected to enter the market in the near future.

Breeders of oilseed hybrids seek to increase the oil content of new hybrids and breeders of confectionary sunflower seek to improve seed size, de-hulling ability, and seed color characteristics. All of these considerations, including those listed in the section above, must be made while striving to improve the heterotic yield potential of newly developed hybrids.

1.4.2 Tools and strategies

Sunflower has been bred as a hybrid crop for 40 years following Leclercq's discovery of cytoplasmic male sterility and fertility restoration genes (Leclercq, 1969). There are tremendous opportunities for yield gain and improvements in disease and pest resistance through conventional breeding that have not yet been made. Additionally, there are several wild annual and perennial species that outcross readily with cultivated *H. annuus* that provide a rich source of novel yield and disease quantitative trait loci (QTL) that might be exploited through conventional breeding. For a detailed review of species that cross readily with *H. annuus*, see "*Sunflower Species of the United States*" (Rogers *et al.*, 1982).

Sunflower breeders have recognized the value of exotic germplasm from wild species for decades and several publicly funded breeding programs such as Fundulea in Romania, the institute at Krasnodar in Russia, and the United States Department of Agriculture's breeding center in Fargo, North Dakota have had active wild species breeding programs for years. These programs

have fostered the improvement of elite germplasm through the identification and incorporation of disease resistance genes from wild species including genes for downy mildew resistance, rust resistance, and *Sclerotinia* tolerance. Improved germplasm from these institutions has been made available to private seed companies through public releases that can be obtained for a nominal fee.

In 1990, the United States Congress authorized the establishment of a National Genetics Resource Program with responsibility to acquire, characterize, preserve, document, and distribute to scientists germplasm of life forms important to food and agriculture. The United States Department of Agriculture's Agriculture Research Service maintains a web-based Germplasm Resources Information Network (GRIN) that lists available germplasm including exotic sunflower accessions that are available to breeders free of charge. For details about this tremendous resource, see the GRIN website at <http://www.ars-grin.gov>. Currently, there are roughly 3000 accessions of *Helianthus* that are maintained at the North Central Regional Plant Introduction Station in Ames, Iowa.

Since about 1970, sunflower hybrid breeders have developed inbreds from two primary heterotic pools: a male pool and a female pool. The male pool is fixed for fertility restoration genes and is typically fixed for recessive branching genes. Recessive branching in males is a desirable trait that extends the pollen shed of males in hybrid production fields ensuring pollen availability when females are flowering. Fertility restoration genes are necessary to restore fertility to hybrids made with sterile females so that the hybrids can self-pollinate to produce harvestable seed. Males are called restorer lines as they restore fertility to cytoplasmic male-sterile females as described above.

From the female pool, breeders develop two analogous single-headed biotypes: a fertile "maintainer" (B line) and a male sterile (A) line. The maintainer line carries the wild-type cytoplasm rather than the sterile one; it is fertile but does not carry fertility restoration genes for the sterile cytoplasm. The male sterile (A) line carries the sterile cytoplasm, but also lacks restorer genes for that cytoplasm. The sterile (A) line is developed by backcrossing the nuclear genotype of the maintainer into the sterile cytoplasm. When

the maintainer line is crossed onto the male sterile line, the offspring are all sterile. To make commercial hybrid seed, the sterile (A) line is planted in alternating strips with a restorer (male) inbred. The hybrid seed is harvested from the sterile strips. The maintainer is needed to multiply, or "maintain" the A line.

Breeders develop new inbreds within the male pool by crossing elite restorers, and within the female pool by crossing elite maintainers. From a line development standpoint, breeding in the male pool requires less time than breeding in the female pool as newly developed lines can be evaluated in hybrid combinations for yield and other agronomic characteristics as soon as they appear to be genetically and morphologically uniform, typically after 4–6 generations of inbreeding following the initial cross. Female inbred development takes longer as the maintainer line must be developed first, then the sterile (A) line analogue must be developed through backcrossing.

To accelerate new product development, breeders typically employ alternate environments to achieve two or more generations per year. If breeding in the northern hemisphere for example, a breeder may exploit a southern hemisphere environment or a low latitude tropical location during winter months to achieve an additional generation. In India, where temperature is not limiting but where natural moisture constrains the growing environment, sunflower breeders exploit the natural fluctuation of rainy seasons in different geographies to achieve two generations per year.

In cases where breeders have selectable markers for desirable traits like herbicide resistance or disease resistance, backcrossing is employed to convert proven elite lines lacking these traits. This is often accomplished in greenhouses or tropical environments where two or even three generations can be obtained per year. Sunflower floral induction and maturity is influenced by day length and by the accumulation of heat units and the combination of these factors differs widely with genotype. In some cases, a breeding cycle can be shortened by manipulating these parameters in a growth chamber or by growing the material in short-day tropical environments like Hawaii. Another tool that is sometimes employed to shorten cycle time is embryo rescue, where immature embryos are excised and grown in tissue

culture, permitting breeders an opportunity to artificially shorten the time required to recover new progeny.

1.4.3 Breeding with molecular markers

There have been considerable discoveries in the public sector that have facilitated the advancement of molecular breeding in sunflower. The first molecular genetic linkage maps of cultivated sunflower were developed by using restriction fragment length polymorphism (RFLP) markers (Berry *et al.*, 1994, 1995, 1996, 1997, 2003; Gentzbittel *et al.*, 1992, 1995, 1999; Jan *et al.*, 1998) and random amplified polymorphic DNA (RAPD) markers (Rieseberg *et al.*, 1993; Rieseberg, 1998; Yu *et al.*, 2003). Subsequently, several genetic linkage maps were constructed using amplified fragment length polymorphisms (AFLP) markers (Peerbolte and Peleman, 1996; Gedil *et al.*, 2001b). RAPDs have been used for tagging phenotypic loci in sunflower such as rust and broomrape resistance genes (Lawson *et al.*, 1998; Lu *et al.*, 2000b). More recently, simple sequence repeat (SSR) markers have been developed for cultivated sunflower that have provided a dense public reference map that could be used to unify independently developed linkage maps and establish a universal linkage group nomenclature (Gedil, 1999; Tang *et al.*, 2002, 2003a, b; Langar *et al.*, 2003; Mokrani *et al.*, 2003; Yu *et al.*, 2003).

Tang *et al.* (2002) constructed the first genetic linkage map of sunflower based on SSR markers and the first dense public genetic linkage map based on single- or low-copy DNA markers (Yu *et al.*, 2003). The map was developed with recombinant inbred lines (RILs) derived from a cross between a public confectionary restorer line and a public oilseed restorer line (RHA280 × RHA801) both released by the United States Department of Agriculture (USDA) (Fick *et al.*, 1974; Roath *et al.*, 1981; Yu *et al.*, 2003). The map utilized the 1089 SSR markers described by Tang *et al.* (2002) and resulted in 17 linkage groups ($x = 17$) with a total length of 1368.3 cM and a mean density of 3.1 cM per locus (Yu *et al.*, 2003).

Subsequently, Yu *et al.* (2003) published a map that integrated the SSR map of Tang *et al.* (2002) and the RFLP maps of Berry *et al.*

(1997) and Jan *et al.* (1998) and added unmapped markers developed at the Oregon State University, CARTISOL, and Advanta (Yu *et al.*, 2003). Detailed description of this work is available elsewhere (Yu *et al.*, 2003).

The discovery and publication of molecular markers in sunflower has facilitated the implementation of whole genome marker applications that permit breeders to define heterotic groupings of inbred lines and allow the exploitation of these groupings in new breeding crosses and untested hybrid combinations. The availability of dense genetic maps has also facilitated the ability of researchers to make marker-trait associations with important disease resistance and other genes and develop high throughput markers that facilitate marker-assisted selection (MAS) for resistant individuals in segregating breeding populations. Finally, the ability to fingerprint inbred lines with SSR markers permits breeders to employ marker-assisted backcrossing that enables a more rapid and thorough recovery of recurrent parent alleles in trait integration and elite line conversion programs.

The future of sunflower breeding holds enormous promise. Given the power of molecular breeding tools and the vast diversity of wild species and novel alleles therein, the combination and exploitation of these resources in tandem should allow breeders to identify and incorporate valuable traits into elite populations faster than ever before. The rising cost of petroleum coupled with the increasing demand for vegetable oil used in the production of biodiesel and the desire for healthier oils by consumers should increase demand for sunflower oil significantly in the years to come. This, in turn, should increase funding in the public and private sectors for the development of improved sunflower hybrids with better yields and agronomic performance and accelerate the rate of genetic gain for the crop overall.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

With the diversity of germplasm available to breeders and the recent development of molecular breeding tools, it is probable that rapid and significant improvements will be made to sunflower germplasm through conventional breeding

that should dramatically improve yield and the profitability of the crop to growers in the next few decades. These trends should stimulate investments in conventional breeding and transgenic breeding alike. Sunflower genomic researchers are discovering more and more new genes that are associated with various phenotypes. With more markers being developed, MAS will become an increasingly important tool in conventional breeding. However, the lack of genetic diversity for certain traits such as *Sclerotinia* resistance in elite sunflower germplasm could limit the improvements through conventional breeding. Other limitations including the complex and time-consuming process of breeding quantitative traits controlled by multiple genes might be overcome through the identification of simpler single gene systems in other organisms and transfer of these genes into sunflower with the tools of biotechnology.

Transgenic breeding has clearly demonstrated its ability to make significant improvements to agriculture. One needs look no further than a corn or soybean field in the United States to find the considerable improvements that have been achieved using transgenics. The major advantage of transgenic breeding over conventional breeding is the vast potential for incorporating desirable traits without the limitations of reproductive barriers between species, or linkage drag that is often problematic with breeding efforts involving wild species. Transgenic breeding is complementary to conventional breeding. The combination of the two breeding approaches has enormous potential to advance the development of new sunflower products.

The center of origin for sunflower is in North America and concerns have been raised in the United States about the potential for transgenic introductions to outcross with related wild species (Snow *et al.*, 2003). These concerns, coupled with a backlash against transgenic food crops in Europe have increased the cost of transgenic deregulation in most countries due to the demand for detailed environmental impact and human health studies prior to approval by the regulatory bodies that govern food safety. In the seed industry, sunflower generates less revenue than crops like corn and soybean. With the high cost of gaining regulatory approval for new transgenic traits, the focus is on crops like corn and soybeans, where high value traits have greater value capture potential than

in sunflower. This presents a major challenge for sunflower industry. As discussed in Section 3, new technology has the potential to solve the gene flow issue associated with transgenic sunflower. The increase in demand for sunflower that is expected as biodiesel and latex production emerge as viable concerns will likely promote increased funding to sunflower research, including research in transgenic breeding.

2. TRANSGENIC TRAIT DEVELOPMENT

To develop transgenic traits in sunflower, plant biotechnologists require a gene encoding a trait, a promoter that directs expression of the gene, and a terminator for stopping the expression of the gene in a delivery and expression vector. Then a transformation method is employed to deliver and integrate the transgene unit into the sunflower genome. Finally the gene activity and the engineered trait are evaluated in the transgenic plants under various laboratory, greenhouse, and field conditions. In this section, we will elaborate the discovery of genes, promoters, and terminators, the methods of transformation, and the development of new agronomic input traits and output quality traits in transgenic sunflower plants.

2.1 Gene, Promoter, and Terminator Discovery

2.1.1 Genes

As indicated in (Table 3), a number of new trait genes have been isolated and characterized in sunflower in the last decade. These genes have a great potential to be used for engineering various traits in sunflower and other crops. For example, the sunflower albumin gene (*SFA8*) can be used to increase methionine content in sunflower and other crop seeds (Wang *et al.*, 2001; Godfree *et al.*, 2004). New technologies have significantly accelerated trait gene discovery in sunflower. For example, genomic approaches have lead to the discovery of several disease defensive genes from sunflower (Hu *et al.*, 2003). Many genes have been isolated from sunflower using degenerate oligodeoxynucleotides deduced from

Table 3 Sunflower genes and potential traits

Genes	Potential traits	References
<i>NBS-LRR</i>	Disease resistance	Plocik <i>et al.</i> , 2004
<i>PLFOR48</i>	Disease resistance	Hewezi <i>et al.</i> , 2006
<i>RGAs</i>	Disease resistance	Radwan <i>et al.</i> , 2003
<i>PKS</i> and <i>RAS</i>	Disease resistance	Bidney <i>et al.</i> , 2003
AAC oxidase	Defense	Liu <i>et al.</i> , 1997
Carbohydrate Oxidase	Defense	Custers <i>et al.</i> , 2004
<i>Defensin SD2</i>	Defense	Sotchenkov <i>et al.</i> , 2005
Pathogen-induced protein	Defense	Mazeyrat <i>et al.</i> , 1998
<i>PR5</i> and <i>SOC</i>	Defense	Hu <i>et al.</i> , 2003
Proteinase inhibitor	Defense	Kouzuma <i>et al.</i> , 2001
<i>SOD</i> and <i>GPX</i>	Defense	Herbette <i>et al.</i> , 2003
<i>Lectin</i>	Pest resistance	Nakagawa <i>et al.</i> , 2000
<i>AHAS</i> 1,2,3	Herbicide resistance	Kolkman <i>et al.</i> , 2004
Guanylate kinase and <i>LytB</i>	Drought or salinity stress	Liu and Baird, 2003
<i>Dehydrin</i>	Drought tolerance	Giordani <i>et al.</i> , 1999
<i>Hahb4</i>	Drought tolerance	Dezar <i>et al.</i> , 2005a, b
<i>TIP</i>	Drought tolerance	Sarda <i>et al.</i> , 1999
<i>Metallothioneins</i>	Detoxification of metals	Chang <i>et al.</i> , 2004
<i>Hsp</i>	Heat shock protein	Coca <i>et al.</i> , 1996
<i>Hahb-10</i>	Light responses	Rueda <i>et al.</i> , 2005
<i>HaHSA9</i>	Embryogenesis	Almoguera <i>et al.</i> , 2002b
<i>Lea</i>	Embryogenesis	Prieto-Dapena <i>et al.</i> , 1999
<i>SERK</i>	Embryogenesis	Thomas <i>et al.</i> , 2004
<i>SF21</i>	Pistil development	Krauter-Canham <i>et al.</i> , 2001
<i>LIM</i>	Pollen development	Baltz <i>et al.</i> , 1999
<i>ORFB</i>	Male sterility	Sabar <i>et al.</i> , 2003
<i>PET1</i>	Male sterility	Horn <i>et al.</i> , 1996
<i>ACP thioesterase</i>	Oil modification	Serrano-Vega <i>et al.</i> , 2005
<i>ACP desaturase</i>	Oil modification	Rousselin <i>et al.</i> , 2002
<i>FAD2</i>	Oil modification	Martinez-Rivas <i>et al.</i> , 2001
<i>Thiolase II</i>	Oil modification	Schiedel <i>et al.</i> , 2004
<i>Ha-LTP5</i>	Lipid transfer and defense	Regente and de la Canal, 2003
<i>Asparagine synthetase</i>	Amino acid composition	Herrera Rodriguez <i>et al.</i> , 2002
<i>Sulfur-rich albumin</i>	Amino acid composition	Wang <i>et al.</i> , 2001

the conserved protein domains of plant genes by polymerase chain reaction (PCR). The sunflower homeodomain gene (Gonzalez *et al.*, 1997) and the nucleotide-binding site-leucine-rich repeat (NBS-LRR) type of disease resistance genes (Bidney *et al.*, 2003; Plocik *et al.*, 2004) are good examples. On the other hand, genes from other organisms can also be used to engineer sunflower. Examples include the wheat oxalate oxidase gene (*OxO*), which confers significant enhanced resistance to *Sclerotinia* in transgenic sunflower (Hu *et al.*, 2003), and genes from the bacterium *Bacillus thuringiensis* (*Bt*) that confer insect resistance in sunflower (Pozzi *et al.*, 2000).

Sunflower genome projects are accelerating sunflower gene discovery. Expressed sequence tag (EST) database is continually increasing in size and

is publicly available. Kozik *et al.* (2003) reported that more than 40 000 sunflower ESTs from multiple complementary DNA (cDNA) libraries have been assembled using the CAP3 program and organized into the Composite Genome Project database (<http://cgpdb.ucdavis.edu/>). This assembly represents 12 000 sunflower unigenes. A total of 12 886 EST sequences were released in July of 2003, and these sequences are available through GnpSeq module sequence databases (<http://genoplante-info.infobiogen.fr>). In a genomic study of sunflower embryogenesis, Tamborindeguy *et al.* (2004) reported 1502 unigenes from sunflower protoplasts, while Fernandez *et al.* (2003) identified a number of organ-specific cDNA clones using subtractive hybridization methods. Ben *et al.* (2005) developed

Table 4 Transgenes and potential traits in transgenic sunflower

Traits	Genes	Promoter	Terminator	Transformation	Reference
<i>Sclerotinia</i> resistance	<i>OXO</i>	SCP1	PinII	<i>A. tumefaciens</i>	Scelonge <i>et al.</i> , 2000
Insect resistance	<i>CryIF</i>	SCP1	PinII	<i>A. tumefaciens</i>	Pozzi <i>et al.</i> , 2000
Broomrape resistance	<i>AHAS</i> (or <i>ALS</i>)	CaMV 35S	CaMV 35S	<i>A. tumefaciens</i>	Slavov <i>et al.</i> , 2005
Metal tolerance	<i>CUP1</i>	CaMV 35S	Nos	<i>A. tumefaciens</i>	Watanabe <i>et al.</i> , 2005
Low stearate oil	<i>ACP desaturase</i>	Hads 10	Hads 10	<i>A. tumefaciens</i>	Rousselin <i>et al.</i> , 2002
High stearate oil	<i>ACP desaturase</i>	Napin	Nos	<i>A. tumefaciens</i>	Bidney <i>et al.</i> , 2002
High methionine	<i>SFA8</i>	CaMV 35S	CaMV 35S	Particle bombardment	Wang <i>et al.</i> , 2001
High methionine	<i>SFA8</i>	NA	NA	<i>A. tumefaciens</i>	Higgins, 1998

expressing profiling tools in their embryogenesis research and identified several thousands of sunflower EST clones that are associated with embryogenesis. Characterization of these ESTs including functional genomic research and EST mapping will link more and more genes with phenotypes or traits and facilitate conventional and transgenic breeding efforts. These gene discoveries will also promote identification and isolation of more tightly regulated promoters.

2.1.2 Promoters and terminators

A promoter is the 5'-flanking region of a gene. Promoters serve very important roles in transgene expression and determine the expression level and expression pattern of genes and their responsiveness to certain environmental signals. Constitutive promoters (Humara *et al.*, 1999; Schenk *et al.*, 1999; Lu *et al.*, 2000; Gago *et al.*, 2002; Bowen *et al.*, 2003), tissue-specific promoters (Domon and Steinmetz, 1994; Nunberg *et al.*, 1994, 1995; Carranco *et al.*, 1999; Prieto-Dapena *et al.*, 1999; Rousselin *et al.*, 2002; Almoguera *et al.*, 2002a; Baltz *et al.*, 2002), and inducible promoters (Avsian-Kretchmer *et al.*, 2004; Dezar *et al.*, 2005b) are available. As more sunflower genes are characterized, more and more promoters will be available for transgenic breeding approaches. Promoters from other crops may be useful in sunflower and sunflower promoters can be used in other crops as well. Synthetic promoters are also useful. For example, UCP3 (the enhancer of maize Ubi1 promoter is fused to the 5' end of the Rsyn7 core promoter) and SCP1 (the enhancer of cauliflower mosaic virus 35 S (CaMV 35S)) promoters are fused to the 5' end of the Rsyn7 core promoter express strong constitutive activities

in sunflower (Lu *et al.*, 2000; Bowen *et al.*, 2003). These results suggest that desirable promoters can be developed using *cis*-acting elements for engineering-specific traits in plants.

A transcription terminator is a nucleotide sequence that acts as a signal for termination of transcription. The nopaline synthase (Nos) terminator from *Agrobacterium tumefaciens* (Bevan, 1984) and terminator sequence from *Solanum tuberosum* proteinase inhibitor II (PinII) (An *et al.*, 1989) as well as CaMV 35S terminator are the most commonly used terminators in transgenic sunflower plants (Table 4).

2.2 Transformation

2.2.1 Tissue culture and regeneration

The development of a transgenic sunflower requires fertile whole plant transformation. Sunflower plants can be regenerated from somatic and zygotic embryos, hypocotyls, mature cotyledons, and protoplasts. The regeneration rate of whole transformed plants depends largely on genotype, explant type, developmental stage, the culture media, and the growing conditions employed (Bidney and Scelonge, 1997). Genetic elements are closely associated with regeneration ability (Deglene *et al.*, 1997; Flores Berrios *et al.*, 2000). High oleic sunflower genotypes can be rapidly and efficiently regenerated (Mohamed *et al.*, 2003). Jambhulkar (1995) developed a rapid embryo-raised plant system for sunflower production from immature embryos. Fiore *et al.* (1997) developed a high frequency regeneration method via somatic embryogenesis has been used with several sunflower genotypes. The embryogenic

frequency of this method can be as high as 72% and the rooting frequency is up to 82%. The somatic embryos could develop into normal plants and produce viable seeds. Shin *et al.* (2000) successfully regenerated plants from the embryo meristem and primordial leaf tissues of 17 different sunflower genotypes. Sujatha and Prabakaran (2001) found that Gamborg basal salt media induced embryogenesis of immature zygotic embryos. Ozyigit *et al.* (2002) could directly regenerate plants from hypocotyls with a high frequency (29–40%). Efficient protocols have also been developed for plant regeneration from leaves (Yordanov *et al.*, 2002) and split embryonic axes (Hewezi *et al.*, 2003). *Agrobacterium rhizogenes* can induce rooting of *in vitro* regenerated shoots and may improve the rooting rate (Devi and Rani, 2002). These results are useful for the improvement of sunflower transformation and gene transfer systems.

A significant bottleneck to sunflower transformation is low transformation efficiency. Optimizing tissue culture and regeneration conditions is fundamental to increasing overall transformation efficiency. There are several transformation methods that can generate transgenic sunflower plants. Herein are presented the four most common and important methods involving *Agrobacterium*, particle bombardment, protoplast, and chloroplast transformations.

2.2.2 *Agrobacterium*-mediated stable transformation

Sunflower is naturally susceptible to *A. tumefaciens* infection (Braun, 1941). Therefore, *A. tumefaciens*-mediated transformation is the most efficient method for transforming sunflower. Bidney and Scelongo (1997) and Pugliesi *et al.* (2000) have reviewed this transformation method. The transformation efficiency depends largely on genotype. Ten sunflower genotypes were evaluated for their suitability for *A. tumefaciens*-mediated gene transfer based on detection of β -glucuronidase (GUS) gene expression. Transformation efficiencies ranged from 0% to 82.7% GUS-positive plants among the tested genotypes, with hybrid genotypes showing higher transformation rates than inbred lines (Gurel and Kazan, 1999). Stable transformation of sunflower can be achieved using nonmeristematic hypocotyls from inbred

line HA300B with 0.1% transformation efficiency (Muller *et al.*, 2001). Hewezi *et al.* (2002) reported a method for genetically transforming recalcitrant split embryonic axes of immature sunflower (*H. annuus* L. var. RHA266). A simple and reproducible *A. tumefaciens*-mediated transformation system was reported by Rao and Rohini (1999). This method produced up to 2% transgenic fertile plants.

Wounding explants by particle bombardment before inoculating meristems with *Agrobacterium* has been shown to dramatically increase transformation frequency (Bidney *et al.*, 1992). Macerating enzymes (0.1% Cellulase R10 and 0.05% Pectinase) treatment can also improve *Agrobacterium*-mediated transformation efficiency (Weber *et al.*, 2002). An endogenous auxin pulse is among the first signals leading to the induction of somatic embryogenesis (Thomas *et al.*, 2002). Transient expression of a gene encoding isopentenyl transferase, involved in the *de novo* synthesis of cytokinins, significantly increases the recovery of *Agrobacterium*-mediated transgenic shoots (Molinier *et al.*, 2002). Modification of the culture conditions, such as the balance of growth regulators, may also increase the transformation efficiency.

Mohamed *et al.* (2004) reported on the optimization of two parameters affecting transfer DNA (T-DNA) delivery and plant regeneration for two high oleic sunflower genotypes subjected to *Agrobacterium*-mediated transformation. The transformation was performed using strain LBA4404 for Capella and strain GV3101 for the inbred lines. Preculturing the explants for 3 days on SIM2 medium before the bacterial inoculation followed by co-cultivation for 72 h increased the percentage of the transgene expression up to 40% in Capella and SWSR2. It also improved overall regeneration. Ikeda *et al.* (2005) reported that small and branching sunflower varieties can be easily transformed and are useful for molecular genetic research.

Matsumura *et al.* (2005) developed a sunflower transformation system using *A. rhizogenes* strain R1000 harboring *rol* genes in four different sunflower varieties. This protocol includes three steps: gene introduction, shoot and plant regeneration, and seed production. They successfully introduced GUS into the sunflower variety Pacino with this method.

2.2.3 Particle bombardment

Bidney (1990) first reported the successful transfer of a transgene into sunflower by particle bombardment. However, the sunflower plants were only partially transformed. Particle bombardment is an important transformation tool that has been used successfully in several plant species. This method is especially important for dicotyledonous plants like sunflower and soybean. The particle bombardment method involves coating DNA onto microparticles such as tungsten or gold and delivering the particles into target cells with acceleration provided by gun-power or high-pressure gas (Hansen and Wright, 1999). The method can be used to transform sunflower (Lucas *et al.*, 2000); however, particle delivery systems have a limited ability to focus bombardment on target cells. A stainless steel focusing device, which fits to the DuPont/BioRad PDS1000/He particle gun apparatus, was developed for solving this problem (Vischi *et al.*, 1999). This device significantly increases the transformation frequency of sunflower cotyledons. More detail information on transformation using particle bombardment can be found in book chapters (Bidney and Scelonge, 1997; Pugliesi *et al.*, 2000).

2.2.4 Protoplast transformation

Methods for isolating protoplasts from sunflower tissues have been established and there are a number of reports on direct gene transfer into protoplasts (Bidney and Scelonge, 1997; Pugliesi *et al.*, 2000). Sunflower protoplasts can take up antibodies (Briere *et al.*, 2004), suggesting that protoplasts might be used as a tool for studying protein and gene function in transgenic research. Binsfeld *et al.* (2000) obtained asymmetric somatic hybrid plants by polyethylene glycol-mediated mass fusion of protoplasts from perennial *Helianthus* species and hypocotyl protoplasts of *H. annuus*. These results indicate that micronuclear induction and asymmetric somatic hybridization can facilitate the transfer of genes into economically important sunflower lines. Plasmid DNA delivery into protoplasts may be achieved with ease. Regeneration from sunflower protoplasts is possible, but the frequency is very low. The challenge with this method lies in the

generation of fertile whole sunflower plants from the transformed protoplasts.

2.2.5 Chloroplast transformation

Chloroplast genomes are maternally inherited and provide a high level of expression with low levels of gene silencing and transgene positional effects. Therefore, chloroplast genomes have been becoming an attractive target for genetic modification and protocols have been developed in some crops (Daniell *et al.*, 2004). Unintended transgene flow via pollen dissemination is a critical issue with genetically modified sunflower plants. Chloroplast transformation is thus of particular interest as a potential means of preventing gene flow from transgenic plants to conventional sunflower or related wild species in field environments. Unfortunately, there is currently no report of sunflower chloroplast transformation. Further efforts are required for more research in this important area for sunflower transgenic breeding.

2.2.6 Selection of transformed tissue

Selection of transformed plant tissues and whole explants are important steps in the development of transgenic sunflower. To facilitate the selection process, selectable markers that can be utilized to identify successfully transformed cells are employed in the process of whole plant regeneration. Genes that confer resistance to selection agents that inhibit growth or kill nontransgenic cells are employed as part of the transformation vector. Bacterial genes encoding resistance to antibiotics or herbicides are commonly used to this end. For example, the *Escherichia coli* *NPTII* gene encodes neomycin phosphotransferase that effectively inactivates kanamycin or neomycin. Kanamycin kills plant cells by inhibiting protein translation. Therefore, in the presence of kanamycin, transgenic sunflower tissues expressing the *NPTII* gene will survive, but nontransgenic cells lacking *NPTII* will be destroyed. Similarly, the *Bar* gene from the bacterium *streptomyces hygroscopicus* encodes phosphinothricin acetyl transferase (PAT) can also be used as a selection marker. PAT converts

the herbicide phosphinothricin into its nontoxic acetylated form and allows growth of transformed plants cells expressing the *Bar* gene in the presence of phosphinothricin. Other commercial glufosinate ammonium-based herbicides such as BASTA™ or Bialaphos can be used for selecting *Bar*-transformed sunflower tissues (Goodwin *et al.*, 2004). Enzymatic-mediated colorimetric reactions such as those observed with wheat OXO (Hu *et al.*, 2003) and GUS (Bidney and Scelonge, 1997) have also been used as positive selection markers.

Transgene integration, expression, and localization in plants need to be confirmed at the molecular level. Southern blot analysis and/or PCR assay can be utilized to confirm the presence and copy numbers of transgenes in the genome of transformed sunflowers. Northern blot assay and/or reverse transcription-PCR (RT-PCR) analysis can be employed to confirm the expression level of a transgene at the transcriptional level, and Western blot and/or enzyme-linked immunosorbent assay (ELISA) or enzymatic analysis may be used to confirm expression at the protein level (Ingham, 2004; Liu *et al.*, 2004; Page and Minocha, 2004).

2.3 Transgenic Improved Traits

Crop yield is the most important trait targeted by crop scientists and farmers alike. The ultimate goal of plant biotechnology is to transfer useful genes into elite germplasm for the improvement of the productivity and quality of crops. As the global human population increases, the food supply will need to expand to meet increased demand and to maintain human nutritional health. In an effort to increase crop yield, plant biologists have applied technologies to generate transgenic plants with improved resistances to diseases, insects, and herbicides as well as enhanced tolerance to abiotic stresses. Most of the efforts to improve sunflower seed quality have been focused on oil and amino acid modifications. In the following sections, we present various transgenic approaches for improving input agronomic and output quality traits in sunflower. In addition, we will also address the potential industrial uses of sunflower and its impact on the sunflower transgenic approach.

2.3.1 Disease resistance

Fungal diseases, such as *Sclerotinia*, *Phomopsis*, rust, downy mildew, and *Alternaria* are the most important disease problems for sunflower production worldwide. Two bacterial diseases in sunflower are widespread and caused by *Pseudomonas syringae* pv. *tagetis* and *Xanthomonas campestris* pv. *helianthi*. Virus diseases are relatively less important in sunflower. Conventional breeding programs have targeted improving resistance to sunflower pathogens for decades. For diseases like downy mildew and rust that can be controlled by single dominant resistance genes, breeders have been very successful in identifying new resistance alleles in wild species and integrating them into elite germplasm (Gulya *et al.*, 1997). However, for more complex diseases like *Sclerotinia*, desirable levels of resistance have often been hard to achieve due to the lack of genetic variations, the complexity inherent to multigenic resistance mechanisms, and the lack of consistent and reliable disease screening methods.

Sclerotinia wilt caused by *S. sclerotiorum* (Lib.) de Bay is the most important fungal disease of sunflower. Under the right environmental conditions, it causes sunflower root rot, stem rot, and head rots (Lu, 2003). The annual crop losses caused by this fungal pathogen are in a range of \$50–\$80 million. Transgenic expression of the wheat *OXO* gene shows great promise for enhancing resistance to *Sclerotinia* (Lu *et al.*, 1998, 2000; Scelonge *et al.*, 2000; Hu *et al.*, 2003). The *OXO* gene was originally named as germin and was isolated from a wheat cDNA library (Dratewka-Kos *et al.*, 1989). Genomic DNA clones encoding *OXO* were also isolated from wheat genomic library (Lane *et al.*, 1993). The *OXO* gene used for transforming sunflower was isolated from a wheat line (Pioneer, 2458) and encodes a protein identical to the wheat germin gf2.8 *OXO* (Lane *et al.*, 1991). *OXO*-transgenic sunflower plants have been generated with a vector utilizing the constitutive SCP1 promoter (Lu *et al.*, 2000), the omega prime messenger RNA (mRNA) leader sequence from tobacco mosaic virus (Gallie *et al.*, 1987), and the potato proteinase inhibitor (PINII) 3' terminator (An *et al.*, 1989) in a pBin19 vector (Bevan, 1984), that was transferred through *Agrobacterium*-mediated transformation (Bidney *et al.*, 1992; Lu *et al.*, 2000; Scelonge *et al.*, 2000).

The *OXO* transgene in sunflower is the full-length coding region of *gf2.8* including the signal peptide for protein expression in the intercellular space. *A. tumefaciens* strain EHA105 was used to transform the sunflower line SMF3 in a modified meristem culture method as described previously (Scelonge *et al.*, 2000). The transgenic plants were identified by an *OXO* enzymatic activity assay (Hu *et al.*, 2003) or by *OXO* ELISA analysis (Scelonge *et al.*, 2000). Whole explants were regenerated from the secondary culture of nodal meristems of transformed plant sectors. The T_0 plants are chimeric. Therefore, most of the bioassays were carried out at T_2 or more advanced generation after confirming positive transgene expression at the protein level (Scelonge *et al.*, 2000; Hu *et al.*, 2003).

When *OXO*-transgenic and nontransformed sunflower plants were subjected to infection by *Sclerotinia*, disease-induced lesions in transgenic sunflower leaves were significantly smaller than those in the leaves of nontransformed controls. Lesion sizes in the transgenic leaves were inversely related to the endogenous levels of *OXO* activity (Hu *et al.*, 2003). *Sclerotinia* spread to the head tissue of the control plants, but the lesions on the transgenic plants were primarily confined to the main stem. These results demonstrated that *OXO* can confer significant *Sclerotinia* resistance in transgenic sunflower (Hu *et al.*, 2003). Greenhouse, screenhouse (cage), and small block field bioassays also showed that *OXO*-transgenic lines exhibited significantly enhanced resistance to mid-stalk rot, root rot, and head rot (Lu *et al.*, 1998; Bazzalo *et al.*, 2000; Scelonge *et al.*, 2000). When crossing the transgenic line with natural *Sclerotinia*-tolerant sunflower lines, the hybrids carrying the *OXO* transgene were more resistant to *Sclerotinia* than the corresponding nontransgenic isogenic hybrids. These experiments clearly demonstrate that the combination of *OXO* with natural tolerance genetic background provides a greater level of *Sclerotinia* resistance than that in the currently available commercial hybrids (Bazzalo *et al.*, 2000). The *SCP1* promoter activity and *OXO* activity are stable over generations and preliminary field assays indicated that *OXO* transgenic expression has a little impact on other agronomic traits at proper level of expression.

Enhanced *Sclerotinia* resistance in *OXO*-transgenic sunflower plants is significant and is the first example of a successful transgene-mediated fungal resistance mechanism in plants. However, the *OXO* enzyme has been found to persist in simulated gastric fluid digestion studies, raising concerns about whether the protein may be a human allergen (Jensen-Jarolim *et al.*, 2002). Wheat *OXO* and *Arabidopsis* germinlike proteins have been produced in transformed tobacco plants. *In vitro* and *in vivo* IgE-binding assays with these proteins indicate that wheat *OXO* and *Arabidopsis* germinlike protein are capable of binding IgE via carbohydrate determinants. IgE binding by these proteins is another indicator that *OXO* may have allergenic properties (Jensen-Jarolim *et al.*, 2002).

For transgenic sunflowers, a major concern is the potential for transgene escape from a transgenic crop to wild relatives near production environments (Snow *et al.*, 2003). Burke and Rieseberg (2003) highlighted an important finding concerning the potential escape of *OXO* in *OXO*-transgenic sunflower plants. They backcrossed the *OXO* transgene from *OXO*-sunflowers to wild species. The resulting progeny were grown in containment cages at field sites in Indiana, North Dakota, and California. The *OXO* transgene was shown to enhance resistance to *S. sclerotiorum*, but it did not significantly impact seed production and reproductive fecundity of the wild species. They conclude that it is not the *OXO* gene transfer *per se* that is important, but rather its potential contribution to the relative fitness in related wild species has the potential to influence the persistence of the transgene in the wild (Heritage, 2003). This type of study is important in demonstrating the safety of biotechnology and provides insight to the concern about gene flow to related species. The results from the Burke and Rieseberg study (2003) suggest that genetically enhanced wild plants will not become more “weedy” since they do not produce more seeds than unmodified controls. These results also indicate that the *OXO* transgene would do little more than diffuse neutrally after its escape in the absence of selection pressure for *Sclerotinia* tolerance. Chapman and Burke (2006) further studied the escape of transgenes from crop \times wild hybridization and showed that natural selection, and not the overall rate of gene flow, is the most

important factor governing the spread of favorable transgene alleles.

Two additional enzymes, oxalate decarboxylase (OXDC) and Oxyl CoA-decarboxylase, can convert oxalate to formate and carbon dioxide. These enzymes have been identified and isolated from fungi and bacteria (Hu and Lu, 2005). The fungal *OXDC* gene has been expressed under the control of CaMV 35S promoter in the cytosol and vacuole of transgenic tobacco, tomato, and lettuce plants. Transgenic plants expressing *OXDC* showed remarkable resistance to *Sclerotinia* (Kesarwani *et al.*, 2000; Dias *et al.*, 2006). These plants had a normal phenotype, and the transferred trait was stably inherited in subsequent generations. The bacterial *OXDC* gene could also be used for oxalate degradation and engineering *Sclerotinia* resistance in plants (Dickman and Mitra, 1992). These results imply that *OXDC* would be a useful candidate for sunflower transformation to combat *Sclerotinia* diseases.

Sawahel and Hagran (2006) reported that overexpression of a human lysozyme gene in sunflower can confer *Sclerotinia* resistance. The lysozyme gene was expressed under CaMV 35S promoter and Nos terminator in a binary vector that contain *NPTII* and *GUS* marker genes (Nakajima *et al.*, 1997). The transgenic sunflower plants were generated by *Agrobacterium*-mediated transformation of particle-wounded hypocotyls explants. The transformed shoots were identified by kanamycin selection and GUS assay, and were further confirmed by Southern, Western, and Northern blot analyses. Two transgenic lines, SH-1 and SH-2, highly expressed the lysozyme and were used for *Sclerotinia* infection assay. The leaf infection analyses indicated that overexpression of the lysozyme significantly reduced *Sclerotinia* infection as indicated by fungal colony formation and colony area.

Sunflower biotechnologists have pursued other transgenic strategies to enhance resistance to sunflower diseases. A number of resistance (R) gene homologues have been isolated from sunflower (Table 3), providing a valuable resource for engineering disease resistance in sunflower. Hewezi *et al.* (2006) reported that TIR-NBS-LRR R genes in sunflower and tobacco have a dual role in plant development and fungal resistance. A number of antifungal protein genes

have been identified (Sauerborn *et al.*, 2002; Lu, 2003; Qi *et al.*, 2005). Induced disease resistance and defense pathways have been identified in plants and have potential to control viral, bacterial, and fungal diseases, as well as parasitic weeds in sunflower (Cao *et al.*, 1998; Dmitriev *et al.*, 2003; Hu *et al.*, 2003). Sotchenkov *et al.* (2005) reported that sunflower defensin *SD2* and modified gene homologues showed similar anti-*Fusarium culmorum* activity. Custers *et al.* (2004) reported that sunflower carbohydrate oxidase can produce hydrogen peroxide and confer resistance against bacterial pathogen *Pectobacterium carotovorum* ssp. in transgenic tobacco. In addition, virus-mediated RNAi (RNA interference) technology has great potential to control viral diseases (Moissiard and Voinnet, 2004) in sunflower.

2.3.2 Insect resistance

Over 150 insect species have been reported to infest sunflower. The most damaging insects of cultivated sunflower include the sunflower moth, banded sunflower moth, and sunflower bud moth (Charlet *et al.*, 1987). Little genetic insect resistance has been obtained through conventional breeding. A common strategy for engineering insect resistance in crops is transformation of sunflowers with *Bt* genes that code for insecticidal proteins. *Bt* is a gram-positive soil bacterium that produces a protein toxic to many insect pests in the family Lepidoptera.

Transgenic expression of the *CryIF Bt* gene in sunflower confers significant control of *Rachiplusia mu* and *Spilosoma virginica*, two important insect pests that impact sunflower production in Argentina (Pozzi *et al.*, 2000). The *CryIF* gene was isolated from the Mycogen/Dow AgroScience *Bt* library. The binary vector used for the sunflower transformation included a constitutive promoter SCP1 (Lu *et al.*, 2000), the omega prime leader sequence of tobacco mosaic virus (Gallie *et al.*, 1987), the *CryIF* gene, and the PINII terminator (An *et al.*, 1989). *CryIF*-transgenic sunflower plants were generated from the line SMF3 through *A. tumefaciens*-mediated transformation (Bidney *et al.*, 1992; Scelonge *et al.*, 2000). Whole plants were regenerated from transformed meristem, and the transformed T₀ plants were identified by *CryIF*

ELISA analysis. T₂ generation plants were used for the initial insect bioassay. Bioassay was performed using transgenic leaf discs and *R. mu* and *S. virginica* larvae at seedling and preflowering stages. The feeding assay clearly exhibited enhanced insect resistance of the transgenic leaf tissues compared to the control. Two field bioassays were carried out during the 1999–2000 seasons in Argentina. In these assays, two *CryIF*-transgenic sunflower events exhibited complete resistance to Argentine looper (*R. mu*) infestation at a high level of insect attack (Pozzi *et al.*, 2000).

A *CryIac*-transgenic sunflower line (CMS-PET1) with a plant-optimized *CryIac* gene from *Bt* under a constitutive promoter CaMV 35S and Nos terminator showed insect resistance. The *CryIac* transgene was crossed with *H. annuus* and the progeny showed significant enhanced resistance to Lepidopteran damage. The transgenic × wild sunflower hybrid produced 55% more seeds than the hybrids lacking the *CryIac* gene (Snow *et al.*, 2003). This study confirms the effectiveness of *Bt* genes in controlling insect pests in sunflower, and raises a concern about “gene flow” to wild species that has the potential to enhance the fitness of weeds in the environment. Preliminary analyses suggested that increased seed production may increase the size of wild population (Pilson *et al.*, 2002). These results imply that wild plants could proliferate as weeds with greater fecundity. However, they are different from the conclusion from *OxO*-sunflower studies (Burke and Rieseberg, 2003), indicating gene flow effects on environments depend on the transgene employed.

Jyoti and Brewer (1999) reported that applications of *Bt* can provide resistance to banded sunflower moth (*C. hospes* Walsingham). Along the same line, there are additional potential insecticidal proteins in other bacteria, fungi, or plants (Legaspi *et al.*, 2004). Chang *et al.* (2003) reported that the *H. tuberosus* agglutinin (*HTA*) gene confers resistance to peach–potato aphid (*Myzus persicae*). Efforts of using lectin or proteinase inhibitor genes to engineer sunflower with insect resistance has been reported (Schuler *et al.*, 1989). However, proteinase inhibitors have been shown to have negative impact on the performance of nonruminants such as poultry and swine, and resistances conferred by proteinase inhibitors are not durable.

2.3.3 Herbicide resistance

There are several important weeds that impact sunflower growth and yield. Wild mustard (*Sinapis arvensis*) and broomrape (*Orobancha cumanna*) are examples of two major weeds that impact sunflower production. Weed control is generally achieved through application of herbicides that affect photosynthesis or the biosynthesis of essential compounds in plants. However, crops are often not resistant to the herbicides. Crops that are genetically transformed with herbicide resistance genes give farmers greater flexibility for the control of weeds. Phosphinothricin and glyphosate have been used in transgenic herbicide resistance. These herbicides have effective toxicity to a broad spectrum of weeds, low toxicity to mammals, and a little impact on environmental systems.

The IMI class of herbicides targets the enzyme acetohydroxy acid synthase (AHAS), which is also referred to as acetolactate synthase (ALS). The ALS enzyme governs the first step in the biosynthesis of essential branched chain amino acids. Three *AHAS* genes were cloned from sunflower by Kolkman *et al.* (2004) providing an opportunity to explore the development of transgenic IMI resistance in sunflower.

IMI resistance in sunflower was identified in a wild population of *H. annuus* in Kansas by Miller and Al-Khatib (2002). The basis for IMI resistance in sunflower is the expression of two naturally occurring mutations in the gene that encodes ALS in sunflower and that gives rise to a conformational change in the structure of the ALS enzyme that prevents herbicide binding. IMI resistance has been incorporated into elite germplasm through conventional breeding and is currently sold in the United States, Argentina, and several European countries under the Clearfield™ trademark owned by BASF. Imazimox (labeled as Beyond™) herbicide was approved for use on Clearfield™ sunflowers in 2003. Clearfield™ sunflower hybrids result from a cross between two IMI-tolerant parents. The original releases were derived through extensive backcrossing to commercial sunflower inbred lines in both female and male heterotic pools. The Clearfield™ sunflower system allows growers to control a wide range of grassy and broadleaf weeds. The susceptibility of conventional sunflower to

ALS-inhibiting herbicides coupled with the long soil residual activity of some IMI herbicides can impact crop rotation in sunflower production systems. Sunflower at early developmental stages are more sensitive to ALS-inhibitors than late developmental stages. Therefore, determining the response to soil residual activity of ALS-inhibiting herbicide is important for managing sunflower crop rotations in fields treated with IMI herbicides (Howatt and Endres, 2006).

While resistance to IMI herbicides was incorporated through conventional breeding methods, some have raised concerns regarding gene flow of the IMI-resistance trait to wild species, regardless of the fact that the trait came from wild sunflower in the first place. Massinga *et al.* (2003, 2005) reported on the fitness of imazamox-resistant common sunflower and prairie sunflower. They created hybrids of domesticated sunflower with both common sunflower and prairie sunflower with and without the imazamox-resistance trait, and carried out greenhouse experiments by measuring the growth of IMI-R and IMI-S common and prairie sunflower hybrids under noncompetitive conditions. The relative growth rate, net assimilation rate, leaf area, and total dry weight were similar in IMI-R and IMI-S common and prairie sunflower, whereas plant height of IMI-S hybrid was greater than that of IMI-R common sunflower hybrids. They also did a replacement series study under field conditions in 2001 and 2002 to evaluate the relative competitiveness of IMI-R and IMI-S common and prairie sunflower. They conclude that IMI-R and IMI-S hybrids of both sunflower species were equally competitive. These results indirectly suggest that the ALS mutant genes can be used for engineering sunflower for herbicide resistance and ALS-transgenic sunflower could be expected to have little environmental impact.

Broomrape has become a potential threat to sunflower production in Europe (Melero-Vara *et al.*, 1989) and to alfalfa, peanut, soybean, tobacco, and tomato in North America (Jain and Foy, 1989). Overexpression of a mutant ALS gene in carrot conferred control of broomrape by imazapyr, an ALS inhibitor (Aviv *et al.*, 2002). This mutant *ALS* gene is an interesting candidate for developing transgenic sunflower plants that might permit the control of broomrape in fields treated with IMI herbicides.

However, Clearfield™ sunflower hybrids treated with Beyond™ herbicide in broomrape infested fields in Turkey have not enabled season long control of this aggressive plant parasite. Slavov *et al.* (2005) transformed several tobacco cultivars with a mutant *ALS* gene for resistance to the herbicide chlorsulfuron (Glean®, DuPont). The mutant gene was isolated from mutant *Brassica napus* (Ouellet *et al.*, 1994) and expressed under CaMV 35S promoter and terminator. The binary construct was transformed into tobacco by *Agrobacterium*-mediated transformation following leaf disc method (Horsch *et al.*, 1985). Transgenic plants were identified by kanamycin selection. The efficiency of the herbicide was demonstrated in greenhouse and field trials. An Orobanche/tobacco growth system was used to evaluate the resistance of the transgenic tobacco plants to chlorsulfuron (Glean®, DuPont). These mutant *ALS* or *AHAS* genes are other potential candidates for the development of transgenic sunflower that might be employed for the control of broomrape.

DuPont announced the release of new sunflower hybrids tolerant to Express® SX Herbicide in Europe on May 25, 2006. These hybrids were derived through conventional breeding and permit the use of Express® SX herbicide (Sulfonylurea) for postemergence broadleaf weed control in sunflower production systems. This offering provides farmers with more weed management flexibility and increased levels of weed control versus existing options. Sulfonylureas are a family of herbicides that were discovered by DuPont scientists in 1972 and first commercialized for in-crop use in wheat and barley crops in 1982. The efficiency and economic benefit of Express® SX herbicide has been proven in cereals and now will benefit sunflower growers (http://www.pioneer.mediaroom.com/index.php?s=press_releasesanditem=160).

2.3.4 Tolerance to environmental stresses

With the alteration to global weather patterns that are expected as a result of global warming, more focus has been given recently to the development of transgenic tolerance to abiotic stresses, such as drought, cold, salinity, extreme temperatures, chemical toxicity, and oxidative stress. Drought

and salinity stress tolerance are likely the most important abiotic resistance traits in sunflower. Although transgenic sunflower plants resistant to abiotic stresses have not yet been developed, research on molecular control mechanisms for abiotic stress tolerance has been reported. Liu and Baird (2003) cloned and characterized five drought-regulated cDNAs and 12 salinity-regulated cDNAs from sunflower. Dezar *et al.* (2005a) reported that *Hahb-4*, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to *Arabidopsis* plants. *Hahb-4* is regulated at the transcriptional level by water availability and abscisic acid. They overexpressed this gene in *Arabidopsis* under CaMV 35S promoter and Nos terminator. Transformed plants were generated by *A. tumefaciens*-mediated transformation with the floral dip procedure (Clough and Bent, 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR assay. The transgenic *Arabidopsis* plants developed shorter stems and internodes, rounder leaves, and more compact inflorescences than the nontransformed controls. Shorter stems and internodes are due to a lower rate of cell elongation rather than changes in the rate of cell division. Transgenic plants were more tolerant to water stress conditions. Under drought condition, the transgenic *Arabidopsis* plants showed improved development, a healthier appearance, and higher survival rates than wild-type plants. These genes and other defense-related genes (Table 3) are exciting candidates for engineering tolerance to environmental stresses through transgenic breeding approaches.

Watanabe *et al.* (2005) introduced a yeast metallothionein gene (*CUP1*) into sunflower and evaluated heavy metal tolerance of transgenic plants at the callus stage taking advantage of the large biomass of sunflower plants. The *CUP1* was isolated from the yeast genome by PCR amplification and expressed in sunflower calli under the CaMV 35S promoter and Nos terminator in an *A. tumefaciens* LBA4404 binary vector. This construct was co-cultured with sunflower calli for 20 min. The transgenic calli were selected based on kanamycin resistance, and further confirmed by PCR and Southern blot analysis. They evaluated the heavy metal (Cd) tolerance of transgenic sunflower calli in terms of 2,3,5-triphenyl-tetrazolium chloride (TTC)-

reducing activity. The TTC method enables one to quantitatively determine the amount of triphenyl formation formed by succinic acid dehydrogenase-mediated TTC-reducing activity. This method was used to select heavy metal-tolerant lines of the transgenic sunflower calli. Several transgenic callus lines showed a high TTC-reducing activity even after treatment with Cd 200 μ m for 10 days. The TTC reducing activity correlates to the expression of *CUP1* gene. These results illustrate the potential for use of transgenic approaches to develop tolerance to abiotic stresses in sunflower.

2.3.5 Modified oil compositions

Sunflower oil is one of the four major sources of edible oil worldwide including soybean (*Glycine max* L. Merr.), sunflower, rapeseed (*Brassica*), and peanut (*Arachis hypogaea* L.). Conventional sunflower oil contains oleic acid (20%), linoleic acid (65%), linolenic acid (less than 1%), and about 12% saturated fatty acid. The main use of sunflower oil is for human consumption. Sunflower oil is very stable against lipid peroxidation and is used for salad dressings, cooking, and frying. Modified oil profiles including high oleic, low linoleic, and even low linolenic fatty acid compositions have been developed successfully through conventional breeding in sunflower (Purdy, 1986; Hammond, 2000). In the United States, traditional linoleic, high oleic, and NuSunTM sunflower oil is available to food processors and to consumers through specialty grocery outlets.

High oleic acid sunflower was originally developed from VNIIMK 8931 by Soldatov (1976) using dimethyl sulfate-mediated mutagenesis. The mutagenized seeds were advanced to M₃ and screened for differing levels of oleic acid. Breeding lines with oleic acid levels as high as 895 g kg⁻¹ were created in 1978. A synthetic population of plants with high levels of oleic acid was obtained in 1982. Plants from this population were selfed to produce the Pervenets lines. A Pervenets female was crossed with the inbred line HA89 to create the first mid oleic or NuSunTM sunflower hybrid (Miller *et al.*, 1987). Genetic studies demonstrated that the mid oleic trait is controlled by at least three genes (Fernandez-Martinez *et al.*, 1989). In a series of field trials conducted in the United States from 1996 to 2000, NuSunTM sunflower

hybrids were shown to have favorable yield and agronomic performance, and they have since been accepted by end users and growers. The adoption of NuSunTM hybrids by growers was so rapid that by 2001 nearly 50% of the oilseed sunflower area in the United States was seeded to NuSunTM hybrids. Further improvements are focused on reducing the level of saturated fatty acids and increasing antioxidant levels in NuSun(tm) oil (Burton *et al.*, 2004). Based on the 2002 US Sunflower Crop Quality Report, NuSunTM mid oleic sunflower oil continue to provide a solution to food processors, food service users, and consumers looking for a cooking oil with excellent taste and performance, and a healthy profile. NuSunTM sunflower oil has excellent oxidative stability without the need for partial hydrogenation. Animal feeding studies conducted by Dr. Robert Bicolost and T. Wilson at the University of Massachusetts indicate that a diet containing NuSunTM sunflower oil can reduce levels of LDL cholesterol without significantly reducing HDL cholesterol. They further demonstrated that consuming mid oleic sunflower oil reduces the risk of cardiovascular disease (Binkoski *et al.*, 2005). The process of developing NuSunTM sunflower oil sheds light on developing transgenic sunflower products with further modifications to the oil profile.

The European Commission supported a project on transgenic nonfood sunflower oil. The aim of the project was to produce plants with high oleic acid content and reduced content of stearic acid as well as plants producing short- and medium-chain fatty acids. Transgenic plants have been produced and introduced into field trials (fifth generation) (<http://www.molecularfarming.com/nonmedical.html>).

The plant oil biosynthesis pathway is well understood (Ohlrogge and Browse, 1995; Voelker and Kinney, 2001). Genes encoding key enzymes involved in fatty acid biosynthesis have been cloned from sunflower and other plants (Voelker and Kinney, 2001; Serrano-Vega *et al.*, 2003). These genes hold promise for exploitation through biotechnology and may permit additional useful oil modifications that could be achieved through targeted alterations of lipid metabolic pathways.

Transgenic sunflower and canola plants that produce high oleic oils have been generated, and the high oleic oil produced by these plants exhibited excellent thermal stability and frying

performance (Dobarganes *et al.*, 1993; Katz, 1997). High stearic oils are targets for delivering trans-fat-free substitutes for hydrogenated oils. New tailor-made (designer) oils are a series of vegetable oils suitable for edible purposes (Sakurai and Pokorny, 2003). High oleic sunflower lines contain relatively high levels of stearic acid (5–6%), which is undesirable in some industrial uses as it elevates the clouding point and adds difficulty to oil processing. δ -9-stearoyl-(acyl carrier protein) desaturase is the key enzyme that converts stearic acid to oleic acid. Oleic acid is generated by specific ACP desaturase. An 18:1-ACP-specific thioesterase then releases oleate for transport to the endoplasmic reticulum (ER) for subsequent triacylglycerol synthesis. A gene encoding δ -9-stearoyl-(acyl carrier protein: ACP) desaturase was cloned from *Ricinus communis* (castor) cDNA library using an Avocado ACP desaturase antibody (Shanklin and Somerville, 1991). Rousselin *et al.* (2002) overexpressed the castor ACP desaturase gene under a sunflower seed-specific promoter Hads10, which comes from the sunflower late embryogenesis abundant gene *hads10*. The Hads10 terminator was used to terminate the transcription of the ACP desaturase transgene in a binary vector. This binary vector was transformed into an interspecific hybrid sunflower line STR by *Agrobacterium*-mediated transformation without additional particle bombardment (Burrus *et al.*, 1996). Transgenic plants were generated by grafting the putative transgenic shoots onto 3-week-old HA300B plants. T₀ plants were selfed and transgenic plants were selected by kanamycin and confirmed by Southern and Northern blot analyses. The transgene was stable from T₁ to T₅. The transgenic plants were evaluated in greenhouse and field over five generations by analyzing the fatty acid compounds of the seed oil. Some transgenic lines showed significant reduction of stearic acid level compared to the nontransformed control plants (Rousselin *et al.*, 2002).

On the other hand, antisense expression of a stearoyl-ACP desaturase gene in sunflower significantly increased saturated fatty acids (Bidney *et al.*, 2002). A sunflower gene encoding stearoyl-ACP desaturase was cloned from sunflower by RT-PCR. The Napin promoter and nopaline synthase (NOS) terminator were used for antisense expression of the *ACP desaturase* gene and the binary vector was transformed into sunflower by

Agrobacterium-mediated transformation (Bidney *et al.*, 1992; Scelonge *et al.*, 2000). The transgenic sunflower lines expressing the antisense stearoyl-ACP desaturase gene increased stearic acid concentrations fourfold in seed and produced over 40% saturated fatty acids. This product has significant industrial uses including coatings, soap, and margarine (Bidney *et al.*, 2002). Antisense technology for increasing saturated fatty acid has also been demonstrated in other crops (Knutzon *et al.*, 1992). Hairpin DNA constructs, containing an intron, can induce posttranscriptional gene silencing (PTGS) with very high frequency in plants. The antisense and PTGS is now commonly called RNAi (Cerutti, 2003). This RNAi technology has been successfully used to modify seed lipids in other oilseed crops (Singh *et al.*, 2000). The new transgenic modified oils show reduced polyunsaturated fatty acids and reduced susceptibility to oxidation. The content of undesirable trans fatty acids in the oil from transgenic plants is approximately one-third lower than that of hydrogenated traditional oils (Singh *et al.*, 2000).

Acyl-ACP thioesterases terminate acyl-chain elongation during fatty acid biosynthesis by hydrolyzing the thioester bond of acyl-ACP (Harwood, 1988). The substrate specificity of ACP thioesterase plays an important role in determining when the acyl chains are cleaved and released from ACPs, thus determining which fatty acids are available for the biosynthesis of storage and membrane lipids in seeds. Serrano-Vega *et al.* (2003, 2005) isolated, cloned, and sequenced a cDNA clone (*HaFatA1*) encoding acyl-ACP thioesterase from sunflower seeds. This gene may be useful for transgenic modification of sunflower lipids.

Fatty acid biosynthesis is a complex process, and the manipulation of a single gene in a pathway may not be enough to obtain the desired oil compositions. Strategies involving gene stacking may be required for achieving desired traits. Kinney *et al.* (2001) summarized and discussed the challenges in the genetic engineering for improving oil quality. The main factors that impact the genetic manipulation efficacy include the fatty acid flux, desaturase gene families, and intracellular transport of desaturase products in plants. There have been success engineering novel fatty acids in transgenic soybean seeds by expressing various

desaturase genes from other plants (Cahoon, 2003; Kinney, 2003). This approach can be used in transgenic sunflower for improving sunflower oil value and producing industrial useful oils for industry.

2.3.6 Amino acids

Sunflower seeds contain significant amounts of storage proteins called helianthinin and 2S albumins. The 2S albumin protein contains 16% methionine and 8% cysteine, and its protein level in sunflower seeds can be as high as 30% of the total albumin fraction (Anisimova *et al.*, 1999). This indicates the 2S gene (designated as *SFA8*) is a good candidate for genetic modification of sunflower and other crop seeds to enhance nutritional qualities. The sulfur-rich amino acids are important limiting factors for ruminant animal nutrition. Feeding ruminant animals with a ration high in sulfur-rich amino acids could increase animal productivity, such as wool growth rates in sheep (Higgins *et al.*, 1989). Transferring the *SFA8* gene encoding sunflower 2S albumin protein into forage plants may permit the development of transgenic forage species that enhance overall ruminant animal health and productivity. For example, the sunflower *SFA8* gene has been used to increase the methionine content in tall fescue (*Festuca arundinacea* Schreb) and *Trifolium subterraneum* subsp. *subterraneum* (Wang *et al.*, 2001; Godfree *et al.*, 2004).

SFA8 was isolated from sunflower by Kortt *et al.* (1991). The amino acid sequence of the purified SFA8 protein was obtained and then screened a sunflower embryo cDNA library using the deduced DNA sequences. To achieve expression in tall fescue, the gene was modified at the C-terminal with the addition of an ER retention signal KDEL. The CAMV 35S promoter was used for constitutive expression in one construct and the wheat cab promoter was used for leaf-specific expression in another expression cassette. The terminator used for both constructs was CaMV 35S terminator. A modified hygromycin (Hm) phosphotransferase gene was used as a selectable marker. Constructs were transferred into tall fescue by particle bombardment. Transgenic cells were selected on Hm (250 mg l⁻¹) and Hm-resistant calli were regenerated into plants.

Western blot analysis indicated that KDEL-target signal significantly enhanced the accumulation of SFA8 protein. The sulfur-rich SFA8 protein accumulated in transgenic plants up to 0.2% of total soluble protein (Wang *et al.*, 2001). The *SFA8* gene was also used to transform *T. subterraneum* subsp. *subterraneum* (Godfree *et al.*, 2004) and lupins (*Lupinus angustifolius* L.) (Molvig *et al.*, 1997). Ravindran *et al.* (2002) evaluated the nutritive value of the transgenic lupin seeds, which contained higher contents of methionine in broiler chickens. The amino acids in transgenic seeds were as digestible as those in conventional lupins. They also evaluated the nutritive value to sheep of transgenic lupin seeds containing the sunflower albumin gene (White *et al.*, 2000). The transgenic lupin seeds contained 2.3-fold higher methionine and 1.3-fold higher cysteine concentrations as compared to wild-type lupin seeds. The transgenic lupin seed increased sheep growth rate by 8% compared to the control. The sulfur concentration of wool and the cysteine concentration of plasma were also higher in the sheep that were fed transgenic lupin seeds. These results demonstrate that *SFA8* can improve the nutritional value of forage crops for ruminant animals like sheep. This is a good example of how sunflower genes may be exploited as valuable resources for improving food and feed value in other plants.

The Commonwealth Scientific and Industrial Research Organization (CSIRO) intends to continue to work toward the commercial release of a line of transgenic subterranean clover with improved nutritional value for enhanced wool growth in sheep. The transgenic clover line expresses the sunflower SFA8 albumin protein that is resistant to breakdown in the rumen of sheep. The selectable marker gene is *NPTII* (neomycin phosphotransferase gene from the bacterium *E. coli*), which confers resistance to the antibiotic kanamycin. The gene expression unit was transferred into clover plants using *Agrobacterium*-mediated transformation (Higgins, 1998). The CSIRO Plant Industry also intended to release a transgenic lupin line expressing SFA8 protein. PAT (from bacterium *s. hygroscopicus*) that confers resistance to the herbicide Basta and GUS were used to select transgenic plants (Higgins, 1997). These transgenic lines exhibited significantly enhanced level of sulfur-amino acid demonstrating their value in animal feed.

While sunflower seed albumin protein (2S or SFA8) shows promise for enhancing the nutritive value of forage crops like clover and lupin, there have been some concerns related to the allergenicity of these proteins. The sunflower SFA8 protein is somewhat homologous to the mature 2S albumin protein of the Brazil nut and shares 34% amino acid residues in common with this allergen. Overexpression of the 2S albumin gene from Brazil nut under the French bean phaseolin promoter and Nos terminator in canola (Altenbach *et al.*, 1992) and sunflower (Scelonge *et al.*, 1993) significantly enhanced the methionine in seed proteins. Kelly *et al.* (2000) examined the potential allergenicity of the sunflower seed 2S albumin protein. Sunflower seed can cause severe anaphylactic reactions in some susceptible individuals. A series of biochemical and molecular analyses demonstrated that several IgE-binding proteins include regions of the high-methionine 2S albumin SFA-8 protein.

2.3.7 Sunflower biodiesel

The biodiesel industry is growing rapidly, and is expected to have a significant impact on vegetable oil production, including sunflower. World consumption of vegetable oils has increased steadily from 62.6 million metric tons (MMT) in 1993 to 87.8 MMT in 2000 (Gobliz, 2002), and demand continues to rise. Demand is being driven by the increased use of edible oils in food preparation and in many industrial products including fuels. *Biodiesel* is a fuel comprised of monoalkyl esters of long chain fatty acids derived from vegetable oils or animal fats. It is a renewable fuel that can be utilized in diesel engines and derived from natural sources of oil like sunflower, canola, or soybean. Biodiesel has much higher density and higher kinematic viscosity than petroleum diesel fuel (Semenov, 2003). A relatively inexpensive and promising method of producing biofuel from renewable plant vegetable oils has been achieved. A mixture of saturated and unsaturated fatty acid methyl esters (ROME) was obtained by esterification of rape oil (Marchenko and Semenov, 1991). High oleic sunflower is particularly attractive for biodiesel since it can produce oil with up to 90% oleic acid, which has high oxidative stability and uniformity. Producing high

concentrations of industrially useful fatty acids in plant seeds has been initiated using biotechnology, and modifications of the fatty acid compositions of vegetable oils can make them more versatile in their uses (Burton *et al.*, 2004). Genes encoding unusual fatty acid biosynthetic enzymes have been isolated from plants. However, the challenge is how to utilize the genes for producing higher concentrations of useful fatty acids in transgenic plants (Jaworski and Cahoon, 2003).

EPOBIO is an international project funded through the European Union's Sixth Framework Programme (FP6) to exploit the economic potential of plant-derived raw materials. One of their projects is to develop sunflower oil for industrial application. Matsumura *et al.* (2005) co-ordinated the research on the production of diesel fuel through ozonation of sunflower oil. To ameliorate some of the negative characteristics of biodiesel fuel including high viscosity, flash point, and pour point, they employed the process of ozonation, which serves in the electroreduction of fatty acid methylesters and breaks long chain fatty acids into shorter molecules. This new method was very effective in improving the viscosity and flash point of sunflower oil, but had no impact on pour point. To increase the economic viability of biodiesel from sunflower, Matsumura *et al.* (2005) have carried out basic research on conversion of waste glycerol to 1,3-propanediol and have been focused on breeding for enhanced oil composition in sunflower seeds. Matsumura *et al.* (2005) point out four issues to be solved in the biodiesel business including price of vegetable oil, a reliable process for continuous production of biodiesel, improving the fuel properties of raw oil, and a technology to convert plant wastes to valuable materials (biorefinery). By comparing various plant oils, they determined that sunflower oil is better suited to biodiesel fuel manufacture than other oils as it is available from mid oleic and high oleic varieties with naturally low levels of saturated fats. Additionally, other parts of the sunflower plant have potential for producing valuable industrial materials. For example, sunflower stems might be used to produce pulp that could be used in paper manufacture. By improving the fuel properties of sunflower through the expression of novel genes from other plants, transgenic breeding has the potential to promote the development of more suitable oils for biodiesel manufacture.

On June 8th of 2006, the European Commission launched the Biofuel Technology Platform and announced the establishment of a Biofuel Task Force within EuropaBio to co-ordinate industry input. Although canola and soybean oils are popularly used in the process of trans esterification, Brazil's new biofuel—H-Bio—is a mixture of cotton, castor bean, sunflower, and soybean oils (BUSINESS LINE, July 02, 2006 Mumbai, July 1, 2006). The biofuel potential of sunflower oil could be a major driver of sunflower biotechnology and transgenic breeding progress in future.

2.3.8 Sunflower rubber

Sunflower is an important global crop that produces latex naturally. It is an ideal plant for producing rubber since sunflower latex is of high quality and some of the taller perennial species have potential for high latex yields. Scientists are attempting to improve the quality and quantity of latex from sunflower plants (Wood, 2002). Rubber manufactured from sunflower latex has the potential to partially replace America's reliance on imported natural rubber and rubber made from petroleum byproducts. Every year, the United States imports million tons of natural rubber at a cost of about 1000 million US dollars (Grow, 2002; Wood, 2002). Most of the natural rubber comes from *Hevea brasiliensis*, more commonly known as the Brazilian rubber tree. Professor John Vederas at the University of Alberta, Canada and his colleagues are working to develop a genetically modified version of sunflower to provide an alternative commercial source of natural rubber. This research was supported by USDA and the National Sciences and Engineering Research Council of Canada (NSERC). Dr. Vederas believes that sunflowers could be genetically modified to produce a reliable source of high quality natural rubber with high quality. The sunflower rubber can be stronger, more resistant to abrasion, less sensitive to cold, and better at heat dispersion than rubber produced from rubber tree latex (Wood, 2002).

The Crop Improvement and Utilization Research of USDA-ARS at Albany, California, has employed sunflower transformation to enhance latex production in sunflower. Their protocol uses both split apices and proximal parts of the cotyledons to incorporate two key genes

in the latex biosynthesis pathway. Herbicide and antibiotic resistance were used for selecting the transgenic plants. Transformants from six experiments were successfully rooted in the greenhouse (USDA-ARS 2005 Annual report). Since transgenic sunflower can cross-pollinate with other cultivated and wild sunflower plants, the group is working on a project designed to evaluate and mitigate the effects of gene flow from transgenic sunflower to wild relatives. The engineering of metabolic pathways in transgenic plants for industrial use has seen exciting progress in the past decade (Capell and Christou, 2004). Transgenic sunflower producing high levels of latex is an important product of this area of research that should be available commercially in future.

2.4 Regulation of Transgenic Sunflower Plants

Biotechnologists, plant breeders, environmental scientists, ecologists, members of society, and government understand that risk assessment procedures are critical for transgenic product development and approval. Processes related to obtaining approval to commercialize transgenic plants are overseen by regulatory authorities operating in different countries under various organizations. All of the transgenic crops that have been commercialized so far have undergone extensive field trials and regulatory studies to determine their stability and safety to humans, animals, and the environment. In the United States, government agencies take primary responsibility for regulating the release of transgenic organisms. Field experiments involving transgenic plants are highly regulated by federal and state laws as well as institutional biosafety committees (IBCs). For greenhouse research with transgenic plants, National Institutes of Health (NIH) has guidelines at four physical containment levels for experiments involving recombinant-DNA molecules (http://www.isb.vt.edu/greenhouse/green_man.section3.htm). These guidelines further categorize experiments according to specific risk criteria and assigns them to one of the four biosafety levels; BL1-P through BL4-P. The purpose of these guidelines is to prevent the transfer of recombinant-DNA from transgenic organisms

in greenhouse experiments to organisms outside the greenhouse. IBCs have been established under the NIH guidelines to provide local review and oversight of nearly all forms of research utilizing recombinant-DNA including transgenic sunflowers. USDA-APHIS (USDA Animal and Plant Health Inspection Service) permits are required for shipping transgenic sunflowers across states, performing greenhouse experiments, and for field evaluations (<http://usbiotechreg.nbii.gov/roles.asp>). All transgenic products require approvals from the EPA (Environmental Protection Agency International Agreements) (<http://www.epa.gov>) and the FDA before commercialization. These approvals require robust data demonstrating that novel transgenic products are safe for human consumption, safe for the environment, and do not negatively impact nontarget organisms. All of the member countries of European Economic Community (EEC) operate under EEC directive 90/220 "on deliberate release into environment of genetically modified organisms." The Organization for Economic Cooperation and Development (OECD) is playing a significant role in coordinating the international harmonization among member countries (OECD, 1986 and 1990), and UNIDO/UNEP/WHO/FAO is contributing to the development and application of common procedures for developing countries (UNIDO, 1991).

In 1992, several governments signed Agenda 21 at the United Nations conference on environment and development in Rio de Janeiro (Taylhardat and Zilinskis, 1992). Part of this agreement commits signatories to fostering the development of an international set of guidelines on safety in biotechnology. Developing countries like China and India are also developing regulatory procedures. In India, the major responsibility for genetically modified organism (GMO) regulation is under the Ministry of Science and Technology and Ministry of Environment and Forests, Government of India (Manjunath, 2005). Two IBC committees and the Review Committee on genetic manipulation are working under the department of Biotechnology (Manjunath, 2005). In China, various regulations have been passed into law by government. One of the agencies responsible for this is the Center for Chinese Agriculture Policy in the Chinese Academy Science (Huang *et al.*, 2002). The assessment of risks to human

health and the environmental risks from the release of genetically modified (GM) plants involves several challenges. Dale *et al.* (1993) described a risk assessment procedure. In sunflower, pollen- and seed-mediated gene flow has already been researched. Field tests have included open-air plots of male-sterile plants and caged plots that containing fertile plants that were pollinated with bees. In some developing countries, sunflower is considered as a minor crop and not much transgenic development work has been done but in other developing countries, some work has been conducted. In the 2000/2001 growing season, there were 0.8 ha transgenic sunflower planted in Chile. However, no further information is available regarding this effort. More information on regulation of transgenic plants is reviewed recently (Lu *et al.*, 2008).

3. FUTURE ROAD MAP

3.1 Expected Products

As discussed in Section 2 and demonstrated in Table 4, both agronomic input and quality output transgenic traits are being pursued in sunflower, but no commercial transgenic varieties are now in the market. *Sclerotinia* is the most destructive fungal disease of sunflower. *OXO* gene conferred significant resistance against *Sclerotinia* in transgenic sunflower (Bazzalo *et al.*, 2000; Lu *et al.*, 2000; Scelonge *et al.*, 2000). However, the *OXO*-sunflowers are not commercialized because *OXO* protein was found to be a potential allergen. Transgenic resistance to leaf herbivory by the Argentina looper was also being explored. Oil modification is another important area for transgenic research in sunflower. Genes encoding sunflower seed albumin have been utilized for transgenic enhancement of sulfur-rich amino acids in other plants for various purposes.

The two primary constraints for transgenic trait development in sunflower are the potential environmental risk of gene flow to related wild species and the cost/benefit relationship of developing and deregulating a novel transgene in the context of its value to stakeholders. Sunflower is open-pollinated and there are in proximity to some 49 related wild species in the United

States. Transgenic sunflower plants can hybridize with plants in naturally occurring wild sunflower populations and transfer the transgene into these populations. This risk is more relevant for input traits including insect resistance, disease resistance, herbicide resistance, and tolerance to abiotic stress. Quality traits, such as oil modification, pose less risk as these traits should have no impact on survival fitness or the fecundity of related weedy species.

Mutation breeding has been a primary driver for the advancement of modified sunflower products with novel traits. High oleic and NuSun™, hybrids with modified oil profiles, Clearfield™, and Express® Herbicide Tolerant Sunflowers are examples of new products on the sunflower market that have exploited mutations for favorable traits and that were developed through conventional breeding methods. These products are not transgenic. The acceptance of these new products by sunflower growers and industry customers alike has the potential to facilitate the acceptance of certain sunflower transgenic traits. Sunflower biodiesel and rubber production are examples with the potential to drive new advances in sunflower biotechnology. New technology like the integration of transgenes into chloroplasts may also stimulate additional investments in sunflower biotechnology by mitigating the risk of gene flow to related species.

Of all of the transgenic initiatives to improve sunflower, transgenic oil modification holds the greatest potential for commercial candidacy. Supporting rationale include the following: first, oil modification genes pose less environmental risk from gene flow to wild populations; second, genes are available for modifying oil composition and increasing oil yield; third, oil is the primary driver for the overall sunflower market; and finally the potential of sunflower oil for industrial applications like biodiesel should foster new investments in research and product development for oil modification and oil yield enhancement in sunflower.

Sunflower oil contains more vitamin E than any other vegetable oil, high levels of monounsaturated and polyunsaturated fats, and low saturated fat levels. This fact determines the health benefit of sunflower oil. Vitamin E consists of two types of compounds: the tocopherols and the tocotrienols.

The α -tocopherol is the most biologically active form of vitamin E. The majority of vitamin E in the diet comes from vegetable oils such as sunflower oil. It functions as an antioxidant nutrient and plays a significant role in protecting the body against damage to cell membranes. Vera-Ruiz *et al.* (2006) mapped a gene that control vitamin E accumulation and scientists at the University of Georgia recently isolated genes for the synthesis of various fatty acids and vitamin E (http://www.csrees.usda.gov/nea/plants/sri/pbpgg_sri_sunfloweroil.html). Their discovery will promote transgenic approaches to develop healthier sunflower oils with longer shelf life and novel vitamin E and industrial useful sunflower oil for biodiesel. Actually, Van Eenennaam *et al.* (2003) reported that overexpression of *Arabidopsis* gene encoding enzymes in the vitamin E synthesis pathway significantly increases the vitamin E level in soybean seeds. These results indicate that high vitamin E sunflower is another promising product in the future sunflower market.

Many trait genes (Table 3) from sunflower and other crops are currently available for transgenic applications to improve crop productivity and quality. The combination of conventional breeding method and transgenic approaches has the potential to drive synergy in sunflower product development. Biotechnology and new molecular technologies hold tremendous potential for improving modern agriculture. Transgenic sunflower varieties with enhanced disease resistance, modified oil profiles, increased grain yield, and improved agronomic performance should therefore be available to growers in the future. Industrial sunflower plants that produce fatty acids for biodiesel production in the seeds and natural rubber in leaves and stems may also be available in the future.

3.2 Risk Assessment

Many objectives of transgenic breeding are the same as those in conventional breeding. The major difference between the two approaches is the expanded gene pool that is available for transgenic research. Genes from unrelated plant species, bacteria, fungi, viruses, and even animals can be transferred into a crop genome

for the expression of favorable traits. For example, *Sclerotinia* resistance can be achieved by inserting a wheat *OXO* gene into the sunflower genome (Lu, 2003), whereas this is not possible through traditional breeding. Introducing novel genes into crops such as sunflower may also pose potential risk. Even in conventional breeding, gene combinations can sometimes give rise to undesirable gene products (Dale *et al.*, 1993). Glucosinolates in *Brassica* species (Thompson and Hughes, 1986) are good examples. Similarly, transgenic insertions may generate undesirable gene combinations with unintended impact on the environment (Chassy *et al.*, 2004).

The development of transgenic crops has been primarily limited to species of high economic importance to the developed world such as maize, soybean, cotton, sunflower, rice, and potato. The relative lack of transgenic research in developing countries is ultimately driven by funding limitations in those countries. One of the advantages of biotechnology is that once a new approach has been developed, it can be applied to other crops. Yield has always been the most important trait target for seed companies and growers. More than 30% of the yield losses experienced in agricultural production systems are due to biotic factors such as predation by insects, diseases, and competition from weeds. Therefore, input traits with the potential to significantly offset yield losses have been the primary focus of biotechnologists to date, and the first four commercialized transgenic crops have been developed for insect or herbicide resistance (Manjunath, 2005).

Sunflower pollen remains viable for several months at room temperature. There are 13 annual species, and cultivated *H. annuus* is an improved form of its wild *H. annuus* counterpart that is a weed in cropping systems. Gene flow from domesticated or transgenic sunflower plants to wild species has been well documented (Daniell, 1999; Faure *et al.*, 2002). For example, the IMI-resistant gene can flow from domestic sunflower to IMI susceptible common sunflower and prairie sunflower (Massinga *et al.*, 2003). A plant ecologist, Allison Snow, and her team studying *Bt* sunflower found preliminary evidence of gene flow from transgenic sunflower to wild plants. This is the major concern and risk for

transgenic sunflower traits. They studied a *Bt* transgene, *cry1Ac*, in backcrossed wild sunflower populations and observed that reduced herbivory allowed transgenic plants to produce an average of 55% more seeds per plant than nontransgenic controls at a field site in Nebraska. A similar but nonsignificant trend was seen at another site in Colorado (14% more seeds per plant). In a greenhouse experiment the transgene had no effect on fecundity, suggesting that it was not associated with a fitness advantage. Snow *et al.* (2003) reported the first experimental evidence indicating that transgenes could move from transgenic crops and introduce novel traits into related species. This potential risk cast a potential shadow over the advancement and realization of biotechnology's full potential in sunflower. However, Burke and Rieseberg (2003) reported that *OXO*-enhanced wild plants did not produce more seeds than the unmodified controls, and that the *OXO* transgene would do little more than diffuse neutrally after its escape. Chapman and Burke (2006) focus on the escape of transgenes from cultivated crops via crop \times wild species hybridization and show that natural selection, and not the overall rate of gene flow, is the most important factor governing the spread of favorable alleles in wild species. Massinga *et al.* (2005) reported that the IMI-resistance gene has little impact on the competitive ability of common and prairie sunflower. In general, the ecological impacts of transgenic sunflower are largely unknown. When addressing the environmental impact of a transgene, the space, time, genetic background, and abiotic stresses need to be taken into account (Chapman and Burke, 2006).

Although greenhouse and field trials have been conducted in the past several years, no transgenic sunflower product has been commercialized to date. Consequently, it is too early to evaluate the impact of transgenic sunflower on humans and the environment. Since the very first transgenic crop was commercialized in 1996, transgenic crops with improved input and quality traits have been planted in 17 countries on 6 continents and represent over 81 million ha (Manjunath, 2005). The impact magnitude of GM crops on the landscape has the potential to reduce biodiversity by increasing agricultural intensification, gene flow, and toxicity to nontarget organisms from herbicide-resistance, insect-resistant, and disease-

resistant crops. Currently GM crops coexist with organic production, and conventional agriculture (Altieri, 2005). Transgenic plants may generate unintended nontarget effects (Pilson and Prendeville, 2004). The escape of transgenes, conferring insect resistance, herbicide resistance genes, and stress-tolerance genes into wild populations via hybridization and introgression, may increase weeds populations by enhancing their fitness in the environment.

Five categories of risks related to the release of transgenic crop plants have been discussed (Hedge and Ellstrand, 2002; Altieri, 2005; Manjunath, 2005). The potential risks include: (1) Gene flow from transgenic crops to wild species and subsequent spread through horizontal transfer, seed or pollen dispersal. Horizontal transfer means the nonsexual transfer of transgenes from one organism to another. This type of transfer is more important for sunflower since sunflower is an open-pollinated crop and has many wild relatives. The gene flow may impact the farming practices in developed countries. (2) Impact of transgenic products on nontargets. Nontargets refer to the organisms that are present in cropping systems such as beneficial insects that are not the intended targets of engineered genes. The impacts include unintended detriment or benefit to the nontargets. (3) Direct risks from the transgenic crops. The transgenic product may have detrimental effects on human health or a transgenic plant may itself become an environmental problem if ecological performance is altered by the transgene. For example, Brazil nut albumin-transgenic soybean seeds have the potential to cause food allergies (Nordlee *et al.*, 1996). A transgenic food product containing antibiotic resistance markers may have risk by reducing the ability of antibiotic drugs to control pathogens causing human diseases. (4) Risks from the increased use of herbicide. This risk includes the exploitation of herbicide resistance traits that enable the use of herbicides on crops that were historically susceptible to a given herbicide. (5) Evolved resistance in the targeted pests. Resistance may occur in organisms targeted by the transgenes and is a potential environmental hazard that requires new control strategies or chemicals. Insect, pathogens, and weeds all have the ability to evolve new resistance. This is an important resistance management consideration.

It is important to keep the potential risks in mind when pursuing the transgenic approach for improving sunflower. However, not all of the risks apply to sunflower or to sunflower-specific transgenic traits. The risks depend on the transgene and the trait target. Clearly, there is much work ahead before the commercialization of a transgenic sunflower product is realized. However, given the enormous adoption and utilization of GM crops without any reported deleterious impacts to humans, it is clear that the benefit to humanity warrants further development in this exciting field.

3.3 New Technologies for Transgenic Breeding

3.3.1 Chloroplast transformation

Organelle inheritance is strictly maternal and genomes of organelles are often inherited uniparentally. Therefore, engineering chloroplast or mitochondrial genomes may provide a practical solution to the gene flow problem. This new transformation method is especially important for sunflower biotechnology. Although not yet reported in sunflower, stable transformation of chloroplasts in other higher plants has been reported (Svab *et al.*, 1990; Daniell *et al.*, 2004; Maliga, 2004). Rieseberg *et al.* (1991) and Rieseberg and Arias (1992) have studied the sunflower genetics of chloroplast genome, and Wills *et al.* (2005) describe chloroplast SSR polymorphisms in the family composite and the mode of organelle inheritance in sunflower. This research provides the best evidence to date that organelle gene containment may be a viable strategy in sunflower.

Gressel and Al-Ahmad (2003, 2004) described molecular solutions for increasing biosafety of transgenic plants. There are many ways to prevent transgene introgression from crops to other species, or to related weeds or wild species (containment strategies). These strategies are of particular importance for sunflowers. Once a transgene escapes, there is potential for it flowing into unintended species unless mitigated by some other containment strategy. To promote the development of transgenes in sunflower and

other crops, attention must be given to technology that can solve the gene flow problem.

3.3.2 Transformation efficiency and selection markers

One bottleneck of sunflower biotechnology is transformation efficiency. Optimization of the conditions for particle bombardment and identification of suitable genotypes and tissues for *Agrobacterium*-mediated transformation should improve overall transformation efficiency. Combinations of these methods may also enhance efficiency (Bidney and Scelonge, 1997; Pugliesi *et al.*, 2000).

For *Agrobacterium*-mediated transformation, systematic studies to identify the optimum tissue type, genotype, *Agrobacterium* strain, wounding method, medium, and binary vector are required for developing an ideal transformation method. Transgenic plants generated through *Agrobacterium*-mediated transformation may contain unwanted oncogene sequence from *Agrobacterium*, which may mediate gene transmission to other organisms that impact water, soil, and even humans. Therefore, the transformation method should include steps to completely eliminate unwanted sequence to prevent the unintended movement of transgenes to other organisms. *Agrobacterium* can be eliminated with antibiotics (Cubero and López, 2004). Extra DNA sequence from *Agrobacterium* may be delivered into transgenic tissues and the sequence can be detected and characterized via PCR assay (Cubero and López, 2004).

Antibiotic and herbicide resistance genes are often used as selectable markers for identifying transgenic plants. These genes have the potential to impact humans and other nontarget organisms or to impact the environment. Therefore, transgenic products should not contain such marker genes. Ebinuma *et al.* (2004) have developed a vector-based method to produce marker-free transgenic plants. The method utilizes the oncogenes of *Agrobacterium* and a site-specific recombination system to eliminate the marker genes. This system can also increase regeneration frequency. Positive selection, such as the OXO-transformation method (Lu *et al.*, 2000; Scelonge *et al.*, 2000), is another way to get marker-free transgenic plants.

3.3.3 Transgene expression

Stable expression of the desired transgene in transgenic plants is critical for success. In general, the promoter determines the pattern and level of the transgene expression. There are constitutive, tissue-specific, and inducible promoters available for developing transgenic sunflower. Limiting transgene expression to targeted tissues can reduce the potential negative impacts of the transgene on other traits. The site of transgene integration in the sunflower genome can also influence the pattern and level of expression. To mitigate these problems, a number of transgenic events are often generated with a given construct and only the event with the best performance is selected for product development. Multiple copies of the transgene can co-exist in a single transgenic event. In general, single copy events are preferentially selected for product development to prevent potential co-suppression of the transgene.

Most transgenic crops contain only one transgene that codes for pest resistance, herbicide tolerance, or other traits. In order to compound the benefits of multiple novel traits, more than one gene is sometimes used in the development of new transgenic products. This can be achieved through the construction of vectors with more than one beneficial transgene or can be achieved by stacking or pyramiding proven transgenes through conventional breeding. Transgene stacking is a useful technology for developing traits that are controlled by unrelated genes. For example, stacking a *Bt* gene and an herbicide resistance gene in corn can produce transgenic corn plants with both enhanced insect and herbicide resistance (Castle *et al.*, 2006). This technology might also be utilized to develop transgenic sunflower plants that produce high levels of latex in the leaves and stems for rubber manufacture and enhanced fatty acid profiles in the seeds for the manufacture of biofuel.

3.3.4 Gene shuffling technology

Gene shuffling is a process of producing a large array of many variants of DNA sequences from a gene or a gene family, and then reassembling the gene from random fragments by recombination (Stemmer, 1994; Castle *et al.*, 2004). The

recombinant genes can be screened in bacterial or other systems to identify the best version of the target gene. This technology has been successfully applied in various areas including herbicide resistance gene (Castle *et al.*, 2004). A weak glyphosate acetyltransferase (*GAT*) was improved 7000-fold through the shuffling technology and the improved *GAT* is being transformed into corn, soybean, and other crops for improved glyphosate herbicide resistance by Pioneer Hi-Bred International Inc., a DuPont company (Castle *et al.*, 2004; Siehl *et al.*, 2005). This optimized *GAT* gene can be transformed into sunflower for achieving a high level of glyphosate herbicide resistance. One of the challenges for oil composition modification in sunflower and other oilseeds is increasing the level of the new fatty acids. This shuffling technology can facilitate improving the activity of various desaturases for producing healthier oils or more useful industrial oils.

3.3.5 RNA interference technology

RNAi technology is a double-stranded RNA-induced and small RNA-mediated gene silencing (Cerutti, 2003), and the RNAi machinery is present in sunflower (Hewezi *et al.*, 2005). As indicated in the oil modification session, RNAi technology is very useful for modifying oil composition (Singh *et al.*, 2000; Bidney *et al.*, 2002). This technology also has great potential for engineering other traits in sunflower including resistance to disease, insect resistance, and herbicide. For example, expression of double-stranded RNA of a broomrape essential gene in sunflower may control the weed broomrape through uptake of specific dsRNA and subsequent silencing of the essential gene in broomrape through RNAi mechanisms.

3.3.6 Molecular marker-assisted selection

Conventional breeding has relied primarily on phenotypic selection of superior progenies from hybridization between two inbred lines. Although this breeding method has been very successful and has resulted in crops with improved yield and other agronomic traits, selection is often complicated

by environmental variance that creates obstacles to the evaluation of complex quantitative traits like yield and disease resistance. Sunflower breeding programs have been significantly enhanced through the use of molecular markers including RFLP, RAPD, SSR, and single nucleotide polymorphism (SNP). Some traits are controlled by single genes but many important agronomic and quality traits are controlled by multiple genes. Following the inheritance patterns of the QTL using molecular markers can assist selection for these loci in a breeding program. This process is called MAS for QTL and has the potential to both increase genetic gain in plants and enable breeders to identify genes contributing to traits like yield. The recent, rapid evolution of marker technology has fostered the identification and sequencing of valuable trait loci and the definition of heterotic groupings in crops where hybrid breeding is established. It has also enabled MAS techniques that permit breeders to identify desirable progeny earlier in the breeding process, and facilitates trait integration programs that permit the conversion of proven elite lines to isogenic sister lines with added traits of value (Tanksley *et al.*, 1998; Rommens and Kishore, 2000; Perez-Vich *et al.*, 2004a, b). MAS technology has been used in sunflower breeding for various disease resistance traits (Lawson *et al.*, 1998; Brahm *et al.*, 2000). With the development of an array of molecular markers and a dense genetic map of the sunflower genome, MAS for both single genes and QTLs is now possible (Babu *et al.*, 2004). MAS in combination with transgenic technology have the potential to greatly accelerate sunflower product development. Precise markers can be designed with transgene sequence that can facilitate the rapid introgression of transgenes into multiple genetic backgrounds. More importantly, MAS can greatly enhance transgene stacking for the development of sunflower products with multiple added-value traits.

3.4 Future Perspectives

1996 was a landmark year in the history of biotechnology. In that year, four transgenic crops with transgenic insect and herbicide resistance received regulatory approvals and were commercially planted in the United States. The transgenic

approval process in general required some 14 years of intensive research to demonstrate that a novel transgenic product is both beneficial to farmers and safe for humans, animals, and the environment (Manjunath, 2005). The crops included corn hybrids that expressed a *Bt*-protein for protection against European corn borer (*Ostrinia nubilalis*); potatoes expressing *Bt*-proteins to protect against the Colorado potato beetle (*Leptinotarsa decemlineata*); cotton expressing *Bt*-proteins to protect against a host of pests including tobacco budworm (*Heliothis virescens*), bollworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiella*); and soybean with transgenic resistance to the broad spectrum herbicide glyphosate (Manjunath, 2005).

During the last 10 years additional crops with improved transgenic insect and/or herbicide resistance and other traits have been developed and commercialized in the United States and other countries. In addition, corn and soybean varieties with multiple transgenic trait stacks have also been commercialized. The area planted to transgenic crops is steadily increasing and more and more countries are issuing approvals that will permit even greater adoption of the technology in the future (James, 2004; Manjunath, 2005; Castle *et al.*, 2006). The planted area of transgenic crops increased from 1.7 million ha in 1996 to 81 million ha in 2004 and the number of countries growing transgenic crops increased from 6 in 1996 to more than 17 in 2004 (James, 2004). The crop that leads in overall cultivated area is soybean with transgenic herbicide resistance. Benefits from these transgenic crops include improved weed and pest management, higher yields, and greater profits to the growers. These benefits and the amelioration of some negative environmental impacts from agriculture, such as soil erosion and wide spectrum pesticides, have significantly promoted the adoption of transgenic crops globally. In 2004, Ford Runge and Barry Ryan summarized the global transgenic research and status: at least 63 countries have initiated transgenic approvals for commercialization of some 57 different crop plants. More and more people are realizing the benefits of transgenic crops. The world population will likely reach 9.3 billion by the year 2050 and food demand will double from its current level as the population grows. Transgenic crops have the potential to help

meet this increased food demand and prevent the risk of an unstable future. The amount of arable land available for agriculture is steadily decreasing each year as industrial, residential, recreational, and other needs lead to conversion of farmland into parking lots, strip malls, and housing complexes. Currently about 852 million people in developing countries suffer from malnutrition. About 1.4 million women (about 22% of world population) suffer from anemia caused by iron deficiency. About 140 million children suffer from vitamin A deficiency. More than 30% of crop losses in agriculture are attributable to biotic stresses such as disease, insects, and weeds and abiotic stresses such as drought, cold, heat, salinity that might be overcome through transgenic research (Manjunath, 2005). There is an increasing need to produce more food that is more highly nutritious from a shrinking arable land base.

A transgene can come from any organism and biotechnology eliminates the reproductive and phylogenetic barriers that have limited success through traditional breeding. The first generations of transgenic crops with enhanced insect or herbicide resistance have already provided beneficial traits to farmers and end users. In the future, transgenic crops with enhanced tolerance to environmental stresses such as drought, and/or with enhanced nutritional value, and/or with improved properties for industrial uses such as biodiesel may be achieved. Hedge and Ellstrand (2002) discussed potential benefits of transgenic crops by comparing them to their conventionally derived counterparts. They listed four primary benefit categories:

1. Benefits from increased yields. Meeting the demand for increased food supplies by a growing human population is considered as one of the major environmental threats of the future (Clout, 1999). Transgenic traits have provided an efficient method for increasing crop yield. *Bt*-corn and *Bt*-cotton are good examples where yield losses from predatory insects have been reduced using transgenic technology. Yield gains from the use of insect-resistant transgenic plants provide indirect benefits to the environment by reducing the need for further exploitation of land that is not currently under cultivation. This is most important in developing countries

(Alexandratos, 1999). Yield enhancement can postpone the conversion of wild lands to agricultural production and maintain balanced ecological environments.

2. Environment protection from agricultural chemicals. One of the major concerns for conventional agriculture is the heavy use of insecticides, fungicides, and nematicides that can pollute water, air, and soil environments. *Bt*-technology can enhance the ability of crops to withstand pests, thereby benefitting the environment by reducing insecticide use. The commercialization and widespread use of *Bt*-corn and *Bt*-cotton has significantly reduced the amount of insecticides used on these crops in the United States and other countries.
3. More efficient use of renewable resources such as land, water, and soil nutrients. Soil erosion in conventional industrial agriculture can occur 100 times faster than the rate of soil formation (NRC, 1989). The rapid rate of soil erosion has enormous potential to reduce the productivity of soils and contributes to the sedimentation in streams and pollution through run off that negatively impacts aquatic ecosystems. The advent of no-till agriculture in the United States has resulted in a number of economic and environmental benefits. The cultivation of transgenic herbicide-resistant corn and soybean has greatly contributed to the increase of no-till agricultural systems that help reduce or eliminate soil erosion. Transgenic plants that have enhanced resistance to root diseases could further increase the prevalence of no-till agricultural systems.
4. Monitoring of environmental pollution using pollution-sensitive transgenic plants. Transgenic plants have the potential to be used for detecting and even remediating environmental pollution. Transgenic tobacco plants can serve as monitors of heavy metal concentrations in soils and may serve as visible indicators of temperature or moisture levels in the environment.

In addition to the benefits described above, transgenic crops can significantly benefit human life by providing more nutritious and healthy foods, desirable fruits, and valuable industrial products. High oleic soybean (Cahoon, 2003; Kinney, 2003) and transgenic tomato (<http://>

www.ars.usda.gov/is/AR/archive/sep00/tomato0900.htm) with long shelf life are good examples that illustrate the benefits of transgenic crops to human life. Sunflower is one of the world's four most important oilseed crops, with an estimated value of \$40 billion per year and provides a source of premium vegetable oil, confectionery food and ingredients for various industrial products. Biotechnology has the potential to help evoke the full potential of this valuable crop. With the increases in demand for petroleum that are expected in the future, it is likely that government and industry will continue to support biofuel development including biodiesel made from sunflower. Latex from sunflower has the potential to be improved and exploited for domestic rubber manufacture and the promise of specialty oils with a myriad of industrial applications will further drive the development of new sunflower products. As new products are developed, transgenic approaches have the opportunity to play a pivotal role in their development.

Public acceptance of genetically modified crops varies and some people are opposed to the use of biotechnology for crop improvement. The reluctance to accept biotechnology in agriculture is often attributed to a lack of sufficient and accurate information in the public sector (Bruening, 2000; Hino, 2002). For example, Hollywood films depict GMOs as terrifying scientific accidents in movies like "Spider Man" and "Corn". These films are not based on science and can mislead the public into believing that any manipulation of DNA is extremely risky and may lead to disaster (Byrne, 2006). The good news is that universities and biotechnology companies have initiated efforts to educate the public with a series of lectures on biotechnology. Like other technologies, it will take time for biotechnology to be dominantly accepted by the public.

Intellectual property protection is another hotly debated topic related to biotechnology. A training handbook is available (<http://www.dfat.gov.au/publications/biotech/index.html>) and a recent review has summarized and discussed intellectual property issues (<http://www.dfat.gov.au/publications/biotech/index.html>) in the regulation of genetically engineered plants and animals. Intellectual property takes important roles in economic growth and advancement in the biotechnology sector. Patents protect researchers'

new ideas and products and provide incentives for private sector investment into biotechnological research and product development.

New technological developments should accelerate the discovery of new trait genes, expression tools, efficacious transformation methods, and methods for breeding transgenes into elite lines. Advancements in sunflower structural and functional genomics have lagged behind advancements in crops such as maize, rice, and soybean, in part due to a lack of financial support for investments in the crop and its relatively large genome size. Fortunately, the technologies and tools that have already been developed in other crops can be easily adapted to sunflower research. Gene flow is a particular issue for sunflower biotechnology. Therefore, efforts should be directed toward developing methods for preventing transgene introgression from sunflower to related weeds or wild species. Chloroplast transformation is one of the relevant techniques that may facilitate this end. Many trait genes from sunflower and other crops are available for improving productivity and quality in the crop. In the sunflower industry, transgenic initiatives like the ones listed in Table 4 have already begun and sunflower biotechnologists have gained considerable knowledge, methodologies, and tools for developing novel transgenic products. Molecular breeding and transgenic approaches can be synergistic in product development. In the near future, sunflower biologists will be able to generate transgenic sunflower varieties that have increased grain yield through enhanced resistance to abiotic and biotic stresses and produce high quality seeds with more useful fatty acid profiles. The great potential of rubber and biodiesel from sunflower will likely advance sunflower biotechnology to a bright new era and foster the development of "industrial sunflower" with enhanced oils for biofuel in the seeds and enhanced latex content in leaves and stems for rubber manufacture; all the while inspiring the imagination of artists with its beautiful floral magnificence.

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Peanut

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1. INTRODUCTION

Peanut, or groundnut, is a New World legume that is believed to be native to South America. Discovered in the 1500s by early Spanish and Portuguese explorers as an extensively cultivated crop of the Indians in the West Indian Islands, Mesoamerica, and South America, peanut was disseminated throughout Europe and eventually traveled to the seaboard of the southeastern United States. Cultivated peanut, *Arachis hypogaea* L., has since become the third most important oilseed grown in the world, serving as an important food source for millions of people living in semiarid and tropical regions. As a significant source of proteins, vitamins, and minerals, peanut is used for human and animal consumption as well as in soaps, detergents, and cosmetics. This chapter discusses the origin and history of the peanut, progress toward improving the crop through traditional breeding, and the role of transgenic breeding in future peanut germplasm improvement.

1.1 History, Origin, and Distribution

There is little doubt that the peanut, *A. hypogaea* L., is of South American origin. Although the crop was referred to by various names, evidence to

support its presence in South American countries is abundant in the 16th and 17th century literature. The first recorded report of the peanut appears to be one written by Captain Gonzalo Fernandez de Oviedo y Valdes, a historiographer of the Indies. In 1535, he wrote of a fruit named “mani”, which was sown, grown underground, and upon harvesting, yielded fruit about the size of pine nuts, but in shells (de Oviedo y Valdes, 1535/1547). The Amerindian name “mani” for peanut is still used in Cuba and Spanish South America today. An extensive review of early post-Columbian records reveals many references to peanut and its cultivation (Hammons, 1973, 1982) indicating the crop’s widespread geographical distribution before the discovery of America. Peanut cultivation was reported in Peru, Hispaniola, Rio de Janeiro, Mexico, Brazil, Spain, the West Indies, and the La Plata basin in the 1500s. By the 17th century, production of peanuts had spread to Jamaica, Barbados, Guadeloupe, the French Antilles, and Pernambuco, with the first published report of cultivation in the Americas occurring around 1640.

Archeological evidence of peanut cultivation also points to a South American origin. The earliest history of the crop is revealed in art and artifacts of the early Peruvian civilization along the eastern slopes of the Andes, dating back to approximately 3900–3750 years before the present

(Hammons, 1994). Well-preserved groundnuts in terracotta jars and funerary vases decorated with well-sculptured groundnut pods reveal the esteem to which the crop was held by those cultures. Bird (1948, 1949) discovered evidence placing the appearance of peanuts in Peru prior to maize and warty squash, somewhere around 1200–1500 BC. The discovery of ethnobotanical remains established the presence of peanuts as a cultigen in the Tihuacan valley of Mexico at approximately 100 AD, although the evidence suggests that it was introduced as a minor crop there. As of yet, phytomorphic representations and ethnobotanical remains of peanuts in Brazil and Bolivia have not been discovered, but the climate in those countries is less favorable for the preservation of plant remains. For the most part, archaeological evidence concerning peanuts is supported by natural historians and other disciplines.

Although the exact geographical origin of peanut is not known, we know that the crop has been under cultivation for more than 3500 years and has been widely dispersed through human migration and crop exchanges. All wild species of the genus *Arachis* are found in South America and are distributed from northeastern Brazil to northwestern Argentina and from the south coast of Uruguay to the northwestern Mato Grosso (Gregory and Gregory, 1979; Gregory *et al.*, 1980). The cultivated peanut, *A. hypogaea*, was first described by Linnaeus (1753) and is believed to have originated in southern Bolivia (Krapovickas, 1968; Cardenas, 1969).

Dissemination of the cultivated peanut from South America began with Europe and spread to both coasts of Africa, Asia, the Pacific Islands, and eventually to the southeastern United States. At least two distinct forms, a 2-seeded Brazilian and a 3-seeded Peruvian type, were distributed (Dubard, 1906). The first introduction of the groundnut into Europe was reported by Nicolas Monardes in 1574 (Hammons, 1994). Early European peanut cultivation was further documented by Krapovickas (1968) based on reports that a Spanish type of seed came from Brazil to Portugal and then to Rome. The Portuguese have been credited with enriching African agriculture by introducing peanut there from Brazil and possibly to other lands as well. Most believe the first introduction of peanuts into the United States occurred when slave ships from

Africa arrived in the 1700s. The earliest full-season peanut reported in the United States was a small-podded runner type (var. *hypogaea*), which was cultivated in the southeast and probably came from Africa, but has characteristics similar to those described for peanuts found in the West Indies (Hammons, 1994). Large-seeded Virginia-type (var. *hypogaea*) peanuts are thought to have originated in Bolivia and were cultivated in the United States as early as 1844. Spanish peanuts (var. *vulgaris*), which were first cultivated in Europe by Spaniards and thus termed “Spanish”, require a shorter growing season and were introduced to the United States from Malaga, Spain, in 1871. Valencia (var. *fastigiata*) peanuts were coined so by Beattie (1911) due to their introduction into the United States in the 1900s from Valencia, Spain. Introduction of the peanut into India, credited to the Spaniards, is believed to have occurred in the middle 1800s, with the first large scale production (4000 acres) being reported in India in 1850–1851.

1.2 Botanical Description

The genus *Arachis* L. belongs to the family Leguminosae and comprises a large and diverse group of diploid and tetraploid taxa in excess of 50 species, which are naturally dispersed in Argentina, Bolivia, Brazil, Paraguay, and Uruguay. The diversity of this genus is evident by the numbers of annuals and perennials it comprises and their varied forms of reproduction. Most *Arachis* species reproduce by seeds, but some are rhizomatous and reproduce largely through vegetative means. Members of the genus are identified and characterized by their alternately attached basal and dorsal anthers, flowers in terminal or axillary spikes, and pinnate stipulate leaves and leaflets. The “peg”, which results from the expansion of the intercalary meristem at the base of the ovule, is unique to the genus and results in a lomentiform carpel (pod), which contains one to five seeds, each comprises two large cotyledons and a straight embryo. The generic description of *Arachis* given by Bentham and Hooker (1867) is as follows:

Cotyledons thick, fleshy; radical very short; low suberect herbs, often prostrate; leaves abruptly bipinnate, leaflets in 2 pairs or rarely trifoliate, estipulate, stipules adnate to the petiole at the base. Flowers crowded into a dense axillary spike,

sessile at the leaf axile or the biauriculate bract or very shortly pedicellate. Bracteoles below the calyx linear. Calyx tube filiform, lobes membranaceous, the 4 upper ones connate, the lower one slender, separate. Petals and stamens inserted at the apex of the tube, standard suborbicular, wings oblong, free, keel incurved, prostrate. All stamens connate into a close tube, occasionally one absent; anthers alternate, elongate, subbasifixed, the alternate ones versatile. Ovary subsessile toward the base of the calyx tube, two to three-ovuled. After the fall of the flower ovary shows toward a stalk, the torus, which elongates and becomes reflexed and rigid and the ovary is continuous with the same, acute at the apex, and after the fall of the style ends in a stigmalike callus; the style is long, filiform with a minute terminal stigma; the pod ripens inside the soil and is oblong, thick, indehiscent, subtorulose but not articulate, and continuous inside. Seeds 1-3, irregularly ovoid.

Cultivated peanut, *A. hypogaea* L., is not known to occur in the wild state but is presently cultivated in over 80 countries in tropical and warm temperate regions of the world. *A. hypogaea* L. was the first *Arachis* species to be described by Linnaeus and is one of four tetraploid ($2n = 4x = 40$) *Arachis* species, the other three being *A. monticola*, *A. glabrata*, and *A. hagenbeckii* (Smartt and Stalker, 1982). Most other *Arachis* species are diploid, having a $2n = 2x = 20$ complement of chromosomes. Cytological characterization of *A. hypogaea* L. reveals two distinct pairs of chromosomes, one termed the A genome, is smaller than any other pair, and the other termed the B genome, has a secondary constriction and is satellited (Husted, 1933, 1936). Thus, the cultivated peanut is an allotetraploid, which combines the A and B genomes. The presence of an A genome in all examined *Arachis* material except *A. batizocoi* was later confirmed (Smartt *et al.*, 1978a, b). The exact origin of the A and B genomes remains unclear. However, four A genome species, *A. cardenasii*, *A. chacoense*, *A. correntina*, and *A. duranensis*, and two B genome species, *A. batizocoi* and *A. impaensis*, have been identified as possible progenitors of *A. hypogaea* L.

A. hypogaea L. is divided into two subspecies, *hypogaea*, which is further divided into four botanical varieties, and *fastigiata*, which is made up of two varieties. The subspecies *hypogaea* has alternate branching, an absence of flowering along the main stem, and either a spreading (prostrate)

or bunching growth habit. One botanical variety within this subspecies, *hypogaea* var. *hypogaea*, encompasses the Virginia and runner peanut market types. Another botanical variety, *hypogaea* var. *hirsuta*, comprises the Peruvian humpback or Chinese dragon types. Two other botanical varieties exist under the subspecies *hypogaea*: var. *aequatoriana* and var. *peruviana*. Seed from the *hypogaea* subspecies requires a dormancy period after maturity before germination. The *fastigiata* subspecies is characterized by sequential branching, flowers on the main stem, little or no seed dormancy, and an erect growth habit. The two botanical varieties within the *fastigiata* subspecies are *fastigiata* (Valencia market type) and *vulgaris* (Spanish market type) (Krapovickas and Gregory, 1994).

Cultivated peanuts are either erect or prostrate and usually 6–24 in. (15–60 cm) in height (Figure 1). Peanut plants produce one well-developed tap root system with many lateral roots that are generally sparsely hairy. Nodules

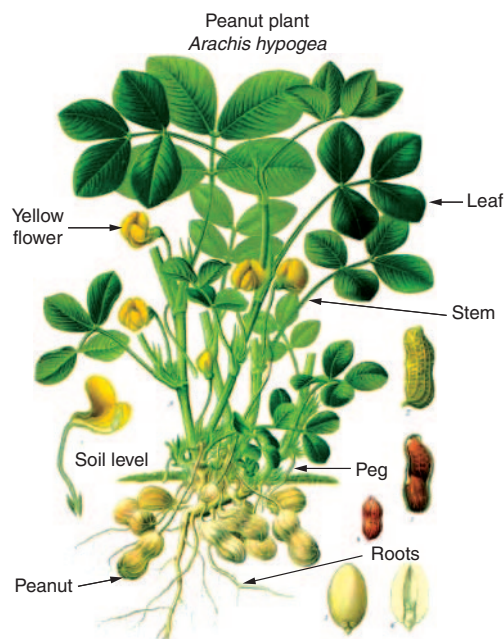


Figure 1 Graphic illustration of a peanut plant [Reproduced from http://www.enchantedlearning.com/.../Peanutplant_bw.GIF. © EnchantedLearning.com]

where nitrogen fixation takes place are formed by the symbiotic bacteria belonging to the genus *Rhizobium* and are normally found on the roots. Arranged alternately on the stems, peanut leaves have four leaflets arranged in opposing pairs. Leaves are normally borne on slender, grooved and jointed rachis, subsessile, elliptic, and stipulate (Ramanatha and Murty, 1994). Flowers occur on inflorescences located in the axils of the leaves and are never born at the same node as vegetative branches. Each flower has five petals at the top of the calyx tube, ten stamens, and a pistil composed of an ovary, a style, and a stigma. The first flowers appear 4–6 weeks after planting. Anthesis and self-pollination usually occur at sunrise with pollination taking place within the closed keel of the flower. After fertilization, a pointed gynophore, commonly known as the “peg”, develops, elongates, and enters the soil where pod growth begins. Spanish and Virginia market types require less time to produce mature seed (90–120 days) compared to runner market types, which generally require 130–150 days after planting (DAP). Boote described the growth stages of peanut in 1982 (Boote, 1982), dividing them into two categories: vegetative and reproductive (Table 1).

1.3 Economic Importance

Peanut is grown on approximately 37 million acres worldwide and is the third major oilseed crop, surpassed only by soybean and cotton. China leads the world in peanut production and exports, producing over 13 million metric tons in 2004 (Table 2). Peanut acreage in China accounts for 25% of that for all oil seed crops. Production there has increased greatly since 1961, when production was reported to be just over 1 million metric tons. Peanuts can be grown in most provinces of China, but most production occurs in the summer and in one of two geographic regions separated by the Yangtze river. At least half of the peanuts grown in China are used for oil where the majority of peanuts grown are of the intermediate runner and Spanish types. Second only to China, India is now the second largest peanut producing nation in the world, harvesting almost 7 million metric tons in 2004. Peanut accounts for approximately 50% of oilseed production in India, and like China, half of the peanuts produced are used for oil production. Five states, namely, Gujarat, Andhra Pradesh, Tamil Nadu, Karnataka, and Maharashtra, account for almost 90% of India's production, where peanuts are grown during the

Table 1 Growth stages of peanut^(a)

	Abbreviated title of stage	Description
Vegetative stages		
VE	Emergence	Cotyledons near soil surface with seedling showing; some part of plant visible
VO		Cotyledons flat and open at or below soil surface
V-1 to V-(N)	First tetrafoliate to Nth tetrafoliate	One to N developed nodes on the main axis (node is counted when its tetrafoliate is unfolded and leaflets are flat)
Reproductive stages		
R1	Beginning bloom	One open flower at any node on the plant
R2	Beginning peg	One elongated peg (gynophore)
R3	Beginning pod	One peg is the soil with turned, swollen ovary at least twice the width of the peg
R4	Full pod	One fully expanded pod, to dimensions of cultivar
R5	Beginning seed	One fully expanded pod in which seed cotyledon growth is visible when the fruit is cut in cross section (past the liquid endosperm stage)
R6	Full seed	One pod with cavity filled by the seeds when fresh
R7	Beginning maturity	One pod showing visible natural coloration or blotching of the inner pericarp or testa
R8	Harvest maturity	Two-thirds to three-fourths of all developed pods showing coloring of pericarp or testa
R9	Over-mature pod	One undamaged pod showing orange-tan coloration of testa or natural peg deterioration

^(a)Reproduced from Boote (1982)

Table 2 Peanut production, 2004^(a)

Countries	Production (t)	Harvest (ha)	Yield (kg ha ⁻¹)
China	13 420 000	5 057 000	2650
India	7 700 000	8 000 000	960
United States	1 879 750	530 950	3540
Nigeria	1 510 000	1 230 000	1230
Indonesia	1 130 000	700 000	1610
Chad	450 000	480 000	940
Senegal	445 000	525 000	850
Congo, Dem Republic of	410 000	520 000	790
Vietnam	400 000	250 000	1600
Sudan	370 000	550 000	670
Argentina	324 000	163 000	1990
Burkina Faso	301 000	331 000	910
Mali	285 000	250 000	740
Ghana	225 000	225 000	1000
Brazil	221 000	98 000	2260
Guinea	210 000	210 000	1000
Egypt	190 000	60 000	3170
Cameroon	100 000	320 000	310
Zimbabwe	45 000	220 000	200
Total	29 405 750	197 199 500	1491

^(a)Source: <http://www.usda.gov/nass/pubs/agr04/04.ch3.pdf>

kharif (rainy) season (<http://www.lanra.uga.edu/peanut/knowledgebase>). Although India is the second largest peanut producer in the world, the inability to rid its crop of aflatoxin accumulation limits the amount of product exported.

The United States ranks third among peanut producing countries, with the majority of its peanut acreage distributed across the southeastern region of the nation. Although the United States does not lead the world in peanut production, it has ranked first in yield per land unit for over 15 years. Peanut is ninth among major row crops being grown in the United States and was second in dollar value per acre prior to the passing of the Farm Security and Investment Act of 2002, but has dropped in the rankings since then due to the elimination of the price support system. Historically, the bulk of peanut production in the United States is concentrated geographically in specific counties of seven states: Alabama, Florida, Georgia, New Mexico, North Carolina, Oklahoma, and Texas. Georgia leads the United States in peanut production acreage cultivating approximately 755 000 acres in 2005 (http://www.nass.usda.gov/Statistics_by_State/Georgia/index.asp). All four market types of

peanut are grown in the United States with the southeast producing mainly runner types, the Virginia–Carolina region producing Virginia and runner types, and the southwest producing Spanish, runner, and Valencia types.

Production of peanuts is generally conducted in the warm temperate regions of the world. In fact, temperature is the major limiting factor for peanut yield since a minimum of 3000 growing degree days (with a base of 50 °F) are required for peanut growth and development (Ketring and Reid, 1995). The seasonal amount of water needed for peanut production is 16–30 in., with the limits depending upon environmental conditions such as air and soil temperature, wind, and radiation. Many peanut production areas receive the required amount of natural rainfall needed for crop production (dry land production) and others must be irrigated.

To begin a growing season with a healthy crop, high-quality seeds with at least 80% germination rate are needed. In the United States, optimal conditions for planting generally occur in early to late spring (Shokes and Melouk, 1995). Adequate soil moisture and a minimum soil temperature of 60 °F are necessary for germination but emergence rapidly increases if soil temperatures are above 70 °F. Growth rates vary with market type but in general, Spanish and Virginia market types require less time to produce mature seed (90–120 days) compared to runner market types, which generally require 130–150 DAP. As the growing season ends, the maturity of peanuts within the pods can be predicted using a hull-blast method developed at the University of Georgia (Williams and Drexler, 1981). When ready to harvest, peanut plants are generally dug, inverted, and left to dry or “cure” in “wind rows” in the field until acceptable moisture content is met. For maximum yield and quality, peanuts should be dug prior to a hard freeze. Freezing temperatures reduce peanut germination, seedling vigor, and metabolic processes as well as reduce the strength of the shoot–root juncture, resulting in yield loss when that connection is severed during digging.

All aspects of peanut production are subject to different types of biotic or abiotic stresses and/or constraints. Biotic stresses vary depending upon the part of the plant affected and the growing region. Biotic agents that affect peanut production include insects, fungi, bacteria, virus, nematodes,

and weeds. A complete list of pathogens affecting peanut as well as the part of the plant and the most common growing region affected has been compiled (Kokalis-Burelle *et al.*, 1997) as well as suggestions on disease management (Melouk and Shokes, 1995). As with most crops, weed control is important during peanut cultivation (Buchanan *et al.*, 1982; Brecke, 1995). Weeds cause yield loss and difficulties during harvest. Most programs for weed control involve a combination of crop rotation and herbicide treatment. Herbicide rotation is also important to prevent the development of resistant weed species, a situation that is becoming increasingly problematic with *Amaranthus* spp. (common name Pigweed) in the United States.

All parts of the peanut plant can easily be utilized. The intact vines of the wild *Arachis* species can serve as excellent forage for horses and ruminant livestock. In fact, in some areas of the United States, peanut is becoming more popular as a forage crop. Florigraze is a perennial peanut cultivar (*Arachis glabrata* Benth.), which is primitive when compared to the common cultivated peanut and produces very few seed in comparison. Released by the University of Florida, Florigraze (Prine *et al.*, 1986) was grown on over 26 000 acres in Florida in 2005. Acreage planted in Florigraze is also on the rise in the coastal plains region of Georgia and across the US gulf coast as far west as Texas. The hay of Florigraze and Arbrook (a more recent University of Florida release; Prine *et al.*, 1990) contains 13–20% crude protein and has amino acid and mineral composition similar to that of alfalfa. The shells or pods of the peanut can also be used as livestock feed, burned as fuel, or made into particle board. Other nonfood products that can be made from peanuts include soaps, medicines, cosmetics, and lubricants.

Most of the peanuts grown in the United States are used for human consumption of the seed. The seed contains 25–32% protein and 42–52% oil and is a reasonably good source of dietary minerals. Peanuts have also been shown to be a good source of resveratrol, an antioxidant that has been linked to reducing heart disease and cancer (<http://www.peanut-institute.org/Plant.Compound.PR.html>). If cleared of aflatoxin contamination, raw peanuts have few antinutritional qualities, most of which

are associated with the testa or “skin” which is commonly removed during peanut processing. Peanut seeds can be used directly for food or can be crushed into oil and high-protein meal. Approximately two-thirds of the world’s peanut crop is crushed for oil (<http://www.lanra.uga.edu/peanut/knowledgebase>), which can be used for cooking, lighting, fuel, and as a food constituent. Recently, there has been a surge in interest in developing peanuts for the production of biofuel or biodiesel (<http://southwestfarmpress.com/news/110106-peanut-biodiesel>). Toward that end, research is underway to develop peanut cultivars that are higher in oil content than those currently available. In the United States, whole peanut seeds are chiefly roasted as shelled or in-shell seeds, used in candy, or made into peanut butter. The large nuts of the Virginia market type are generally sold roasted in the shell, where as the runner and Spanish market-type peanuts are used for candies, salted cocktail nuts, and peanut butter. Valencia peanuts are usually marketed as medium-sized in-shell nuts.

1.4 Traditional Breeding

Regardless of the crop, the goal of any plant-breeding program is to direct heredity in ways that improve the overall performance of the plant. In the past, this goal has proven difficult for peanut breeders due to the lack of genetic diversity among cultivated peanut lines. Simpson *et al.* (1975) listed the major obstacles to rapid progress in peanut breeding as being (1) lack of genetic and cytological information, (2) the complex nature of the species, (3) limited genetic variability, and (4) specific standards for commercial acceptance. Progress has been made since that time to lessen the burden of many of those obstacles, in part due to the increasing utility of molecular techniques with regard to peanut.

1.4.1 Breeding objectives

Objectives of peanut breeding programs have not changed drastically since they were outlined by Branch (1979) but have been re-examined by others (Knauff and Wynne, 1995). The goals of peanut breeders can be placed into

categories based on the intended beneficiary who may be a grower, a processor, or a consumer. Growers desire peanut cultivars with traits such as high yield, pest resistance, early maturity, and environmental stress tolerance. Processors want peanut cultivars with traits that streamline their practices. Such characteristics include uniform maturity, shelling, and blanching properties. Consumers and peanut product manufacturers prefer seed with improved nutritional qualities along with a pleasing color, shape, texture, and aroma. Recently, manufacturers have begun to demand that seed also have an oleic acid content of at least 70% (of the total oil), a quality that extends the shelf life of their products.

1.4.2 Yield, quality, and uniformity

Growers have historically yearned for peanut varieties with a high yield per land unit, but this need is becoming increasingly more urgent due to the rise in production costs (especially fuel) and the elimination of the price support system in the United States. Yields per acre in the United States have increased by 33% since 1995 (<http://www.usda.gov/nass>) but have remained steady worldwide in the last 3 years. Increasing yield in a peanut variety encompasses many different facets including developing disease resistance, shortening the required growing period, and increasing tolerance to environmental stresses such as drought. Three physiological processes have been identified as crucial to peanut yield (Duncan *et al.*, 1978): (1) partitioning of nutrients between vegetative and reproductive parts, (2) rate of fruit establishment, and (3) length of filling period. Much progress has been made toward increasing the percentage of assimilate directed toward reproductive plant parts. Percentage of fruit assimilate, which were at 41% for the first peanut cultivar, are now estimated at 98% for more recent releases (Norden *et al.*, 1982). In countries such as the United States, producers willingly participate in a “check-off” system where they place a percentage of their annual profits into programs coordinated by organizations such as the National Peanut Board (http://www.nationalpeanutboard.org/document_206.asp) to support further research on cultivar development and production improvement. Research aimed

toward the development of high-yielding cultivars is crucial to profitable peanut production as evidenced by the fact that peanut production in 2004 for the United States averaged 3540 kg ha⁻¹ compared to China's 2650 kg ha⁻¹ and India's 960 kg ha⁻¹ (Table 2).

1.4.3 Disease resistance

An important component of strengthening the production of any crop is the development of cultivars with disease resistance. Without the yield loss or input expenses due to disease and disease management, peanut production would be extremely profitable without a price support system in place. Peanut production can be inhibited by many pathogens such as fungi, virus, insects, bacteria, mycoplasma, and nematodes (Kokalis-Burelle *et al.*, 1997). The type of disease threat varies greatly upon the geographic location of the growing region. Still, only a few of the diseases caused by these pathogens are of worldwide importance and are the focus of peanut breeding programs.

Important fungal diseases infecting plant foliage include early leaf spot caused by *Cercospora arachidicola*, late leaf spot caused by *Cercosporidium personatum*, and rust caused by *Puccinia arachidis*. Soil-borne fungi of economic importance affecting peanut include *Sclerotinia minor* Jagger, which causes *Sclerotinia* blight, *Sclerotium rolfsii*, which causes southern stem rot, *Pythium myriotylum*, which causes *Pythium* pod rot, and *Cylindrocladium crotalariae*, which causes *Cylindrocladium* black rot (CBR). Greenhouse and laboratory tests for resistance to these fungal diseases have been developed and used to screen peanut germplasm prior to field testing and/or use in breeding programs (Melouk *et al.*, 1992; Shokes *et al.*, 1996; Chenault *et al.*, 2003). Through these research efforts, peanut varieties have been developed that demonstrate levels of resistance to fungal infection (Goldman *et al.*, 1995; Shew *et al.*, 1995). A molecular marker for *S. minor* resistance in runner, Spanish, and Valencia peanut market types has been developed which may be used for marker-assisted selection (MAS) to increase the efficiency of resistance breeding (Chenault and Maas, 2005). No cultivar has been developed with complete resistance and efforts continue toward

that end. Current management strategies for soil-borne and foliar fungal diseases include both cultivar selection and fungicide application.

Aflatoxin contamination of peanut caused by *Aspergillus flavus* infestation is a widespread serious problem in most producing countries where the crop is grown under rain-fed conditions. Aflatoxin contamination does not affect crop productivity but it makes the end product unfit for consumption as toxins are injurious to health. Aflatoxin contamination continues to be a severe pre and postharvest problem in the southeastern United States and in India, where it limits product export (<http://www.lanra.uga.edu/peanut/knowledgebase>). Screening for *A. flavus*-resistant germplasm has identified several sources of resistance (Mixon *et al.*, 1973; Mixon, 1979) among germplasm accessions. Efforts continue to mine the US national peanut germplasm collection (over 9000 accessions) for resistance to *A. flavus* (Holbrook *et al.*, 1997, 2000a, c; Xue *et al.*, 2004). Identification of *A. flavus*-resistant germplasm will always be a high priority of the peanut industry due to the fact that countries importing peanuts restrict the permissible limits of aflatoxin contamination of products.

Peanut is susceptible to a number of viruses, but tomato spotted wilt virus (TSWV) is the one with the largest economic impact. Prevalent on peanut in the southeastern United States, India, and other countries across the world, TSWV has a host range of over 400 plant species and is vectored by small insects called thrips (thysanoptera: thripidae). Losses incurred due to TSWV on peanut reached over \$40 million in Georgia alone in the late 1990s (<http://www.tomatospottedwiltinfo.org/peanut/management.htm>). Control of TSWV spread through insecticide application is not feasible and thus host plant resistance is the only viable disease management tool. Much success has been achieved in breeding peanut for TSWV resistance. The University of Georgia has developed a TSWV risk index to aid producers in that area in identifying and avoiding high-risk situations (Brown *et al.*, 2001). Although no available cultivar demonstrates complete resistance, moderate to high levels of field resistance to TSWV has now been incorporated in several peanut cultivars of different market types (Yancy, 2002). Several species of the root-knot nematode, *Meloidogyne*, inhibit peanut production in the United States and worldwide. The predominant

species affecting peanut is *Meloidogyne Arenaria*, which can cause yield losses exceeding 30% (Rodriguez-Kabana and King, 1985). Nematode attack damages the plant root system, which results in an overall decline of plant vigor and fruit production. Additionally, in this weakened state, the plant becomes more susceptible to other diseases such as soil-borne fungi. Host resistance to nematode infection was documented in wild *Arachis* species in the 1980s (Baltensperger *et al.*, 1986; Stalker and Moss, 1987; Nelson *et al.*, 1989) but was not documented in cultivated peanut until later (Holbrook and Noe, 1992; Holbrook *et al.*, 2000b). Since that time much progress has been made in breeding for resistance to *M. arenaria* including the development of molecular markers to identify resistant peanut germplasm (Burow *et al.*, 1996; Chu *et al.*, 2006) for use in breeding programs. This type of MAS has greatly enhanced the efficiency of breeding for nematode resistance. Peanut germplasm (Stalker *et al.*, 2002; Anderson *et al.*, 2006) and cultivars COAN and NemaTAM with nematode resistance (Simpson and Starr, 2001; Simpson *et al.*, 2003) have been recently developed.

1.4.4 Drought tolerance

In areas where natural rainfall does not meet the required amount needed for successful peanut production, fields must be irrigated to ensure crop survival. Energy costs for irrigation are a significant cost in peanut production, and availability of underground water is sometimes limited where peanut is cultivated. Thus, breeding for drought tolerance in peanut is a priority in regions where peanuts are not irrigated. Holbrook and Stalker have recently identified key areas of research regarding drought tolerance (Holbrook and Stalker, 2003). Germplasm with increased drought tolerance has been identified for use in breeding programs (Reddy *et al.*, 2001), and the expression of peanut genes in response to drought stress has been reported (Jain *et al.*, 2001).

1.4.5 Early maturity

In regions where growing seasons are shortened by environmental conditions such as freezing temperatures, and limited rainfall, peanut cultivars that mature early are a necessity. In general, most

Spanish cultivars mature at about 120–130 DAP, but few varieties of the other peanut market types mature before 140–150 DAP. The components of early maturity and their inheritance in cultivated peanut have been documented (Bailey and Bear, 1973; Upadhyaya and Nigam, 1994). Early maturing germplasm (Upadhyaya *et al.*, 2002a, b) and runner-type cultivars maturing at 125–130 DAP have been developed (Gorbet, 2003). A recent cultivar release “Wilson” by Mozingo *et al.* (2004), is described as a large-seeded Virginia-type peanut that matures at 135 DAP.

1.4.6 High oleic acid content

High oleic/low linoleic acid content in peanut seed has recently become a breeding priority worldwide. Lower linoleic acid concentrations benefit peanut processors by substantially increasing product shelf life and higher oleic acid concentrations benefit consumers by reducing blood low-density lipoprotein (LDL) cholesterol levels and increasing product flavor. The first cultivar producing a high oleic product was SunOleic 95R (Gorbet and Knaft, 1997) with seed that contained about 80% oleic acid compared to the 50% contained by most other peanut varieties at that time. A patent on the high oleic trait in peanut is held by the University of Florida (Knaft *et al.*, 2000), but for a licensing fee, the technology is available to all breeding programs. Much work has been done to reveal the genetics behind the high oleic acid trait. The two fatty acids are synthesized in the same biochemical pathway with oleic acid being the precursor to linoleic production. Moore and Knaft (1989) reported that two loci controlled the oleic/linoleic ratio in Virginia and runner-type peanuts. Lopez *et al.* (2001) determined that the inheritance of this trait is similarly controlled in Spanish market-type peanuts. Studies are underway to better understand the genetics behind the high oleic trait in peanut and to develop molecular markers linked to this trait for use in marker-assisted breeding.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

One limitation of traditional plant breeding is the lack of available genetic diversity due to the fact that only genes from within one species or

several closely related species or wild species can be utilized. As previously mentioned, cultivated peanut has very limited genetic diversity and thus an extremely limited gene pool. This narrow genetic base has created a peanut crop, which is vulnerable to damage by pests and environmental stress. Therefore, the most important tool available to traditional peanut breeders is that of hybridization. Norden (1973, 1980) noted that peanut breeders must rely on the use of introductions, hybridization, and pure line selection to produce new and improved cultivars with value-added traits.

Peanut breeders have at their disposal germplasm collections such as the US peanut collection which contains over 9000 plant accessions collected from around the world. Plant accessions in these collections include cultivated and exotic (wild) species. A plethora of genetic diversity exists among the wild *Arachis* species, the majority of which are diploid. Although hybridization between the cultivated subspecies is routinely performed, interspecific crosses in the genus *Arachis* are not easily achieved. Thus, the abundant genetic diversity available in wild species accessions is not easily moved into cultivated peanut. Singh *et al.* (1980) stated that the two major constraints to utilization of wild species are differences in ploidy level and incompatibility between some wild species and *A. hypogaea*. Successful crosses between cultivated and wild species have been reported since the 1940s but few useful cultivars have resulted from interspecific crosses (Simpson, 2001).

Another major limitation of traditional breeding is the time that is required to develop a cultivar. After the initial cross is performed, selections must be made over a period of at least six generations before replicated tests for agronomic performance can begin. Depending upon the amount of testing performed, traditional cultivar development can take from 10–20 years. Processes such as greenhouse or winter nursery use can accelerate the advancement of breeding lines, allowing for the growth of more than one generation per year. Recently, genetic linkage maps of cultivated and wild peanut species have been developed (Halward *et al.*, 1994; Burow *et al.*, 2001; Moretzshohn *et al.*, 2005) and the identification of molecular markers associated with phenotypic traits has begun (Burow *et al.*, 1996; Garcia *et al.*, 1996; Herselman *et al.*, 2004). Using current molecular techniques such as simple sequence repeat (SSR),

mirosatellite and single nucleotide polymorphism (SNP) analysis, polymorphism in cultivated peanut is being revealed (Ferguson *et al.*, 2004, 2005; Moretzshohn *et al.*, 2005). Thus, constraints to breeders due to the lack of genetic information available on peanut are quickly disappearing so that MAS breeding will become routine (Stalker and Mozingo, 2001). However, even with the use of MAS in breeding programs, traditional breeding is hindered by the number of years leading up to cultivar release.

Transgenic technology provides the means to partially overcome the limitations of traditional peanut breeding. Methods have already been developed to transform peanut via microprojectile bombardment or *Agrobacterium tumefaciens* methods (e.g., Ozias-Akins *et al.*, 1993; Schnall and Weissinger, 1993; Eapen and George, 1994; McKently *et al.*, 1995; Cheng *et al.*, 1996; Sharma and Anjaiah, 2000; Ozias-Akins and Gill, 2001). Via genetic engineering, useful genes from outside the *Arachis* genus can be inserted into and expressed in the *A. hypogaea* genome, thus overcoming the limitation of genetic diversity available using traditional methods. Many genes for disease resistance have already been transferred into cultivated peanut (Li *et al.*, 1997; Chenault *et al.*, 2002; Higgins *et al.*, 2004; Livingstone *et al.*, 2005). The second limitation of traditional breeding, time required for cultivar development, can be partially overcome using transgenic methods. Although peanut transformation has been achieved, the cultivated species is recalcitrant to the regeneration process. From beginning to end, a realistic time frame for generating a useful transgenic peanut cultivar ranges from 7 to 10 years. Although still lengthy, transgenic methods can reduce the time required for peanut cultivar development by 30–50%.

2. DEVELOPMENT OF TRANSGENIC PEANUT

2.1 Donor Genes

2.1.1 Expression of reporter and selectable marker genes

As in many of the early studies to develop efficient plant transformation systems, the first

transgenes introduced into peanut were reporter or selectable marker genes that conferred resistance to antibiotics or herbicides, and all of these genes were controlled by the constitutively expressed cauliflower mosaic virus (CaMV) 35S promoter along with the nopaline synthase (*nos*) terminator. For example, the bacterial genes coding for β -glucuronidase (GUS) (*uidA*) and neomycin phosphotransferase II (*nptII*) were stably incorporated into peanut callus (Lacorte *et al.*, 1991; Clemente *et al.*, 1992; Franklin *et al.*, 1993) (Table 3). Soon afterwards, Ozias-Akins *et al.* (1993) introduced the bacterial hygromycin resistance gene, *hph*, into peanut, this time obtaining stably transformed plants. The firefly luciferase (*luc*) reporter gene was used with peanut to monitor the transition from transient to stable expression in callus (Livingstone and Birch, 1995), as well as for aiding in selective subculturing of transgenic callus (Livingstone and Birch, 1999).

Brar *et al.* (1994) were the first to transform peanut with something other than the CaMV 35S promoter alone, introducing the *uidA* gene behind the CaMV 35S promoter followed by the alfalfa mosaic virus (AMV) 5' untranslated leader. The *bar* gene, conferring resistance to herbicides such as BASTA, was similarly constructed, but instead of the *nos* terminator, it contained the soybean rubisco small subunit terminator (Brar *et al.*, 1994). The mannopine synthase promoter was shown to be effective in driving GUS expression in leaflets from plants at the 5-leaf stage (McKently *et al.*, 1995), and Lacorte *et al.* (1997) evaluated GUS expression controlled by the 2S albumin promoter from Brazil nut and found it to be less effective than the CaMV 35S promoter. The effectiveness of monocot promoters, *Emu* and *actin 2*, also has been evaluated for their ability to express GUS transiently in peanut leaf tissue (Kim *et al.*, 1999). However, the most extensive evaluation of GUS expression driven by a heterologous promoter in peanut was performed by Wang *et al.* (1998) in which a soybean vegetative storage protein B gene (*vspB*) promoter was utilized. In this work, GUS was strongly expressed in peanut anthers, pod walls and stems, to a lesser extent in leaves, and only weakly in roots. Recently, transient expression of two fluorescent protein mutants, enhanced green fluorescent protein (EGFP) and enhanced yellow fluorescent protein (EYFP), driven by the CaMV 35S promoter have been reported, along

Table 3 Summary of peanut transformation studies in reverse chronological order

Cultivar/market type	DNA delivery method	Explant	Transgenes			References
			Reporter/marker gene	Gene of interest	Promoter	
Georgia Green, MARC-1/runner	Microprojectile bombardment	Somatic embryos	<i>Hph</i>	<i>EGFP, EYFP</i>	Double CaMV 35S with AMV leader	Joshi <i>et al.</i> , 2005
Wilson, Perry, NC-7/Virginia	Microprojectile bombardment	Somatic embryos	<i>Hph</i>	Barley oxalate oxidase	Double CaMV 35S with AMV leader	Livingstone <i>et al.</i> , 2005
TMV 2/Spanish	<i>A. tumefaciens</i> strain EHA105	Plumule of embryonic axes	<i>nptII</i>	Rinderpest virus hemagglutinin (H)	CaMV 35S	Khandelwal <i>et al.</i> , 2003
Okrun/runner	Microprojectile bombardment	Somatic embryos	<i>hph</i>	Tomato spotted wilt virus	CaMV 35S	Chenault and Payton, 2003
Okrun/runner	Microprojectile bombardment	Somatic embryos	<i>hph</i>	nucleocapsid	Dual-enhanced CaMV 35 S	Chenault <i>et al.</i> , 2002
TMV 2/Spanish	<i>A. tumefaciens</i> strain LBA4404	Embryo axis attached to one cotyledon	<i>nptII</i>	Rice chitinase; alfalfa glucanase	CaMV 35S	Rohini and Rao, 2001
VC-1/Virginia, AT120/runner	Microprojectile bombardment	Somatic embryos	<i>hph</i>	Class I tobacco chitinase	CaMV 35S	Magbanua <i>et al.</i> , 2000
TMV 2/Spanish	<i>A. tumefaciens</i> strain LBA4404	Embryo axis attached to one cotyledon	<i>nptII</i>	Tomato spotted wilt virus	CaMV 35S	Rohini and Rao, 2000
JL-24/Spanish	<i>A. tumefaciens</i> strain C58	Cotyledon	<i>nptII</i>	nucleocapsid <i>uidA</i>	CaMV 35S	Sharma and Anjiah, 2000
TMV 2/Spanish	<i>A. tumefaciens</i> strain LBA 4404	Cotyledon	<i>nptII</i>	<i>uidA</i> , Indian peanut clump virus coat protein	CaMV 35S	Venkatachalam <i>et al.</i> , 2000
VRI 2, TMV 7/Spanish	<i>A. tumefaciens</i> strain LBA 4404	Hypocotyls, cotyledons, cotyledonary nodes	<i>nptII</i>	<i>uidA</i>	CaMV 35S	Venkatachalam <i>et al.</i> , 1998

(continued)

Table 3 Summary of peanut transformation studies in reverse chronological order (*continued*)

Cultivar/market type	DNA delivery method	Explant	Transgenes			References
			Reporter/marker gene	Gene of interest	Promoter	
Florunner, Georgia Runner, MARC-1/runner	Microprojectile bombardment	Somatic embryos	<i>hph</i>	<i>uidA</i>	Soybean vegetative storage protein (<i>vsp B</i>), CaMV 35S	Wang <i>et al.</i> , 1998
Florunner, Georgia Runner, MARC-1/runner	Microprojectile bombardment	Somatic embryos	<i>hph</i>	Tomato spotted wilt virus	CaMV 35S	Yang <i>et al.</i> , 1998
MARC-1, Florunner/runner, Toalson/Spanish	Microprojectile bombardment	Somatic embryos	<i>hph</i>	nucleocapsid <i>cryIA(c)</i>	CaMV 35S	Singsit <i>et al.</i> , 1997
New Mexico Valencia A/Valencia	<i>A. tumefaciens</i> strain EHA105	Leaf sections	<i>nptII</i>	Tomato spotted wilt virus	Double CaMV 35S with AMV leader	Li <i>et al.</i> , 1997
Tatu/Valencia	Microprojectile bombardment	Cotyledon with or without embryos	—	nucleocapsid	CaMV 35S	Lacorte <i>et al.</i> , 1997
New Mexico Valencia A/Valencia	<i>A. tumefaciens</i> strain EHA105	Leaf sections	<i>nptII</i>	Brazil nut 2S albumin, <i>uidA</i>	CaMV 35S	Cheng <i>et al.</i> , 1996
Florigiant, NC7/Virginia, Florunner/runner	<i>A. tumefaciens</i> strain EHA101	Embryo axes	<i>nptII</i>	<i>uidA</i>	Mannopine synthase	McKenty <i>et al.</i> , 1995
F43SAT/Spanish	Microprojectile bombardment	Embryonic leaflets	—	<i>luc, uidA</i>	CaMV 35S	Livingstone and Birch, 1995
Robut/virginia, Gajah, McCubbin/Spanish, NC 7/Virginia	ACCELL particle bombardment	Shoot meristems of embryo axes	<i>uid A</i>	<i>bar</i> , tomato spotted wilt virus	CaMV 35S plus AMV leader	Brar <i>et al.</i> , 1994
Florunner/runner, Florigiant/Virginia	Microprojectile bombardment	Somatic embryos	—	nucleocapsid <i>hph, uid A</i>	CaMV 35S	Ozias-Akins <i>et al.</i> , 1993

with production of stably transformed plants containing EGFP controlled by a double CaMV 35S plus AMV enhanced promoter sequence (Joshi *et al.*, 2005). As of the writing of this chapter, a promoter from a peanut gene has not been used to control expression of a transgene.

2.1.2 Transgenes of potential agronomic and therapeutic value

Most peanut transformation experiments have focused on introducing transgenes for increased disease resistance, particularly virus and fungal resistance. TSWV is a major pathogen of peanut. Although TSWV field-tolerant cultivars are available, no cultivars are immune. Consequently, genetic engineering for TSWV-resistant peanut has been a major thrust of several programs. The approach used has been expression of either a sense or antisense TSWV nucleocapsid (N) gene. Both Brar *et al.* (1994) and Chenault and Payton (2003) introduced an N gene from a Hawaiian TSWV isolate controlled by the CaMV 35S promoter and Nos terminator into runner cultivars important to the southeast and southwestern United States, respectively. Li *et al.* (1997) also introduced the coding region of the N gene of the Hawaiian TSWV isolate, this time behind an enhanced double CaMV 35S promoter plus the AMV 5' untranslated leader sequence along with the tumor morphology large (tml) terminator, into the Valencia market type New Mexico Valencia A. An N coding region of a lettuce isolate of TSWV (TSWV-BL) was introduced into runner cultivars Florunner, Georgia Runner, and MARC-1 (Yang *et al.*, 1998). Magbanua *et al.* (2000) is the only study to use an N gene derived from a peanut TSWV isolate, and it was introduced in the antisense orientation behind a double CaMV 35S promoter into cultivars VC1 and AT120. Other reports of transgenic peanut generated for virus resistance include the introduction into Indian cultivars of a coat protein gene of the Indian peanut clump virus (IPCVcp) driven by the CaMV 35S promoter and Nos terminator to confer resistance to clump disease (Sharma and Anjaiah, 2000) and the coat protein gene of peanut stripe virus (Higgins *et al.*, 2004).

The fungus, *A. flavus*, is an important pathogen of peanut because it produces the carcinogenic

compound, aflatoxin. It had been shown that damage to peanut caused by the lesser cornstalk borer (LCB), increases aflatoxin contamination. Consequently, to reduce injury by the LCB, and therefore subsequent *A. flavus* invasion, a *Bacillus thuringiensis cryIA(c)* gene controlled by the CaMV 35S promoter was introduced into transgenic peanut plants (Singsit *et al.*, 1997). To confer resistance to the fungal pathogen *C. arachidicola*, the causal agent of peanut leaf spot, a class I tobacco chitinase gene driven by the CaMV 35S promoter along with the selectable marker gene, *nptII*, regulated by the Nos promoter were stably expressed in plants (Rohini and Rao, 2001). *Sclerotinia* blight of peanut, caused by the fungus *S. minor* Jagger, can result in severe yield losses up to 50%. To combat this fungus, transgenics containing either a rice chitinase gene, an alfalfa B-1,3 glucanase gene (Chenault *et al.*, 2002), or a barley oxalate oxidase coding region behind a double-enhanced CaMV 35S promoter (Livingstone *et al.*, 2005) have been generated.

In all of the above studies, the introduction of a transgene was performed with the goal of improving the peanut crop. There is one report of transgenic peanut being produced, not to enhance peanut, but to use it as a means to deliver an immunogenic protein for use in oral vaccination (Khandelwal *et al.*, 2003). Rinderpest or "cattle plague" is a ruminant disease caused by an enveloped virus called rinderpest virus that produces two surface glycoproteins, one of which is hemagglutinin H. The gene encoding hemagglutinin H was cloned under the control of the CaMV 35S promoter and used to transform the cultivar TMV-2 for eventual use as a fodder to immunize animals susceptible to rinderpest.

2.2 Transformation Strategies

2.2.1 *Agrobacterium*-mediated transformation

Early attempts at developing an *Agrobacterium*-mediated transformation protocol involved infecting peanut with wild type, virulent strains. Daimon *et al.* (1990) produced transgenic hairy roots of peanut following infection with wild-type strains of *Agrobacterium rhizogenes*. However, *A. rhizogenes*-mediated transformation of peanut has

only resulted in the formation of composite plants with transformed roots on nontransformed shoots (Akasaka *et al.*, 1998).

A. tumefaciens-mediated transformation of peanut has met with more success. Lacorte *et al.* (1991) showed that strain A281 was the most virulent of the four *A. tumefaciens* strains tested on peanut, capable of inducing tumors that demonstrated both GUS and *nptII* activity on various Brazilian cultivars. Shortly thereafter, Mansur *et al.* (1993) obtained transformed calli following co-cultivation of seedling hypocotyl explants. Leaf explants have been utilized in several studies to produce transgenic plants. When leaves from young seedlings were co-cultivated with *Agrobacterium*, primary transformants were produced, however these had limited fertility (Cheng *et al.*, 1994; Eapen and George, 1994). The first reproducible *Agrobacterium*-mediated transformation protocol resulting in stably transformed and fertile transgenic peanuts was achieved when leaf section explants of New Mexico Valencia A were incubated with a tobacco leaf extract-treated strain EHA105 containing the binary vector pBI121 (Cheng *et al.*, 1996, 1997). This same group also was able to successfully introduce a TSWV N gene using the same protocol modified to obtain transgenics more quickly by reducing the concentration of selection agent in the regeneration medium (Li *et al.*, 1997). Egnin *et al.* (1998) reported that the same cultivar, New Mexico Valencia A, was more efficiently transformed by *A. tumefaciens* than the runner market types examined. Therefore, it appears that *Agrobacterium*-transformation, particularly using leaf explants, is highly genotype dependent in peanut. This fact remains the major constraint regarding *Agrobacterium* transformation of peanut.

Transformation frequencies using wounded cotyledonary node, cotyledon or hypocotyl explants from 7-day-old seedlings were compared for Indian cultivars VRI-2 and TMV-7 following co-cultivation with LBA4404 (Venkatachalam *et al.*, 1998). Following shoot bud regeneration, cotyledonary nodes were reported to be superior to the other explants with a transformation frequency of 58% when selected on 75 mg l⁻¹ kanamycin compared to 45% for hypocotyls and 40% for cotyledons (Venkatachalam *et al.*, 1998). When cultivar TMV-2 was used with the same *Agrobacterium* strain, cotyledons were

shown to be efficiently transformed following co-cultivation and subsequent somatic embryogenesis (Venkatachalam *et al.*, 2000). Cotyledon explants were also successfully used by Sharma and Anjaiah (2000). However, their protocol called for the cotyledons of JL-24 to be longitudinally bisected prior to cultivation with strain C58, followed by the promotion of adventitious bud formation.

Zygotic embryo axes of mature seeds have been used by several groups to obtain transgenics via *Agrobacterium*-mediated transformation. The first study to use these explants was McKently *et al.* (1995). Although transformants were obtained, they were largely chimeric in the T₀ generation. Mature zygotic embryos with one of the cotyledons removed of cultivar TMV-2 were precultured for 2 days before being wounded and then co-cultivated with LBA4404 that had previously been treated with tobacco wounded leaf extract (Rohini and Rao, 2000, 2001). In these studies, transformants expressing GUS (Rohini and Rao, 2000) or tobacco chitinase (Rohini and Rao, 2001) were obtained in the primary and subsequent generations tested. Precultured shoot apices obtained from embryonic axes of mature seeds of TMV-2 were inoculated with strain EHA 105 and T₀ and T₁ plants were obtained that expressed the rinderpest virus hemagglutinin H protein (Khandelwal *et al.*, 2003).

2.2.2 Microprojectile bombardment

Without question, the most widely used and genotype-independent method of transforming peanut has been through direct DNA delivery using microprojectile bombardment followed by selection on hygromycin-containing medium (Figure 2). Initially, leaflets from mature zygotic embryos were used as target tissues and transformed callus was obtained (Clemente *et al.*, 1992). However, the most successful targets for direct DNA delivery have been embryogenic cultures. This system was first reported by Ozias-Akins *et al.* (1993), and the embryogenic tissue cultures were obtained from immature zygotic embryos of the cultivars Toalson (Spanish) and Florunner (runner) isolated from seeds harvested 3–4 weeks after their pegs had penetrated the soil. This work was the first to describe transgenic peanut plants that stably expressed a transgene. Using

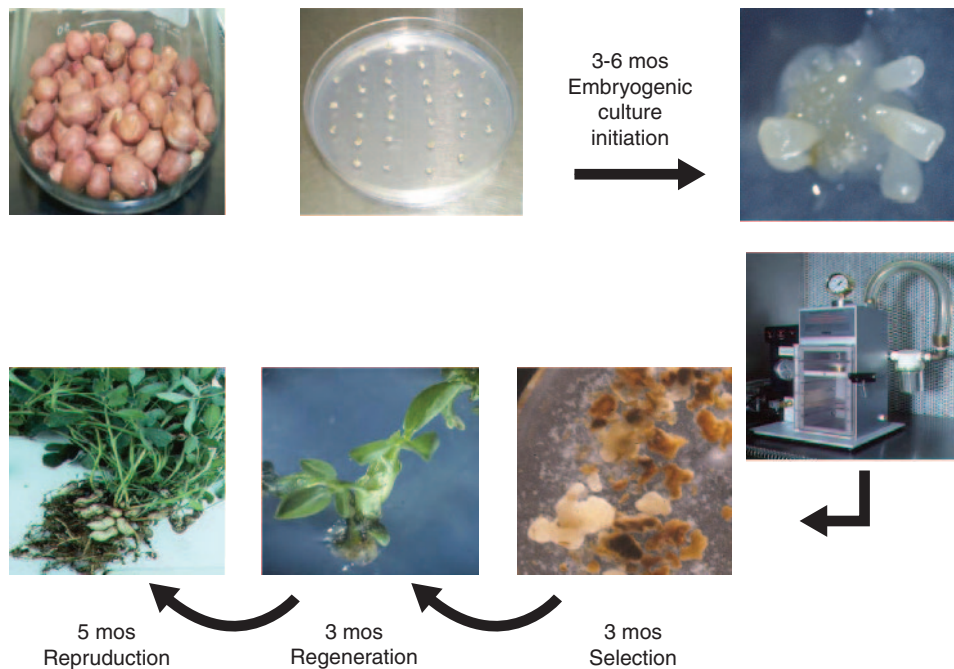


Figure 2 Steps to producing a transgenic peanut plant by the most common method, microprojectile bombardment

this system, the same group and others were able to effectively generate transgenic plants expressing *cryIA(c)* (Singsit *et al.*, 1997), GUS (Wang *et al.*, 1998), hydrolases (Chenault *et al.*, 2002), and TSWV N genes (Yang *et al.*, 1998; Chenault and Payton, 2003).

To overcome the issue of limited availability of immature seed, Livingstone and Birch (1999) produced embryogenic callus from the plumule portions of embryo axes excised from mature seeds and used those cultures as targets to introduce reporter genes into both Virginia (NC-7) and Spanish (Gajah) market types important to Australia and Indonesia, respectively. Using the same explant source, transgenic peanuts have been obtained expressing a variety of transgenes including a TSWV N gene (Magbanua *et al.*, 2000), an oxalate oxidase gene (Livingstone *et al.*, 2005), and several fluorescent reporter gene constructs (Joshi *et al.*, 2005), in a number of different market types.

The major constraint of producing transgenic peanuts via microprojectile bombardment is the amount of time involved from beginning to end. Assuming seed for embryo excision is readily

available, producing quality embryos at the correct developmental stage for bombardment requires 6–9 months. Selection in liquid and then on solid media takes another 3–4 months. Generation of shoot and root systems can take up to an additional 3–4 months. As is the case for any transgenic culture system, a major challenge can exist in maintaining sterile conditions for the period prior to soil containment. Once the T_0 plant is placed into soil and grown to maturity, another 3–4 months may be required. Conservatively, producing one generation of transgenic peanut via this method involves a minimum time period of 15–19 months.

2.3 Selection of Transformed Tissue

For *Agrobacterium*-mediated transformation of peanut, the selectable marker gene, *nptII*, has been exclusively used. Selection has been on kanamycin in the range of 50–300 mg l⁻¹. Kanamycin has been successful in these *Agrobacterium*-mediated transformation protocols because the explants used undergo shoot regeneration where kanamycin

resistance is quite effective at eliminating escapes. The only selectable marker gene used for microprojectile bombardment transformation of peanut has been *hph*, which affords recipients hygromycin resistance. Efficient selection of transformed embryogenic cultures has been with hygromycin B at concentrations of 10, 20, or 40 mg l⁻¹. While most reports have used a stepwise selection scheme (from 10 to 20 mg l⁻¹) with initial selection on liquid medium based on the protocol described by Ozias-Akins *et al.* (1993), others have used one higher concentration of hygromycin (40 mg l⁻¹) throughout the selection process until transfer to antibiotic-free medium for regeneration (Livingstone *et al.*, 2005).

2.4 Regeneration of Plants

Using *Agrobacterium*-mediated transformation methods and kanamycin selection, reported transformation rates have varied widely from 0.2% to 55%. Cheng *et al.* (1996) were able to obtain 52 transgenic plants from five independent transformation events with a transformation rate of 0.2–0.3%. Venkatachalam *et al.* (1998) reported that a 3-day preculture of explants on regeneration medium prior to *Agrobacterium* co-cultivation increased regeneration of transgenic plants. After evaluation of plants using GUS histochemical analysis and PCR, Rohini and Rao (2000) obtained a 3.3% transformation rate following an *Agrobacterium*-mediated nontissue culture protocol which called for 16 h of co-cultivation, 18 h of exposure to cefotaxime and then germination and growth for 16 days prior to transfer to the greenhouse. A transformation rate as high as 55% was reported for cotyledon explants following *Agrobacterium* inoculation with transgenic plants being rooted in approximately 120–150 days after the start of an experiment (Sharma and Anjaiah, 2000). The highest transformation rate reported thus far has been 67% with a survival rate of 90–95%. These results were obtained by Khandelwal *et al.* (2003) after preculturing shoot apices for 2 days prior to co-cultivation and selection on 100 mg l⁻¹ kanamycin.

As mentioned earlier, the first report on the regeneration of transgenic peanut plants was by Ozias-Akins *et al.* (1993). In that study, approximately 1% of the embryogenic calluses

bombarded and selected for hygromycin resistance yielded a stably transformed cell line, and 100 primary transformants were obtained in total. Brar *et al.* (1994) were able to recover only 11 primary transgenics following bombardment of 800 shoot meristems of embryonic axes following screening for GUS activity. In the method described by Livingstone and Birch (1999), three to six independent transformants per bombardment of embryogenic callus could be obtained, and this level of recovery appears to now be standard when using hygromycin as the selection agent. Recently, Livingstone *et al.* (2005) obtained 200 primary transgenic plants from three cultivars following this transformation and selection scheme, while another report on the use of fluorescent reporter genes for visual selection of transformed peanut plants resulted in a low level of transformation, with less than one line obtained per bombardment (Joshi *et al.*, 2005).

2.5 Testing of Transgenic Peanuts

As is the case with other plants, peanut transgenics obtained via *Agrobacterium*-mediated transformation tend to have single or low copy number insertions with progeny segregating in a 3:1 ratio (Cheng *et al.*, 1997; Sharma and Anjaiah, 2000; Rohini and Rao, 2001), whereas those obtained from microprojectile bombardment tend to have variable copy number and can exhibit a variety of rearrangements as evidenced by Southern blot analysis (Ozias-Akins *et al.*, 1993; Yang *et al.*, 1998). Additionally, primary peanut transformants can have reduced fertility and lower yields than their nontransformed controls. Rohini and Rao (2001) reported that T₀ plants expressing tobacco chitinase grew more slowly when compared to nontransformed controls. Also, T₀ plants expressing oxalate oxidase did not have vigorous growth and their fertility was variable, but these results were not correlated with expression of the transgene. Normal growth was restored in the T₁ generation (Livingstone *et al.*, 2005). Particularly when embryogenic cultures are used for transformation, the culture period prior to bombardment should be short to increase the chances of obtaining fertile plants. Livingstone and Birch (1999) recommend using callus cultures that are less than 9 months old to increase fertility.

Unfortunately, the minimum time required to generate embryos useful for bombardment is 6–9 months.

Molecular characterization of T_1 lines has shown stable integration and high levels of gene expression for a number of transgenes. These transgenes have shown efficacy against target insects like the LCB (Singsit *et al.*, 1997), viruses such as TSWV (Magbanua *et al.*, 2000), and fungi, particularly *S. minor* (Chenault *et al.*, 2003; Livingstone *et al.*, 2005). Peanut transformation has developed into an effective strategy to improve the crop.

2.6 Regulatory Measures

To date, no transgenic peanut cultivar has been released for commercial production. Genetic modification of organisms is feared by the general public, especially when the end product is one of food for human consumption, resulting in the development of a network of regulatory systems around the world. The cost of meeting such regulatory requirements for globally traded crops is estimated at \$20–\$30 million per product (McElroy, 2003) which has limited the release and production of transgenic crops to large multinational corporations. Most regulatory agencies focus on the specific DNA construct involved in the transformation rather than the beneficial trait itself, a fact widely debated by those in the scientific community (Bradford *et al.*, 2005; Parrott, 2005; Ponti, 2005).

A risk-based strategy has been proposed for regulating transgenic crops, breaking them down into classes based upon true risks associated with gene function and based on scientific criteria (Barton *et al.*, 1997; Strauss, 2003). Following this strategy, *low risk* crops include those where the transgene involved imparts a trait which is functionally equivalent to that manipulated by traditional breeding and no novel biochemical functions are manipulated (i.e., changes in homologous gene expression achieve a commercially useful trait). The *moderate risk* category involves crops producing plant-made pharmaceutical proteins, that have very low human and environmental toxicity or nonfood crops that have low nontarget ecological impact. Those transgenic crops at *high risk* include

those producing transgene products that have a documented likelihood to cause significant harm to humans, herbivores, or the environment.

In the United States, laws currently used to regulate transgenic products are the Plant Protection Act (PPA), the Federal Food, Drug, and Cosmetic Act (FFDCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and the Toxic Substances Control Act (TSCA). The current system in place in the United States involves three government agencies (<http://www.usbiotechreg.nbii.gov>), each with a specific role in regulation. The USDA-ARS Animal and Plant Health Inspection Service (USDA-ARS APHIS) controls the entire development of the transgenic crop until nonregulated status is granted because nonpest status has been shown. USDA-APHIS also regulates the transportation, importation, field testing, and disposal of all transgenic plants. The Environmental Protection Agency (EPA) regulated the distribution and sale, use, and testing of any pesticidal substance, including transgenic crops. The Federal Drug Agency (FDA) serves to ensure that plant-derived foods or drugs are properly labeled for consumer use.

Although adoption of transgenic crops has rapidly progressed in the United States, Argentina, and Canada (Ponti, 2005), the general public in Europe still resists their importation and use. This adversity is believed to be based on consumer concerns regarding the safety of transgenic crops with regards to long term effects on the human population and the environment. Much debate continues on the utility of transgenic crops considering the quasi moratorium placed on transgenic products in the European Union (EU).

3. FUTURE ROADMAP

3.1 Expected Products

3.1.1 Disease resistance

3.1.1.1 Fungal resistance

Much of the crop improvement using transgenes in peanut has been directed toward disease resistance. This is the typical trend for first-generation transgenic crops. Clearly significant

economic benefit can be gained by the growers when host resistance to pests and diseases is available. When host resistance reduces pesticide input, a significant benefit to the environment also would be anticipated as a consequence. Chemical input to control leaf spot disease is costly. In Georgia alone, the cost of control for 2005 was estimated to be approximately \$36 million, which involved primarily the cost of fungicide application (Kemerait, 2006). Host plant resistance would reduce both costs of production and chemical release into the environment. The consumer does not typically embrace a transgenic crop when its primary benefit is to the economic well being of the grower. The simultaneous benefits of reduced chemical use to the environment and consumer exposure often are overlooked but should be an important consideration for deregulation of transgenic crops.

Fungal resistance is a phenotype category for which there are no crops yet deregulated by USDA-APHIS in the United States, or corresponding regulatory agencies in other countries (database of genetically modified organisms (GMOs) deregulated in the United States at <http://www.isb.vt.edu> and biotech crop database at <http://www.agbios.com/main.php>), in spite of the fact that more than 700 field trials have been conducted for putative fungal resistance in the United States alone (database of US field tests of GMOs at <http://www.isb.vt.edu>). The slow release of fungal-resistant transgenic cultivars may be due to a number of factors but probably is largely associated with the economics of targeting only one or a few pathogens per transgene combined with incomplete efficacy. Deregulation has become a costly endeavor and can be justified

at this time only for major effect genes. As previously discussed, multiple federal agencies typically are involved in the process of deregulation through a coordinated framework (<http://www.usbiotechreg.nbii.gov/index.asp>), which extends the time and costs associated with product approval. Nevertheless, given the intensive use of fungicides in peanut, the greatest impact on peanut production and a safer environment could be achieved with fungal-resistant cultivars.

Genes that currently are being explored to achieve fungal resistance potentially can target one or more of the following pathogens including, *S. minor*, white mold (*S. rolfsii*), early and late leaf spot (*C. arachidicola* and *C. personatum*, respectively), and *Aspergillus flavus/parasiticus* (Table 4). None of these are classical R genes that would act in a gene-for-gene resistance scenario (Dangl and McDowell, 2006), but rather are acting downstream in some part of the inducible or constitutive host resistance pathway. There are many such genes that could act to inhibit fungal growth or invasion or to activate further plant defense responses (Punja, 2001), but the resistance achieved typically is incomplete. A broad-spectrum antimicrobial gene potentially would have the greatest economic impact by conferring disease resistance to multiple pathogens; however, it might also have the greatest potential to disturb the beneficial symbiotic relationship between peanut and *Rhizobium*, a bacterium. The *Rhizobium*-legume symbiosis benefits the plant by providing a source of fixed nitrogen and reduces the cost and pollution potential of inorganic nitrogen application. Genes that are in various phases of research and development are listed in Table 4.

Table 4 Putative antifungal genes engineered into peanut

Genes of interest	Gene sources	Pathogens target	References
Chitinase	Tobacco	<i>Cercospora arachidicola</i>	Rohini and Rao, 2001
Chitinase	Rice	<i>Sclerotinia minor</i>	Chenault <i>et al.</i> , 2005
		<i>Aspergillus flavus</i>	Chenault <i>et al.</i> , 2004
β -glucuronidase	Alfalfa	<i>Sclerotinia minor</i>	Chenault <i>et al.</i> , 2005
		<i>Aspergillus flavus</i>	Chenault <i>et al.</i> , 2004
Oxalate oxidase	Barley	<i>Sclerotinia minor</i>	Livingstone <i>et al.</i> , 2005
Chloroperoxidase	<i>Pseudomonas pyrocinia</i>	<i>Aspergillus flavus</i>	Niu <i>et al.</i> , 2004
Lytic peptides	D4E1	<i>Aspergillus flavus</i>	Weissinger <i>et al.</i> , 2001
Ribosome inactivating protein	<i>Zea mays</i>	<i>Aspergillus flavus</i>	Weissinger <i>et al.</i> , 2004
Bcl-xl	Human	<i>Aspergillus flavus</i>	Chu <i>et al.</i> , 2005

3.1.1.2 Virus resistance

Virus diseases also cause significant losses to the peanut industry. In the United States, the viral disease of most concern is tomato spotted wilt, a tospovirus disease that is vectored by thrips. Crop losses due to viral infection approach the value of fungal disease control (Kemerait, 2006). There is no chemical control for TSWV although there are management strategies, the most important being host plant resistance (<http://www.sacs.cpes.peachnet.edu/spotwilt>). Host plant resistance has been enhanced with pathogen-derived resistance where a gene from the virus, when expressed in the host plant, can confer resistance to the virus from which the gene originated and sometimes related viruses (Beachy, 1997). This approach has been explored in peanut where the N protein gene from TSWV, in sense or antisense orientation, was constitutively expressed in the plant (Li *et al.*, 1997; Yang *et al.*, 1998; Magbanua *et al.*, 2000), and one line expressing protein was shown to have elevated levels of resistance over multiple years of field testing (Yang *et al.*, 2004). The costs of deregulation and freedom to operate have been prohibitive for further development of this transgenic line as a commercial cultivar. Future approaches in peanut could exploit the RNA silencing/RNA interference (RNAi) pathway in plants whereby any viral gene could be targeted for degradation by introducing an inverted repeat of part the gene into the host genome (Helliwell and Waterhouse, 2003; Dunoyer and Voinnet, 2005). Homology dependent gene silencing should operate in most transgenic lines unless the virus has a suppressor of silencing that functions within the same pathway (Goldbach *et al.*, 2003; Moissiard and Voinnet, 2004). Viruses other than TSWV are of minor concern in the United States. However, in Africa, India, or Southeast Asia, groundnut rosette, peanut clump, and peanut stripe can be serious diseases in addition to tospovirus diseases. Transgenic lines expressing a peanut clump virus coat protein gene (Sharma and Anjaiah, 2000) or a peanut stripe coat protein gene sequence (Higgins *et al.*, 2004) have been produced and hold promise for virus resistance in peanut, particularly in developing countries where a more favorable environment for release of GMOs may exist.

3.1.2 Pest resistance

3.1.2.1 Insect resistance

Insect pests are sporadic problems on peanut and most can be effectively managed with curative rather than preventative sprays (Funderburk and Brandenburg, 1995). Many of these insect pests are foliage feeders. One notable exception is the LCB, typically a pod feeder. LCB can scarify or penetrate pods when feeding and thereby increase the vulnerability of peanut to invasion by *A. flavus/parasiticus*. Both of these fungi produce the carcinogenic mycotoxin, aflatoxin, which is a frequent contaminant of peanuts, corn, cottonseed, and some tree nuts grown under drought stress. Toxin levels are strictly controlled in the United States but not in developing countries where acute aflatoxicosis in humans and animals has been recently observed. Aflatoxin levels in seeds of peanut have been consistently correlated with insect-damaged pods (Lynch and Wilson, 1991). Insect resistance genes in peanut germplasm have not been extensively surveyed although some have been identified (Stalker and Simpson, 1995). One future strategy might be to introduce resistance genes from outside of the genus. Insect resistance in transgenic plants has been achieved primarily through the introduction of insecticidal crystalline protein genes from the soil bacterium, *Bacillus thuringiensis* (*Bt*) (Christou *et al.*, 2006). *Bt* toxins are considered to be highly selective for the target pest species with no direct detrimental effect on nontarget beneficial insects, although there can be an expected indirect effect due to prey availability or quality (Romeis *et al.*, 2006). The *Bt* toxin, *cryIA(c)*, was shown to effectively reduce feeding of LCB on foliage and pods of peanut when the protein was constitutively expressed in the plant (Singsit *et al.*, 1997; Ozias-Akins *et al.*, 2002). In spite of *Bt* toxin efficacy, freedom to operate with *Bt* genes was in litigation at the time the initial *Bt*-peanut research was conducted and resolution was not encouraging for peanut. In addition, the widespread adoption of *Bt* cotton across the peanut belt precluded the release of *Bt* peanut for resistance management reasons. The concern is that continuous exposure of an insect pest to a single *Bt* toxin will select for toxin-resistant insect populations (Tabashnik,

1994). One resistance management strategy is the planting of nontransgenic refugia next to fields of transgenic crops (Gould, 1998). A second strategy is to introduce multiple *Bt* toxin genes, some of which have different modes of action. A “stacked” resistance with more than one *Bt* gene is considered a better approach to reduce the probability that insects will become resistant to the plant-expressed protein. Monsanto has followed this approach in their development of YieldGard[®] Plus corn where genes for two different *Bt* toxins (Cry3Bb1 and Cry1Ab) were combined as plant-incorporated protectants to confer resistance to European corn borer and corn rootworms (http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_006430-006484.htm). Although other insect resistance genes have been identified, such as cowpea trypsin inhibitor (Hilder *et al.*, 1987), none are as effective or selective as *Bt* toxin genes (Christou *et al.*, 2006). However, the combination of cowpea trypsin inhibitor and *Bt* toxin genes has shown additive effects in some species and antagonism in others (Oppert, 1999). The combination of *Bt* and a native resistance gene in soybean is more effective than either alone (Walker *et al.*, 2004) and the same is true in potato (Westedt *et al.*, 1998).

3.1.2.2 Nematode resistance

The main nematode pest in peanut is the root-knot nematode, *M. arenaria*. As discussed earlier, a high level of resistance has been found in several of the wild diploid relatives of peanut (Holbrook and Noe, 1990) and that resistance has been introgressed into cultivated peanut through interspecific hybrids (Simpson and Starr, 2001; Simpson *et al.*, 2003). The released cultivars were developed for the southwestern United States where TSWV is not a problem on peanut; therefore, further cultivar development to combine nematode with TSWV resistance in genotypes adapted to the southeastern United States is in progress. Cultivar development for the nematode resistance trait is being facilitated by MAS (Chu *et al.*, 2006). Given that most nematode resistance (R) genes cloned to date are in the NBS-LRR class of R genes, it is possible that a resistance gene analog (Bertioli *et al.*, 2003; Yuksel *et al.*, 2005) for nematode resistance could be cloned

from *Arachis* spp. and engineered into cultivated genotypes. The choice to pursue this genetically modified (GM) alternative to traditional breeding would depend on the economics of the situation. A particularly intriguing mechanism of resistance to root-knot nematode is the use of the RNA silencing mechanism in plants to knockdown a signaling pathway that is required for the nematode to communicate giant cell formation by the plant (Huang *et al.*, 2006). Stacked resistance where the pest would encounter mechanistically different defense strategies is always preferable when it can be achieved because it will reduce the likelihood for the pest to evolve resistance.

3.1.3 Abiotic stress tolerance

3.1.3.1 Drought tolerance

The most significant abiotic stress for peanut is drought, not only because of the direct effect on plant productivity, but also because of the indirect effect on aflatoxin production. A plant under stress apparently stimulates infecting *Aspergillus* fungi to synthesize aflatoxin. Irrigation is an effective management tool for aflatoxin reduction; however, it is not always feasible. Drought-tolerant peanut genotypes have been shown to be less susceptible to aflatoxin production than drought-sensitive genotypes (Holbrook *et al.*, 2000a). The genetic component for drought tolerance is complex and transgenic technologies might provide an alternative route for enhancing drought tolerance. Abiotic stresses commonly result in osmotic and oxidative stress and strategies to mitigate the associated cellular damage can lead to stress tolerance (Wang *et al.*, 2003). The most effective approaches up to now involve the manipulation of signaling pathways or the up-regulation of structural protectants such as osmolytes or antioxidants that can confer some degree of tolerance. One component of drought stress signaling is phospholipase D (PLD), an enzyme that hydrolyzes phospholipids to produce phosphatidic acid. In peanut, PLD is more highly expressed in response to drought in drought-susceptible cultivars (Guo *et al.*, 2006). Its expression may contribute to phospholipid membrane breakdown; therefore, its suppression could promote drought tolerance.

3.1.3.2 Herbicide tolerance

Since chemical stress is a component of abiotic stress, herbicide tolerance falls in this category. Herbicide tolerance is the most popular genetically engineered trait in deregulated crops (James, 2005). Roundup Ready (glyphosate tolerant) cotton and soybean have been developed and are widely grown in the United States. Since peanut typically is grown in rotation with cotton, it would not be ecologically sound to release glyphosate-tolerant peanut. Weeds resistant to this herbicide (e.g., pigweed) already can be a serious problem for cotton cultivation. There are numerous weed pests in peanut, and preplant and pre or postemergence chemical control is an important component of weed management (Brecke, 1995). A broad spectrum of herbicides is registered for use on peanut. Herbicide resistance genes other than EPSPS (for glyphosate resistance) have been identified (Duke, 2005) and could be engineered into peanut, provided that the chemical company with ownership of the herbicide would consider registration for use on peanut to be an economically feasible endeavor. These costs would be in addition to the costs of deregulating the transgenic crop.

3.1.4 Product quality

3.1.4.1 Oil composition

Peanut is classified as an oilseed crop because the seeds are rich in oil (~50%). In the United States, however, a premium price is paid for confectionary quality peanuts and the price for oilstock peanuts is relatively low. Oil composition of the peanut seed affects end quality for both oil and confectionary items. A high ratio of oleic to linoleic acid (high-OL) is desirable for prolonged shelf life and reduced rancidity of oils (<http://www.foodproductdesign.com/archive/2000/0500pp.html>). Since a ratio of 40:1 oleic to linoleic acid has been achieved in spontaneous mutants of the δ -12-oleoyl desaturase genes in peanut (Moore, 1999; Knauff *et al.*, 2000), it is less likely that a transgenic approach would be used for the purpose of high-OL in peanut even though the relevant desaturase genes have been characterized (Jung *et al.*, 2000).

As is the case for most other oilseed crops, peanut could be engineered to produce specialty or industrial oils (Dyer and Mullen, 2005; Murphy, 2006; Scarth and Tang, 2006) if the industry were committed to segregating the crop and preserving identity to the end use. The economy of specialty oil production in peanut versus other commodities (soybean, cottonseed, rapeseed/canola, sunflower, palm, corn) also would have to be taken into account. The potential of designer oil crops has not been realized in part due to the complexity of fatty acid biosynthesis, cellular compartmentalization of novel fatty acids, and insufficient yields for economic viability (Murphy, 2006).

The ω -3 fatty acids reduce risk of coronary heart disease and fatty fish serve as a major source of this nutrient. Land-based plant sources are being proposed although currently the main ω -3 fatty acid in plants, α -linolenic acid (18:3), is undesirable in oils because of its susceptibility to oxidation. Most oilseeds are being developed to produce more stable oils that contain high levels of monounsaturated fats such as oleic acid (18:1), which also have health benefits. The most effective forms of dietary ω -3 fatty acids are fish-derived eicosapentaenoic and docosahexaenoic acids, and the α -linolenic to eicosapentaenoic intermediate stearidonic acid (18:4) has been proposed for increasing the bioconversion efficiency of plant-derived ω -3 fatty acids (Ursin, 2003). Further proof of concept for manipulation of ω -3 fatty acid content in foods would be more efficiently done in species that are easier to transform than peanut (Zhang *et al.*, 2005), and the most feasible strategy subsequently could be adopted for peanut.

Peanut oil was the original oil proposed for the manufacture of biodiesel by Rudolf Diesel (<http://www.cyberlipid.org/glycer/biodiesel.htm>). The current cost of peanut production using management practices that will yield a large and high-quality crop for human consumption likely would be prohibitive for biodiesel purposes. In Georgia, the cost of peanut oil exceeds that of corn, soybean, or rapeseed (<http://www.agecon.uga.edu/~caed/biodieslrpt.pdf>). However, peanut cultivars that have reasonable yields with minimal input probably could be developed with a combination of traditional breeding and GM technologies. These cultivars would need to incorporate drought tolerance for dryland production and multiple resistances for host plant

rather than chemical control of diseases and pests. Additionally, the oil content of peanut is known to vary among genotypes (Isleib *et al.*, 2004) and the trait for high oil production could be manipulated either through traditional breeding and selection or using transgenic technologies (Dyer and McMullen, 2005).

3.1.4.2 Proteins

Peanut fruit, although botanically a legume, not a nut, contains proteins that cause allergic reactions in humans of the same order of magnitude as several tree nuts (Sicherer *et al.*, 1998). The majority of the allergic reactions to peanut can be attributed to three seed storage proteins, Ara h 1 (vicillin), Ara h 2 (conglutin), and Ara h 3 (glycinin). Ara h 1 also is known as conarachin and Ara h 3 as arachin. There has been considerable debate regarding the value of searching for genotypes naturally devoid of one or more of these allergens or of suppressing the levels of one or more allergens through gene silencing (Ozias-Akins *et al.*, 2006). Since all three allergens are seed storage proteins, they have a function in the support of seed germination and seedling development. To what extent the absence of one or more of these three proteins would affect seed viability or peanut flavor is not yet known. There is precedent, however, for eliminating seed storage proteins in other crops through the discovery of natural variation (Burow *et al.*, 1993; Joseph *et al.*, 2006), or gene silencing (Herman, 2005). Since peanut is a relatively recent polyploid, and has undergone an evolutionary bottleneck (Kochert *et al.*, 1996), the probability to discover natural allergen-free variants within the cultivated species is low. Screening for induced mutations is one non-GM strategy (Ozias-Akins *et al.*, 2006). The GM strategy, allergen gene silencing using RNAi, might eventually gain consumer acceptance if the silencing were shown to be stable, but in the interim silencing through RNAi is a valuable means to test the effect of altering protein composition of the seed on seed viability. Seeds with altered protein content also could be tested for allergen sensitization and reaction in animal models such as mouse and pig (Li *et al.*, 2000; Helm *et al.*, 2002). The allergen, Ara h 2, also has been shown to

possess trypsin inhibitor activity (Maleki *et al.*, 2003) and trypsin inhibitors are reported to have insecticidal or antifungal activities (Gatehouse and Gatehouse, 1998; Chen *et al.*, 1999). A possible negative consequence of reducing such an allergen could be increased susceptibility to pests or pathogens. Conversely, a consequence of engineering enhanced pest or pathogen resistance could be increased allergenicity. This consequence should be considered on a case-by-case basis depending on the protein gene that would be introduced.

Legume proteins are deficient in methionine, hence vegetarian diets include a balance of cereals with legumes to obtain essential amino acids. Peanut seeds consist of approximately 25% protein, the majority of which is arachin and conarachin, or 11S and 7S globulins, respectively. A methionine-rich protein has been identified in peanut (Mazhar and Basha, 2004), and it is conceivable that its content could be increased through genetic engineering. Other means for improving protein quality include transformation with a synthetic peptide gene that encodes incorporation of essential amino acids (Jaynes and Derrick, 1998; Beauregard and Hefford, 2006). Scenarios for enhancing the sulfur-containing amino acids in soybean have been proposed and would be applicable to peanut (Krishnan, 2005). Protein quality goals may differ for edible peanuts versus oilcake protein destined for animal feed.

3.1.4.3 Vitamins and other bioactive compounds

Peanut seeds are naturally high in vitamin E and folate (McKevith, 2005), and their consumption by peanut-tolerant individuals is recommended as part of a folate-rich diet during pregnancy (<http://www.marchofdimes.com/professionals/14332.1819.asp>). Folate is known to reduce the chance of neural tube defects, one consequence of which is spina bifida. Metabolic engineering for increased folate has not yet been attempted in peanut and so far has been able to achieve only modest increases in other plants (Rebeille *et al.*, 2006). Folate metabolism in plants is complex but now well characterized with different parts of the biosynthetic pathway being housed in different subcellular compartments (chloroplasts,

mitochondria, cytosol) (Basset *et al.*, 2005). There is considerable flux in folate levels during growth and development due to its importance to multiple biochemical pathways and more than one rate-limiting step in its biosynthesis. Although it may be premature to attempt the enhancement of folates in peanut, the natural ability to produce significant levels of folate in the peanut seed may predispose this crop to successful engineering of even higher folate levels.

Vitamin E is a collective term for several tocopherol and tocotrienol compounds and is an essential component of mammalian diets. The highest vitamin E activity can be found in α -tocopherol, and vitamin E is partitioned into the oil fraction that is extracted from oilseeds. Vegetable oils are an important source of vitamin E in the human diet. Vitamin E biosynthesis has been genetically well characterized in the model dicot, *Arabidopsis thaliana*, and facilitated by earlier genetic and biochemical analyses in the cyanobacterium, *Synechocystis* (DellaPenna and Last, 2006). Engineering of the oilseed, soybean, to increase vitamin E activity fivefold, but not the total tocopherol content, was accomplished by the combined overexpression of two enzymes in the biosynthetic pathway. These were VTE3 which is unique to the γ - and α -tocopherol branch of the pathway and VTE4 which catalyzes the last step in converting γ - to α -tocopherol or δ - to β -tocopherol (Van Eenennaam *et al.*, 2003; Sattler *et al.*, 2004). Peanut seeds already have a considerably higher α -tocopherol content than soybean, but perhaps a similar strategy could enhance the vitamin E activity of peanut.

Polyphenols include secondary metabolites such as some phytoalexins and flavonoids among other substances. Their roles in plants and human health are multifunctional (Parr and Bolwell, 2000). Engineering their composition could fortify functional foods, but since they also play a role in plant defense, the opportunity exists to benefit the plant as well (Treutter, 2006). Some specific goals for peanut might be to alter the quantity or distribution of resveratrol or insecticidal flavonoids. Resveratrol is a stilbene phytoalexin that also is prevalent in grapes and may be responsible for one of the health benefits of red wine (Cordova *et al.*, 2005). Resveratrol was implicated in protecting peanut seeds from *Aspergillus* growth (Wotton and

Strange, 1987), and was negatively correlated with disease incidence in transgenic tobacco expressing a heterologous stilbene synthase gene (Hain *et al.*, 1993). A secondary product that reduces aflatoxin formation by *Aspergillus* is gallic acid, a component of hydrolysable tannins in seed coats of certain walnut cultivars (Mahoney and Molyneux, 2004). Insecticidal effects have been attributed to the flavonoids quercetin and rutin that are present in the foliage of a wild peanut species but not cultivated peanut (Mallikarjuna *et al.*, 2004). The potential for modifying phytoalexins and flavonoids for dual benefit is broad but complex.

3.1.4.4 Therapeutics

Peanut could serve as a vehicle for the manufacture of protein therapeutics just as many other crop commodities (Vitale and Pedrazzini, 2005). The primary issue for crop choice for plant-made pharmaceuticals is the chance for mixture with product intended for human consumption (Horn *et al.*, 2004; Ma *et al.*, 2005). Maize has been promoted for plant-made pharmaceuticals although the growth of transgenic therapeutic-producing genotypes in high corn production areas is not considered by many to be an acceptable scenario because the risk for contamination of maize grown for feed or food would be too great. Peanut seeds contain abundant (~25%) protein and likely would be suitable for the production and extraction of therapeutic proteins, although if cultivated in close proximity to other cultivars, some risk of contaminating other crop sectors would be encountered as with corn. With peanut, the risk would be primarily through seed mixture rather than cross-pollination.

In regions where peanut may be grown for animal fodder, the possibility exists to engineer protein production for animal vaccination. The rinderpest virus causes serious disease in wild and domesticated ruminants. As previously noted, oral immunization is being attempted through the expression of the hemagglutinin protein of rinderpest virus in peanut (Khandelwal *et al.*, 2003).

3.2 Risks and Concerns

The main impediments to the use of transgenic peanut in the commercial arena are the negative

sentiment toward transgenic crops in general in the EU (Ponti, 2005), a major export market for peanut, and the costs associated with deregulation and freedom to operate. Peanut is a subtropical crop and is not a major crop in many developed countries; therefore, less funding has been available for its improvement.

Cultivated peanut is tetraploid and its wild relatives are primarily diploid. Peanut also is a self-pollinated crop. Crosses between diploids and tetraploids have not been documented in nature. There is a low risk of transgene escape into the environment because of peanut's pollination biology and with the exception of South America where peanut is endemic, its lack of tetraploid congeners. Only domesticated forms of peanut are widespread in the southern United States, and they do not compete in the wild over multiple generations. A similar pattern would be expected in other regions of the world where environmental conditions would not be favorable for persistence.

The potential negative ecological or health impacts of traits introduced into peanut would rarely be unique to the crop. On the contrary, the low risk of transgene escape compared with many other crops would be more favorable for peanut. The same issues of pest resistance to plant-incorporated pesticides or antifungals would plague peanut along with other crops and are best resolved on a trait-by-trait or gene-by-gene basis rather than by commodity. Interestingly, liability for impeding the release or adoption by industry of a transgenic plant that would have a health benefit has been discussed as a likely scenario counter to the current trend of not accepting transgenic plants because of unknown risks (Miller *et al.*, 2006).

3.3 Expected Technologies

While peanut transformation is reproducible, it still is low in efficiency and more research to improve the efficiency would be justified, particularly as a functional genomics tool. While hygromycin resistance is a very effective selection method for peanut, there is a trend to eliminate antibiotic resistance genes from the plant transformation toolbox. Alternative selectable marker genes have been proposed and tested in other plants (Miki and McHugh, 2004), but their effectiveness in peanut remains to be examined. The promoter toolbox

available for dicots also provides a resource for peanut. There may be instances where the cost of constitutively expressing a defense protein, for example, is too great to plant productivity. Targeted expression by manipulating regulatory regions is feasible and likely to be more extensively implemented in the future.

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Flax

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Flax (*Linum usitatissimum* L.) is an annual, self-fertilizing plant grown either for its fiber (flax, fiber flax) or for its seed oil (oilseed flax, seed flax, linseed). The species is believed to have originated in either the Middle East or Indian regions (Vavilov, 1951; Durrant, 1976) and spread throughout Asia and Europe, prior to its introduction into the New World. Flax was one of the first crops domesticated by Man around 7000 BC, and archaeological evidence shows the presence of seeds of its wild progenitor *Linum bienne* in human habitats as long ago as 8000 BC (van Zeist and Bakker-Heeres, 1975). Utilization of both the stem fiber and the seed oil can be traced back to early civilization in Egypt and the Middle East, where linen (the fabric made from flax fiber) was worn as clothing and used to wrap mummies, and the seed oil was used in embalming (Durrant, 1976).

Flax is widely adapted to temperate climates of the world, although a degree of regional specialization has occurred. Fiber flax cultivars are taller and less branched and are grown in cool-temperate regions throughout the northern hemisphere, especially in North Asia and eastern Europe. Fiber flax remained the principal vegetable fiber in western nations throughout history until the beginning of the last century when cotton took its place. Nowadays fiber flax

is cultivated on approximately 500 000 hectares worldwide, principally in China, the Russian Federation, and Western Europe (Table 1). Annual global production of around 860 000 tons of fiber accounts for only 3% of the 25 million tons global supply of plant fiber and makes flax now only the third most important plant fiber behind cotton and jute. Linseed cultivars are shorter, more branched, and larger seeded and are grown over a wider area in warmer regions such as Canada, India, China, United States, and Argentina. Linseed is currently cultivated on over 3 million hectares worldwide, producing about 3 million tons of seed (Table 1). This represents a 50% decline compared to the 6 million hectares that linseed was grown on in the early 1970s. The oil equivalent of current production is around 1 million tons, which is around 1% of total world vegetable oil production.

1.2 Economic Products

Flax fiber is obtained from the fibrous bundles, primarily composed of cellulose, that run the full length of the tall fiber flax stem. The individual fiber cells range up to 4 cm in length and are organized as bundles of overlapping strands. Flax fiber is released by a process known as retting and is stronger, more durable, and more moisture absorbent than cotton or wool. The best grades are used for linen fabrics such as damasks, lace, and sheeting. Coarser grades are used for the manufacturing of twine and rope. The stems of

Table 1 Area and production for the top five fiber flax and linseed flax producing countries and world totals for the year 2005^(a)

Crop type	Country	Area (hectares)	Production (tons)
Fiber flax	China	161 000	470 500
	Russian Federation	89 210	55 890
	France	78 644	90 000
	Belarus	71 000	50 000
	Ukraine	23 600	12 700
	Other countries	82 318	180 353
	World total	505 772	859 443
Linseed flax	Canada	803 000	1 082 000
	India	636 000	230 000
	China	570 000	480 000
	USA	386 480	500 280
	Ethiopia	215 000	125 907
	Other countries	502 869	440 231
	World total	3 113 349	2 858 418

^(a) Source: FAOSTAT (<http://faostat.fao.org/site/340/default.aspx>)

the shorter oilseed flax types are also sometimes processed for fiber, especially for use in the high quality paper industry such as in banknotes and cigarettes papers where they provide a combination of high strength and light weight.

In the late 19th and early 20th centuries the decline in fiber flax cultivation was paralleled by increasing cultivation of linseed as an industrial oilseed crop. Industrial uses for linseed oil are based on its high content (>50%) of α -linolenic acid (9,12,15-octadecatrienoic acid). The polyunsaturated nature of linolenic acid imparts a high susceptibility to oxidation and polymerization, making it a valuable drying agent for use in paints, inks and other surface coatings, and as the main ingredient of linoleum. Industrial use of linseed oil peaked during the 1960s when oil-based paints predominated. However, the development of synthetic products from the petrochemical industry resulted in a shift to acrylic paints and vinyl floor coverings, and a consequent decline in demand for linseed oil for industrial purposes. This decline has stabilized somewhat recently with a renewal of interest in natural products and increasing concern over the level of volatile organic compounds released from synthetic coatings.

α -linolenic acid is also an essential fatty acid in the human diet and belongs to the ω -3 group of fatty acids that are increasingly being recognized

as having beneficial effects on a range of health and well-being concerns (Simopoulos, 2003). However, its oxidative instability severely restricts the use of extracted linseed oil in food products and cooking applications. As a result, human consumption of linseed oil has mainly been as a dietary supplement protected from oxidation either by encapsulation or by storage under nitrogen in small volume bottles. Whole linseed grain has also increased in popularity as a means to obtain α -linolenic acid in a more naturally protected form. It is used in a range of wholegrain products, such as multigrain breads, muesli, and breakfast cereal toppings. Ground flaxseed is also used as component in breads (Pohjanheimo *et al.*, 2006). Consumption of the whole seed or ground seed has an added nutritional benefit because it is one of the richest sources of lignans, compounds that have been shown to have strong anticancer properties (Westcott and Muir, 2003).

Recently, a modified form of linseed oil that is very low in linolenic acid was developed by plant breeding to expand the opportunities for the oil in the food industry (Green and Dribnenki, 1994). The new form, called LinolaTM (LinolaTM is a registered trademark of CSIRO, Australia), is similar in composition to sunflower and safflower oils and its considerably improved stability compared to conventional linseed oil, has enabled it to be used successfully in margarines and salad oils.

The meal remaining after extraction of oil from the seed contains about 33% protein and is commonly used as an animal feed, especially for ruminant animals such as cattle and horses. Although the meal can be used for nonruminant animals, such as pigs and poultry, the maximum inclusion levels are relatively low due to its high content of indigestible mucilage and the presence of linatine, an antinutritional factor that inhibits vitamin B6.

1.3 Taxonomy, Cytogenetics, and Genomics

The *Linum* genus belongs to the family Linaceae and has been divided into five sections (*Linum*, *Dasylinum*, *Cathartolinum*, *Linastrum* and *Syllinum*) based on chromosome number, cytology, and floral morphology (Nagao, 1941; Simmonds, 1976). *L. usitatissimum*, along with its proposed progenitors *L. angustifolium* and *L. bienne*, belongs to the section *Linum*, a grouping

of species that is distinguished from the other four sections by having homostylous rather than heterostylous flowers. Comparisons of *Linum* species for fatty acid, tocopherol and plastochromanol composition of seed oil (Rogers, 1972; Green, 1984; Velasco and Goffman, 2000) show distinct patterns between sections that support the classical taxonomic treatment of the genus.

Basic chromosome numbers in the genus include $x = 8, 9, 10, 12, 14, 15$ and 16 , with $x = 9$ and $x = 15$ being the most frequently occurring. Cytogenetic studies of interspecific crosses within the $n = 9$ and $n = 15$ groups of species within section *Linum* revealed essentially homologous chromosomes with only a limited number of chromosome translocation differences between species (Gill and Yermanos, 1967a). *L. usitatissimum* has a chromosome number of $2n = 30$ and behaves cytogenetically as a diploid species. It is considered to have originated by an ancient tetraploidy event from the basic $n = 9$ to give rise to $n = 18$, followed by chromosomal reorganization and aneuploidy to $n = 15$ (Gill, 1987). This hypothesis is supported by the regular occurrence in flax of traits controlled by duplicate gene loci, such as those controlling the $\Delta 15$ -desaturase gene in developing seeds (Green, 1986b; Vrinten *et al.*, 2005).

Extensive studies of crosses between flax and other *Linum* species revealed the presence of significant crossability barriers correlated to the sections of the genus (Allan Green, unpublished data). *L. usitatissimum* is separated from other species in section *Linum* by postfertilization barriers that resulted in hybrid embryo degeneration, whereas it is separated from species from other sections by prefertilization barriers, such as failure of pollen germination or rejection of pollen tubes in the style. These prefertilization barriers may be a consequence of the heterostylous self-incompatibility mechanisms that are prevalent in these sections of the genus.

1.4 Conventional Flax Breeding Methods

1.4.1 Selection within existing genetic variation

The flax plant is predominantly self-pollinating, with outcrossing rates generally below 1%, although rates as high as 6% have been reported

for particular genotypes and environments (Robinson, 1937; McGregor, 1976; Free, 1993). Commercial flax varieties are, therefore, usually pure lines developed through a variety of breeding and selection strategies typically employed for self-pollinating crops. In the early 1900s mass selection or single plant selection from within genetically variable accessions was the main way of developing pure line varieties. Subsequently, hybridization of pure lines having complementary traits followed by pedigree selection of desirable recombinant lines from within the segregating progeny became established as the predominant breeding strategy (Culbertson, 1954; Kenaschuk, 1975).

Pedigree selection schemes commence with the intercrossing of inbred lines, followed by selfing of the F_1 hybrid plants to produce a large F_2 seed population. A population of several thousand F_2 plants is then grown in the field, usually on soil that is heavily infected with *Fusarium oxysporum*, the causal organism of flax wilt, which is the major disease of concern for flax in most production regions. Surviving plants are scored for major traits with high heritability, such as flower color, flowering time, and seed color. The selected F_2 plants are subsequently retested in the wilt nursery as F_3 progeny rows to identify homozygous wilt resistant lines. In parallel, F_3 plants may be inoculated with specific strains of flax rust (*Melampsora lini*) under glasshouse conditions to identify rust resistant lines. Seed harvested from selected F_3 lines is used to establish F_4 progeny rows and selection continues. Typically, selection for desirable plant phenotype and performance will begin in the F_4 generation with the harvesting of a number of single plant selections from within each line, along with bulk seed of the whole F_4 line. In the case of oilseed flax, data on seed quality attributes such as oil quality, oil content, and protein content are obtained from samples taken from the bulk seed lots, and used to further truncate the population of desirable lines. In the following F_5 generation, the bulk seed is used to establish replicated small plots to enable between-line selection for seed yield, and the single plant selections are grown as progeny rows. Superior F_5 lines are identified on the basis of yield and quality performance in the replicated plots and sublines of these are used to establish the subsequent F_6 generation. Depending on the genetic divergence between the initial parents, this cycle of

between-line and within-family selection may need to be repeated for another one or two generations until uniform pure lines with elite performance have been derived. Selected pure lines are advanced into multilocation yield trials to compare their performance and adaptability with that of existing cultivars.

Single-seed descent is a popular modification of the pedigree selection that enables the development of recombinant inbred lines to be accelerated. It typically involves harvesting a single capsule per plant from a large F_2 or F_3 plant population (that may have been grown in a wilt nursery). Seed from each capsule is planted in a single pot, grown to maturity under glasshouse conditions, and a single capsule harvested from each pot. This inbreeding cycle is continued until the F_5 or F_6 generation, after which, seed of each line is increased and subsequently evaluated in replicated field plots. This procedure is best suited for crosses between highly adapted parents where the number of inferior offspring lines is expected to be low. The generation cycle time can be further reduced by growing the single-seed descent generations under conditions of high plant density and restricted nutrition, essentially aiming to produce only a few capsules per plant.

The development of inbred lines can be further accelerated through the use of doubled haploid breeding methods, which not only avoid the lengthy process of selfing but also increase the frequency of recombinant genotypes in the resulting population. In flax, haploid plants were first identified as haplo-diplo twins in polyembryonic seeds, which occur at low frequency (<1%) in many cultivars (Rajhathy, 1976) and are readily converted to diploids by chromosome doubling through the application of colchicine to decapitated stems (Plessers, 1965). Green and Salisbury (1983) developed lines with up to 32% haplo-diplo twin seedlings in a cross between cv. Rocket and cv. Avantgarde, creating a highly efficient, though genotype specific, system for doubled haploid generation in flax. More recently, haploid plants have been routinely produced by microspore culture in a less genotype-dependent manner (Chen and Dribnenki, 2004) and this may prove to provide a more broadly applicable system of doubled haploid breeding in flax.

Backcross breeding is useful for the transfer of simply inherited characters from unadapted donor

parents into elite recipient lines with minimum disturbance to the performance (Kenaschuk, 1975). It involves repeated cycles of crossing to the recipient line (recurrent parent) followed by selection for the trait being transferred. Backcross breeding was first used in flax to develop a set of rust differentials by transferring individual rust resistance alleles into the cv. Bison (Flor, 1955). These differentials have been extensively utilized in flax rust research, and as a source of resistance genes for backcrossing into elite flax varieties to overcome their susceptibility to new races of flax rust. More recently, backcross breeding played an important role in the development of the initial low linolenic flax (Linola) varieties. The low linolenic flax mutants (*ln1* and *ln2*) created in the “Zero” genotype following EMS (ethylmethane sulfonate) mutagenesis of the Australian linseed cv. Glenelg (see below) were initially associated with several undesirable background mutations that reduced plant vigor. Four generations of backcrossing the “Zero” genotype to its parent Glenelg fully restored plant performance and resulted in the development of the Linola cv. Eyre (Green and Dribnenki, 1994). Likewise, four generations of backcross breeding was sufficient to introgress the *ln1* and *ln2* mutations and a yellow seed color marker gene into the elite Canadian flax cultivars McGregor and Norlin, to develop the first Canadian Linola cultivars, Linola 947 (Dribnenki and Green, 1995) and Linola 989 (Dribnenki *et al.*, 1996), respectively, with yields equivalent to their recurrent parents.

Recurrent selection is a variation of backcross breeding, where selection for performance is practiced within consecutive segregating progeny generations after the population has been truncated for the major trait being transferred. Several high-performing lines are crossed back to the recurrent parent to initiate another cycle – there can be up to five cycles of hybridization back to the recurrent parent. This strategy allows for the transfer of a major trait, but also provides the possibility of increasing overall performance above that of the recurrent parent through contribution of favorable genes from the donor parent.

The potential value of F_1 hybrid breeding methods in flax is signified by reports of heterosis levels generally in the range of 25–40% (Kumar and Singh, 1970) and as high as 231% in some

crosses (Dubey and Singh, 1969). Although flax was one of the first crops in which an effective genetic system of cytoplasmic male sterility and fertility restoration was identified (Chittenden, 1927; Gairdner, 1929), the system has never been employed for the commercial production of hybrid varieties. The main impediment has been that male-sterile flax flowers generally have closed corollas, which fail to open and thus prevent cross pollination. However, male sterile plants with more open corollas have been reported (Kumar and Singh, 1970; Thompson, 1977) and simulation experiments have shown up to 13% seed set from open-pollination of flax flowers that were emasculated and had their petals removed (Rykova, 1973). Hybrid varieties clearly offer considerable potential for a quantum increase in yield in flax, however, further research is needed before an efficient, productive and cost-effective system of hybrid seed production will be available.

1.4.2 Interspecific hybridization to access genetic variation in related *Linum* species

Interspecific hybridization has occasionally been proposed as a means to access genetic variation in wild *Linum* species, especially where the extent of natural variation within *L. usitatissimum* has been insufficient to achieve flax breeding objectives. For example, flax shows only limited variation for fatty acid composition of the seed oil, being predominantly composed of linolenic acid (45–65%), which prevents the development of new fatty acid profiles by conventional selection (Green and Marshall, 1981). In contrast, wild *Linum* species show a much greater range in composition, including species such as *Linum tenuifolium* having over 80% linoleic acid and only 3% linolenic acid, and *Linum mucronatum* that contains 5% of the hydroxylated ricinoleic acid (Green, 1984). However, *L. usitatissimum* is reproductively isolated from most other *Linum* species by either prefertilization or postfertilization barriers depending on the degree of taxonomic separation of the species (Allan Green, unpublished data). There are no reports of successful rescue of nonviable embryos from interspecific *Linum* hybrids using tissue culture techniques that have been effective in other genera.

To date, fertile interspecific hybrids have only been achieved between flax and a limited number of species that are from the same section of the genus (section *Linum*) and have the same chromosome number ($2n = 30$) including *L. angustifolium*, *L. bienne*, *L. nervosum*, *L. pallescens*, *L. africanum*, *L. corymbiferum*, *L. decumbens*, *L. hirsutum*, *L. floccosum* and *L. tenue* (Gill and Yermanos, 1967b; Bari and Godward, 1970; Seetharam, 1972). Unfortunately all species from the section *Linum* so far analyzed show similar fatty acid composition to flax (Green, 1984) and therefore, do not represent a useful genetic resource for modification of this character. However, it is possible that those closely related species that are accessible by conventional hybridization may harbor other traits that could be useful for flax breeding, such as novel alleles for disease resistance genes, or wider variability for morphological characteristics.

1.4.3 Mutation breeding to create new variation

In situations where there is insufficient genetic variation available to achieve breeding objectives, new genetic variants can often be created by induced mutation techniques, using either physical or chemical mutagenic agents. A wide range of induced mutations have been reported in flax, including those causing chlorophyll deficiencies (Seetharam, 1971a; Rai and Das, 1975), increased oil content (Larter *et al.*, 1965; Rath and Scharf, 1968; Seetharam, 1971b), altered seed weight (Seetharam, 1971b), increased seed yield (Bari, 1971; Srinivasachar *et al.*, 1972), and improved disease resistance (Srinivasachar and Seetharam, 1971). Although useful for basic genetic studies, most of these mutations have not made a significant contribution to flax improvement.

In contrast, mutation breeding has proved highly valuable in generating altered fatty acid composition profiles in flax seed oil (Table 2). Green and Marshall (1984) used EMS-mediated chemical mutagenesis of the Australian linseed cv. Glenelg to produce two independent mutant lines, M1589 and M1722, in which linolenic acid content was reduced from 43% down to 29%. Subsequent recombination of these mutations resulted in

Table 2 Fatty acid composition (% of total fatty acids) of linseed cvs. Glenelg and McGregor and mutants derived from them by EMS mutagenesis

Genotype	Palmitic (16:0)	Palmitoleic (16:1)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
Glenelg ^(a)	6		3	19	22	50
M1589 ^(a)	7		4	21	35	33
M1722 ^(a)	6		4	24	33	33
“Zero” ^(a)	6		3	17	70	3
M6193 ^(b)	10		6	43	38	2
M440 ^(b)	29	6	3	25	35	2
M606 ^(b)	21	2	6	29	40	2
McGregor	7		4	18	16	54
E1747 ^(c)	7		3	12	75	2
E67 ^(d)	28		4	14	6	43

^(a)Green, 1986c^(b)Green, unpublished data^(c)Rowland, 1991^(d)Rowland and Bhatti, 1990

almost complete removal of linolenic acid from the seed oil (down to only 2%), with an equivalent increase in the content of its precursor linoleic acid, up from 14% to 51% (Green, 1986a). Genetic and biochemical analysis revealed that the mutations were in two independent genes (*Ln1* and *Ln2*) that encode the $\Delta 15$ -desaturase enzyme responsible for converting linoleic acid to linolenic acid (Green, 1986b; Tonnet and Green, 1987; Stymne *et al.*, 1992). Similar changes were subsequently reported in the Canadian cv. McGregor by apparently simultaneous EMS-induced mutations in two independent genes (Rowland, 1991), most likely the same genes as were mutated in Glenelg. These genes were subsequently cloned, confirmed to belong to the *Fad3* family of microsomal fatty acid $\Delta 15$ -desaturases and designated as *LuFAD3A* and *LuFAD3B* (Vrinten *et al.*, 2005). The low linolenic mutants obtained in Glenelg were used to create a new form of linseed oil referred to as LinolaTM with greatly improved oxidative stability and suitability for widespread food use, as an alternative to sunflower and safflower oils (Green and Dribnenki, 1994). Linola has become successfully established as a specialty form of flax, especially in Canada where it accounts for a significant proportion of the flax crop.

EMS mutagenesis was successfully used to produce additional variations in fatty acid composition (Table 2) including lines with elevated oleic acid and palmitic acid contents (Rowland and Bhatti, 1990; Rowland, 1991; Saeidi and Rowland,

1997; Allan Green, unpublished data). Mutation breeding is particularly useful for blocking biosynthetic pathways in order to prevent the synthesis of particular compounds. As well as the above changes in fatty acid biosynthesis pathways, mutation breeding may have potential in the future to remove other undesirable compounds from flaxseed, such as cyanogenic glycosides and linatine.

1.4.4 Limitations of conventional breeding methods

Conventional breeding techniques have been employed in flax for over a century and have been particularly successful in equipping the crop with durable resistance to the major wilt and rust diseases, improving lodging resistance, adapting crop phenology to match regional growing seasons, and developing varieties with greater yield stability across environments. However, over recent decades the rate of overall yield improvement in flax has lagged behind that was achieved in other oilseeds, particularly soybean and rapeseed (canola). This is no doubt partly due to the relatively small size of the flax crop globally and the resulting lower resources and attention given to flax improvement. However, the restricted genetic variation within the species, inability to incorporate genetics from related species, and

importantly, the lack of development of hybrid production systems, contribute to the lack of major progress. Mutation breeding has resulted in significant achievements in the diversification of product quality, however, even this technique is limited in its suitability for the further quality improvements needed in the future, and is unlikely to be suitable for achieving significant increases in yield.

Gene technology has proven to be highly successful in improving several oilseed crops, especially through the transgenic introduction of herbicide tolerance and insect resistance genes that allow more effective and cheaper weed and pest control, and the introduction of novel genetic systems for hybrid seed production. These achievements highlight the improvements that can be realized in flax through genetic transformation with similar novel traits. Additionally, many of the future opportunities for development of new and improved products in flax will require metabolic engineering approaches that involve genetic transformation, either by genes that encode functional enzymes for additional biosynthetic pathways, or gene silencing constructs that can restrict the inactivation of unwanted endogenous genes to specific target tissues (e.g., seed for oilseed flax, and stem for fiber flax). Transgenic technologies, therefore, offer substantial potential to achieve improvements in flax that cannot be attained by conventional breeding methods.

2. FLAX TRANSFORMATION

Because flax is a relatively minor crop in global terms, transgenic applications have not been pursued as much as in larger crops (e.g., maize, soybean, canola, cotton). However, flax has proved to be very amenable to genetic transformation and some novel agronomic (input) traits, such as herbicide tolerances, initially developed in other crops, have now been transferred to flax. In addition, new and improved product quality (output) traits specific for flax have been pursued, including attempts to modify stem fiber quality of fiber flax varieties and to diversify oil composition in oilseed flax (linseed) varieties (Table 3).

2.1 Transgenic Traits Developed

2.1.1 Herbicide tolerance

The first trait engineered into flax was tolerance to the herbicide glyphosate (Roundup[®]), a potent inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in higher plants. A gene encoding a glyphosate-insensitive form of the EPSP enzyme cloned from a complementary DNA library of a glyphosate-tolerant *Petunia hybrida* cell line (MP4-G) was shown to confer glyphosate tolerance to normally sensitive *Petunia* cells when expressed transgenically under the control of the constitutive cauliflower mosaic virus 35S promoter (Shah *et al.*, 1986). Subsequently, this *EPSP* gene was transformed into flax by *Agrobacterium*-mediated transformation and the resulting transgenic flax lines were shown to be resistant to 5 mM glyphosate (Jordan and McHughen, 1988a).

Subsequently, genes encoding tolerance to other herbicides were developed and successfully introduced into flax. A mutant acetolactate synthase gene isolated from *Arabidopsis* was shown to confer tolerance to the sulfonylurea herbicide chlorsulfuron (Glean[®], DuPont) in plants (Haughn *et al.*, 1988). Transfer of this gene under the control of the native *Arabidopsis* regulatory signals to flax led to the development of lines with chlorsulfuron tolerance. Transgenic lines grew vigorously in soil containing 20 g ha⁻¹ chlorsulfuron whereas the growth and development of the untransformed control were severely inhibited (McHughen, 1989). Transgenic flax plants have also been developed to have tolerance to bialaphos, a broad-spectrum antibiotic based herbicide marketed as Basta[®] and Liberty[®] (AgrEvo). This was achieved by introduction of the phosphinothricin acetyl transferase (PAT) gene from *Streptomyces viridochromogenes* under the control of cauliflower mosaic virus (CaMV) 35S promoter. The introduction of the PAT enzyme allows the flax plants to breakdown glufosinate ammonium herbicide, applied at the level of 600 g ha⁻¹, without suffering any toxic effects (McHughen and Holm, 1995a).

The bialaphos and chlorsulfuron tolerant flax lines were evaluated in field trials and the introduced traits were shown to perform well under field conditions and to incur no agronomic

Table 3 A catalog of engineered novel traits in flax (donor species for the gene transferred is indicated in brackets)

Gene(s) introduced	Novel trait developed	References
EPSP synthase (<i>Petunia</i>)	Glyphosate resistance	Jordan and McHughen, 1988a
Mutant acetolactate synthase (<i>Arabidopsis</i>)	Sulfonylurea resistance	McHughen, 1989
Phosphinothricin acetyl transferase (<i>Streptomyces viridochromogenes</i>)	Glufosinate resistance	McHughen and Holm, 1995a
$\Delta 6$ and $\Delta 5$ desaturases (<i>Phaeodactylum tricornutum</i>) and $\Delta 6$ elongase (<i>Physcomitrella patens</i>)	Long chain polyunsaturated fatty acids arachidonic and eicosapentaenoic in seed oil	Abbadi <i>et al.</i> , 2004
3-ketothiolase, acetoacetyl-CoA reductase and polyhydroxybutyrate synthase (<i>Ralstonia eutropha</i>)	PHB polymer in stem tissue	Wrobel <i>et al.</i> , 2004

penalties to the crop (McHughen and Holm, 1995b). Submissions were made to US FDA (Food and Drug Administration) for release of the chlorsulfuron-tolerant variety “Triffid”, however, due to sustained pressure from the Flax Council of Canada and resistance from EU customers the variety was removed from the market in 2001.

2.1.2 Fiber quality

The use of flax fibers in textile industries has been in decline due to severe challenge from cotton and synthetic polymers. In order to enhance the thermoplastic properties of flax fibers, transgenic plants synthesizing polyhydroxybutyrate (PHB) in their stem tissue have been generated. PHB is a nontoxic biodegradable thermoplastic agent that is soluble in water and has similar chemical and physical properties to the petroleum-derived polymer polypropylene. In various species of bacteria, for example, *Ralstonia eutropha*, PHB acts as a source of carbon and energy and is synthesized by the concerted action of 3-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase enzymes. Wrobel *et al.* (2004) transformed flax with the three genes, isolated from *R. eutropha*, encoding these enzymes. The genes were expressed under the control of a stem-specific promoter (14-3-3, GenBank Acc No: AY070220) and were targeted to the plastid to avoid possible deleterious effects of PHB accumulation. The transgenic flax plants were shown to accumulate up to 70-fold higher PHB than nontransgenic flax and were shown to display significant modifications in stem mechanical properties. For example the Young’s modulus E , an average measure of stem tissues resistance to tensile loads, increased up to twofold

in transgenic plants producing PHB than in non-PHB containing controls.

2.1.3 Oil quality

ω -3 long-chain polyunsaturated fatty acids (ω 3 LC-PUFA), such as EPA (eicosapentaenoic acid, C20:5) and DHA (docosahexaenoic acid, C22:6) and are now widely recognized as having important benefits for human health. Whilst the human body is capable of synthesizing them from dietary α -linolenic acid (ALA, C18:3 $\Delta^{9,12,15}$), the conversion rate is very inefficient (Voss *et al.*, 1991) and direct dietary intake of EPA and DHA is therefore recommended for good nutrition. Fish are the current principal sources of these fatty acids, but fish supplies are diminishing and novel sources of EPA and DHA are being sought. EPA and DHA are synthesized by a variety of microorganisms and lower plants, but they are not naturally produced in higher plants. Because flaxseed is one of the richest sources of ALA, it has been regarded as an attractive starting point for genetic engineering to produce a plant source of EPA and DHA.

Metabolic engineering of yeast and higher plants to produce EPA and/or DHA has recently been attempted using genes from organisms that naturally synthesize and accumulate LC-PUFA by a series of alternating elongation and desaturation reactions. Seed-specific expression in flax of cDNAs encoding a set of front-end fatty acid desaturases and a $\Delta 6$ fatty acid elongase (Abbadi *et al.*, 2004) resulted in significant $\Delta 6$ -desaturation of ALA to form stearidonic acid (C18:4) and to a much lesser degree, further elongation and $\Delta 5$ -desaturation to EPA. Additionally, two $\omega 6$ LC-PUFA, γ -linolenic acid

(GLA, C18:3^{Δ6,9,12}) and arachidonic acid (ARA, C20:4^{Δ5,8,11,14}) were synthesized as a result of the action of the same introduced enzymes on linoleic acid (LA, C18:2^{Δ9,12}). Whilst the levels of EPA synthesized were only low in this experiment, recent developments in the area of engineering LC-PUFA in other oilseeds are promising for the eventual development of flax oil with substantially higher amounts of nutritionally important ω 3 LC-PUFA (Napier *et al.*, 2006).

2.2 Transformation Methods

The successful regeneration of transgenic plants requires an effective DNA delivery system to transfer the gene of interest into the cells of the target explants and an efficient, reproducible system to regenerate plants from the transformed cells. The four most established DNA delivery systems are mediated by either *Agrobacterium*, polyethylene glycol (PEG), particle bombardment, or microinjection (Potrykus, 1991). Three of these approaches have been successfully adopted in *Linum* species to regenerate transgenic plants. The first demonstration of successful integration of transfer DNA (T-DNA) into the flax genome was reported in 1983 and involved the transformation of flax epicotyls by a nopaline strain of *Agrobacterium tumefaciens* T37 harboring an oncogenic plasmid pTi37 (Hepburn *et al.*, 1983). Four years later, the first successful regeneration of transgenic flax plants was reported. In that study, flax hypocotyl segments were transformed with another nopaline *A. tumefaciens* strain C58C1 carrying a disarmed (nononcogenic) Ti plasmid vector pGV3850 (Basiran *et al.*, 1987).

One year later, transgenic flax plants were regenerated from transformation of cotyledons with various *A. rhizogenes* strains A4, 1855 and TR7 containing root-inducing (Ri) plasmid pRiA4, pRi1855, and pRiTR7, respectively (Zhan *et al.*, 1988). In order to overcome some of the problems encountered with *Agrobacterium*-mediated transformation techniques and capitalize on the efficient protoplast culture system, direct transformation of protoplast of *Linum suffruticosum* mediated with PEG was developed and transgenic plants were regenerated (Ling and Binding, 1997). In order to develop a rapid and simple means of transforming intact cells and tissues, particle bombardment-mediated transformation technique was also established (Wijayanto and McHughen, 1999).

Subsequently research efforts in different laboratories around the world have been primarily focused on *A. tumefaciens*-mediated transformation due to the potential for high transformation efficiency and production of transgenic plants with simple transgene insertion patterns and high stability of the transgene in the progeny (Jordan and McHughen, 1988a; Mlynarova *et al.*, 1994; Bretagne-Sagnard and Chupeau, 1996; Chen and Dribnenki, 2003). Since the processing and transfer of T-DNA from *Agrobacterium* to plant cells have been demonstrated to be regulated by the activity of the *vir* genes located in the resident Ti plasmid and by the specific interactions between Ti plasmid and *Agrobacterium* chromosomal background (Gelvin, 2003), various types of *Agrobacterium* strains, including octopine, nopaline, and succinamopine type strains have been tested in flax in different laboratories (Table 4). However, no experiments have been designed

Table 4 Type of *Agrobacterium* strains used in flax transformation

Strain	References
A208	Jordan and McHughen, 1988a; McHughen <i>et al.</i> , 1991
A281	Polyakov <i>et al.</i> , 1998
AGL1	Bretagne-Sagnard and Chupeau, 1996; Chen and Dribnenki, 2003
C58C1	Basiran <i>et al.</i> , 1987; Zhan <i>et al.</i> , 1988; McHughen, 1989; Wrobel <i>et al.</i> , 2004
C58C1Rif [®]	Dong and McHughen, 1993a; Bretagne-Sagnard and Chupeau, 1996; Ling and Binding, 1997
C58C1 ATHV	Drexler <i>et al.</i> , 2003
GV2260	Ling and Binding, 1997; Polyakov <i>et al.</i> , 1998
LBA4404	Mlynarova <i>et al.</i> , 1994; Polyakov <i>et al.</i> , 1998
GV3101:pPM90RK	Rakousky <i>et al.</i> , 1999
GV3101:pMP90	Qiu <i>et al.</i> , 2002; Chen <i>et al.</i> , 2008

to directly compare the effect of *Agrobacterium* strains on transformation in flax. In one study, two *Agrobacterium* strains, AGL1 and C58C1 Rif[®] (pGV2260), were used but the binary vector introduced into AGL1 carried a spectinomycin resistance gene, whereas that introduced into C58C1 Rif[®] carried a kanamycin resistance gene. The conclusion from that study was that spectinomycin was a more effective selective agent than kanamycin for flax (Bretagne-Sagnard and Chupeau, 1996). However, the strain differences may have also contributed to the different transformation efficiencies obtained.

Agrobacterium cell density at time of co-cultivation appears to have significant effect on shoot regeneration and transformation. Co-cultivation of explants with *Agrobacterium* strain AGL1 at OD₆₀₀ of 1.2 had more prevalent overgrowth, resulting in lower frequency of shoot regeneration and lower efficiency of transformation than co-cultivation at OD₆₀₀ of 0.3 (Chen and Dribnenki, 2003). Because activity of the *vir* genes present on the resident plasmid of an *Agrobacterium* strain have been shown to be induced by wounding or phenolic compounds such as acetosyringone and related molecules (Stachel *et al.*, 1985), application of these phenolic compounds have been used to increase transformation efficiency of some crop species (De Clercq *et al.*, 2002; Hoque *et al.*, 2005). However, different Ti plasmid, plant species and plant tissues have been shown to require different levels or different types of phenolic compounds for optimal induction of the *vir* genes (Messens *et al.*, 1990). Although sinapinic acid had been shown to specifically activate the *vir* region of the Ti plasmid (Stachel *et al.*, 1985), there was no noticeable effect observed in flax when it was added to the growth medium for *A. tumefaciens* strain C58C1 before co-cultivation at concentration of 100–200 μ M (Zhan *et al.*, 1988). Likewise, the addition of acetosyringone at concentrations from 50 to 200 μ M to co-cultivation medium with AGL1 did not increase transformation efficiency in comparison with co-cultivation medium containing no acetosyringone (Chen *et al.*, 2008). Co-integrative transformation vector systems were used in earlier research experiments (Basiran *et al.*, 1987; Zhan *et al.*, 1988; Jordan and McHughen, 1988a), however, more recent studies have almost exclusively used

binary vector systems (Dong and McHughen, 1993a; Mlynarova *et al.*, 1994; Bretagne-Sagnard and Chupeau, 1996; Chen and Dribnenki, 2003).

2.3 Selection of Transformed Tissue

Regardless of which DNA delivery system is used, it is crucial to have an effective system for selecting the limited number of transformed cells from amongst an overwhelmingly larger number of nontransformed cells. The ideal selection condition is to achieve the optimal growth of transformants and minimal survival of non-transformants. Antibiotic or herbicide resistance conferred by selectable marker gene has commonly been used for this purpose. In *Agrobacterium*-mediated transformation, additional antibiotics, such as carbenicillin, augmentin, timentin, or cefotaxime are used to kill the *Agrobacterium* after a period of co-cultivation with explants.

The first transgenic flax plants were regenerated from transformed cells expressing the selectable marker gene *nptII* (neomycin phospho-transferase II), which confers resistance to aminoglycoside antibiotics, such as kanamycin (Basiran *et al.*, 1987). This selectable marker gene has since been used in many transformation studies and other selectable marker systems have also been developed (Table 5). For *nptII* selectable marker, other aminoglycoside antibiotics, such as geneticin (G418) and paromomycin besides kanamycin, have been used as the selective agents in the selection medium (Dong and McHughen, 1993a; Bretagne-Sagnard and Chupeau, 1996). Compared to kanamycin, lower concentrations of geneticin are needed to kill nontransformed shoots. However, kanamycin at concentrations from 50 to 200 mg l⁻¹ proved to be more effective in selecting transformed shoots than geneticin (Dong and McHughen, 1993a). Paramomycin was not an effective selective agent for flax as concentration up to 200 mg l⁻¹ failed to inhibit shoot regeneration from untransformed cells (Bretagne-Sagnard and Chupeau, 1996). Despite successful reports of using *nptII* as the selectable marker, some laboratories have reported difficulty in regenerating transgenic plants using kanamycin as the selection agent in the medium (Zhan *et al.*, 1988; Bretagne-Sagnard and Chupeau, 1996). In those studies, thousands or tens of thousands of

Table 5 Type of selectable marker genes in flax transformation

Marker gene	References
<i>nptII</i>	Basiran <i>et al.</i> , 1987; Jordan and McHughen, 1988a; Zhan <i>et al.</i> , 1988; McHughen <i>et al.</i> , 1991; Dong and McHughen, 1993a; Mlynarova <i>et al.</i> , 1994; Bretagne-Sagnard and Chupeau, 1996; Ling and Binding, 1997; Polyakov, <i>et al.</i> , 1998; Wijayanto and McHughen, 1999; Drexler <i>et al.</i> , 2003
<i>aadA</i>	Bretagne-Sagnard and Chupeau, 1996; Chen <i>et al.</i> , 2008
<i>hph</i>	Ling and Binding, 1997; Rakousky <i>et al.</i> , 1999; Chen and Dribnenki, 2003
<i>pat</i>	McHughen and Holm, 1995a

explants were inoculated with the same strain of *Agrobacterium* containing the same vectors that have been reported to produce transgenic shoots in flax. The difference in genotype of explants and transformation protocol may partially account for the difficulty encountered with these two reports. Alternatively, this may indicate that kanamycin is a problematic antibiotic to work with in flax perhaps due to poor penetration to the regenerating cells (Bretagne-Sagnard and Chupeau, 1996).

Subsequently, the selectable marker gene *aadA* encoding the enzyme aminoglycoside-3'-adenyltransferase, which confers resistance to a structurally unique aminocyclitol antibiotic spectinomycin, has been successfully used in selecting transformed flax cells and plants. Selection concentration of spectinomycin at 50–100 mg l⁻¹ proved to be effective (Bretagne-Sagnard and Chupeau, 1996; Chen *et al.*, 2008). Spectinomycin appears to be more mobile than kanamycin and thus is spread more homogeneously among the transformed tissue, which makes for more accurate and effective selection (Bretagne-Sagnard and Chupeau, 1996).

The third antibiotic resistance selectable marker system developed in flax transformation was *hph* or *hpt* encoding the enzyme hygromycin B phosphotransferase, which confers resistance to aminoglycoside antibiotic hygromycin B. This selectable marker has been successfully used to obtain transformed cells and plants from protoplasts, hypocotyl explants and anther cultures. Selection at concentrations from 5 to 30 mg l⁻¹ hygromycin B proved to be effective in various experiments in different laboratories (Ling and Binding, 1997; Rakousky *et al.*, 1999; Chen and Dribnenki, 2003).

The incorporation of antibiotic resistance genes in commercial transgenic plants has come to be regarded as undesirable due to public perceptions

that it could accelerate the development of antibiotic resistant microorganisms. Herbicide resistance may, therefore, be a better choice than antibiotic resistance. In earlier studies, antibiotic resistance selection was used to select transformed cells for the expression of herbicide resistant traits, such as glyphosate resistance conferred by the expression of 5-enolpyruvylshikimate-3-phosphate (*eps*) synthase gene (Jordan and McHughen, 1988a) or sulfonylurea herbicide resistance encoded by a modified acetolactate synthase (*als*) gene (McHughen, 1989). Resistance to the herbicide phosphinothricin (10 mg l⁻¹) was used to select transformed cells and shoots that expressed phosphinothricin acetyltransferase gene (*pat*) in addition to antibiotic resistance selection (McHughen and Holm, 1995a).

The level of selectable agents in selection medium has a significant effect on transformation efficiency and escape frequency. Dong and McHughen (1993a) demonstrated that increase in kanamycin concentration from 50 mg/l to 200 mg/l in selection medium reduced the total number of shoots regenerated, but increased the total number of explants producing transgenic shoots and the total number of transgenic shoots. This was an ideal situation since high selection pressure reduced the laborious effort to identify true transgenic plants within a large population of regenerated shoots encountered with low selection pressure. However, selection at too high concentration or for an extended period of time can eventually reduce recovery of transgenic plants since regeneration from transformed cells, particularly those expressed at low-to-moderate levels, could be inhibited. Selection at 10 mg l⁻¹ hygromycin B in callus induction medium for 2 weeks before transfer to a shoot regeneration medium had higher frequency

of shoot regeneration and greater efficiency of transformation than the selection for 4 weeks (Chen and Dribnenki, 2003).

2.4 Plant Regeneration

2.4.1 Hypocotyl explant culture

An efficient plant regeneration system is a prerequisite for the development of an efficient transformation system regardless of DNA delivery methods used. The first successful *in vitro* regeneration of flax plants from cultured explants was reported over 30 years ago (Rybczynski, 1975; Gamborg and Shyluk, 1976). A range of different types of cultured explants have subsequently proven to be amenable to regeneration, either directly or through an intermediate callus phase, with hypocotyl segments being more easily regenerated than other tissues (Mathews and Narayanaswamy, 1976; Bretagne *et al.*, 1994; Dedicova *et al.*, 2000). Not surprisingly, the first successful regeneration of transgenic plants was achieved using hypocotyl segments as the explants for co-cultivation with *Agrobacterium* (Basiran *et al.*, 1987). Transgenic plants have since been regenerated from various explant types, including cotyledons, protoplasts, plastocytes, and anther cultures (Zhan *et al.*, 1988; Ling and Binding, 1997; Chen and Dribnenki, 2003). However, scientists are challenged to develop reproducible transformation protocols as the initial protocols developed in one laboratory could not easily be reproduced in other laboratories.

In the first report, hypocotyl segments were transformed by an *Agrobacterium* strain harboring a vector containing a chimeric *nptII* gene as a selectable marker and the nopaline synthase gene as a scorable trait. Transgenic status of kanamycin resistant calli was determined by Southern analysis, nopaline production and NPTII activity. The transgenic status of shoots regenerating from resistant calli was only demonstrated by nopaline production in these shoots and by their rooting ability in medium containing kanamycin, with no molecular analysis or progeny analysis being conducted on these plants (Basiran *et al.*, 1987). The same *Agrobacterium* strain C58C1 harboring the same disarmed Ti plasmid pGV3850 with the same *nptII* selectable marker gene

as Basiran *et al.* (1987) was used in another study where several thousand hypocotyl segments were used as explants for co-cultivation (Zhan *et al.*, 1988). Transgenic status of kanamycin resistant calli appearing on the cut ends of inoculated hypocotyl segments were demonstrated by Southern analysis and nopaline production. Nopaline production was detected in both bleached and green shoots appeared on kanamycin resistant calli, and Southern analysis of DNA from two of the shoots which were produced in kanamycin containing medium showed that they were not transformed (Zhan *et al.*, 1988). Such nontransformed regenerants were considered to have been derived from nontransformed cells that had survived exposure to the selection agent by cross-protection from adjacent transformed cells (Jordan and McHughen, 1988b). The different results observed between the early study by Basiran *et al.* (1987) and these two subsequent studies by Zhan *et al.* (1988) and Jordan and McHughen (1988b) might be explained by the differences in genotype of explants since almost identical medium and tissue culture procedure were used. Jordan and McHughen (1988a) were able to regenerate true transgenic flax plants when hypocotyl segments were sliced along the top surface, or where strips of epidermis were removed from hypocotyl segments before co-cultivation with *Agrobacterium*. In that study, transgenic status of regenerated plants was confirmed by a leaf callus assay on medium containing the selective agent, nopaline assay on leaf-derived calli, and transmission of selectable marker gene and gene of interest to the next generation (Jordan and McHughen, 1988a). This transformation protocol proved to be repeatable in the same laboratory and was further improved by including the additional step of preculture of hypocotyl segments for a period of time before co-cultivation (McHughen *et al.*, 1989). A preculture period of 9–12 days prior to co-cultivation was found to be optimal for recovery of transgenic shoots. The beneficial effect of preculture was considered to be due to the physiological and developmental shift of hypocotyl cells to become competent for regeneration during the preculture period (McHughen *et al.*, 1989). This improved transformation protocol was used to produce transgenic plants conferring sulfonylurea herbicide resistance (McHughen, 1989). The first report of molecular

analysis of primary transformants was in the study of Dong and McHughen (1993a) where transgenic status of primary transformants was confirmed by polymerase chain reaction (PCR) amplification of antibiotic selectable marker *nptII* gene and by histochemical assay and progeny analysis of the scorable marker gene *gusA* that encodes β -glucuronidase (GUS). Under optimal conditions, transformation efficiency defined as the percentage of independent explants producing at least one transgenic shoot was 13%, and escape frequency defined as the percentage of nontransgenic shoots surviving the selection process was 83% (Dong and McHughen, 1993a). Chimera frequency defined as the percentage of putative transformants showing less than expected transgene positive plant and more than expected transgene negative plants in progeny analysis was approximately 45% (Dong and McHughen, 1993b). The high escape frequency could result from the cross protection of a large number of nontransformed shoots of epidermal origin by transformed callus tissues at the cut ends (Jordan and McHughen, 1988b). Regeneration from cells on the boundaries of peeled and unpeeled region of hypocotyls segments was proposed as the reason for producing high frequency of chimera (Dong and McHughen, 1993b). This study provided the most complete picture regarding overall transformation efficiency since it included transformation efficiency, escape frequency, and escape frequency with a reasonable number of explants for initial co-cultivation with *Agrobacterium*. However, this transformation protocol has a number of drawbacks including tedious and laborious removal of epidermis from hypocotyl segments under the microscope in a flowhood, high frequency of escapes in regenerated plants, and high frequency of chimera in putative transformants. In an effort to overcome the problems encountered with previous protocols, Mlynarova *et al.* (1994) isolated calli formed on the surface of the hypocotyl segments and placed them on fresh selection medium for shoot regeneration. This tedious removal of calli from each hypocotyl segment may help to reduce direct regeneration of plants from epidermal cells and thereby potentially reduce escapes. This could potentially reduce chimera frequency as this protocol does not include the process of epidermis stripping from the explants. The transformation efficiency of about 25% was an improvement over the previous report

by Dong and McHughen (1993a) of 16% based on the number of transgenic shoots obtained per inoculated explant. However, the calculation by Mlynarova *et al.* (1994) was based on GUS activity and PCR analysis of primary transformants, with no progeny analysis conducted. The number of explants used for this study was low and there was no data regarding escape and chimera frequency. In addition, calculation of transformation efficiency based on number of transgenic plants without Southern analysis to determine the independent status of plants is usually an overestimate of true transformation efficiency as some of them are clones due to callus fragmentation and handling errors. Despite the successful results of these reports, Bretagne-Sagnard and Chupeau (1996) were not able to produce any transgenic plants with or without preculture and epidermis removal when over 25 000 hypocotyl segments were inoculated with C58C1Rif[®] (pGV2260) harboring the binary vector p35SGUSINT, the same construct used by Dong and McHughen (1993a). They were however successful in regenerating transgenic plants at the transformation efficiency of around 15–20% when spectinomycin was used as the selective agent in the medium. Transgenic plants derived from transformation of hypocotyl segments with *A. tumefaciens* were characterized by Southern analysis for the first time in flax and progeny analysis was also followed (Bretagne-Sagnard and Chupeau, 1996). Chimera frequency was low based on analysis of a limited number of putative transgenic plants. However, escape frequency was not discussed in that study (Bretagne-Sagnard and Chupeau, 1996). Further modification of transformation using hypocotyl segments as the explants was the development of particle bombardment-mediated DNA delivery system. Particle bombardment mediated-transformation of hypocotyl segments reduced escape frequency and chimera frequency to a certain degree, but transformation efficiency was lower than that in *Agrobacterium* mediated-transformation (Wijayanto and McHughen, 1999).

2.4.2 Cotyledon culture

Even though cotyledons were the first cultured explants to regenerate *in vitro* plants (Rybczynski,

1975), there has been little transformation research with cotyledons as the explants due to technical difficulty to regenerate in subsequent studies (Bretagne *et al.*, 1994; Dedicova *et al.*, 2000). Successful regeneration of transgenic plants as demonstrated by Southern analysis was achieved by co-cultivation of cotyledons with *A. rhizogenes* in one report (Zhan *et al.*, 1988). But there is no report regarding successful transformation of cotyledon explants with *A. tumefaciens*. Development of an efficient transformation protocol using cotyledons as the explants might be possible since high frequency of shoot regeneration from cotyledon explants was recently reported (Rutkowska-Krause *et al.*, 2003).

2.4.3 Protoplast culture

The first successful regeneration of flax plants from protoplast culture was achieved in 1983 (Barakat and Cocking, 1983). The regeneration of transgenic plants from protoplasts by PEG-mediated transformation or plastocytes co-cultivated with *A. tumefaciens* was obtained 14 years later (Ling and Binding, 1997). *Agrobacterium*-mediated transformation of plastocytes apparently reduced escape frequency but its transformation efficiency was much lower than transformation using hypocotyl segments as the explants due to inefficient plant regeneration from transformed calli (Ling and Binding, 1997).

2.4.4 Anther culture

Successful shoot regeneration from anther culture was initially reported in fiber flax (Sun, 1979) and later in oil flax (Nichterlein *et al.*, 1991; Chen *et al.*, 1998a). Anther culture protocol

was subsequently optimized for doubled haploid production and transformation (Chen *et al.*, 1998b, 2003; Chen and Dribnenki, 2002, 2004). A high throughput transformation protocol was developed in flax using anther cultures as the recipient explants. Transgenic status of plants was confirmed by PCR analysis of selectable marker genes, histochemical analysis of a reporter gene, and progeny analysis. Under optimal conditions, transformation efficiency, defined as number of independent calli producing at least one transgenic shoot, was 66%, with escape frequency of 27%, and chimera frequency of 8% (Chen and Dribnenki, 2003). More recently, hundreds of transgenic plants derived from several independent experiments were analyzed by Southern analysis to determine the independent status of transgenic plants from the same callus and integration pattern of transgene. Transformation efficiency based on the number of independent shoots/per inoculated explant was at least twice as much as that calculated based on the number of independent calli producing at least one transgenic shoot per inoculated explant (Chen *et al.*, 2008). This transformation protocol represents a significant improvement over any other published transformation protocols for all transformation parameters: transformation efficiency and escape frequency (Table 6), as well as chimera frequency (Table 8).

2.5 Transformation Stability

In earlier studies of flax transformation, opine expression, rooting of shoots, and positive leaf callus assay on medium containing a selective agent followed sometimes by progeny analysis were used independently and in combination to provide the evidence of transformation (Basiran *et al.*,

Table 6 Transformation efficiency and escape frequency in flax transformation

Study	Transformation efficiency (%)	Escape frequency (%)
Dong and McHughen, 1993a ^(a)	13	83
Mlynarova <i>et al.</i> , 1994 ^(b)	25	60
Bretagne-Sagnard and Chupeau, 1996 ^(b)	15	NA
Chen and Dribnenki, 2003 ^(a)	66	27

^(a)Transformation efficiency was defined as the number of independent explants producing at least one transgenic shoot/100 inoculated explants

^(b)Transformation efficiency was defined as the number of transgenic shoots obtained per 100 inoculated explants

Table 7 Southern analysis of primary transgenic flax plants produced by *Agrobacterium tumefaciens* transformation

Explant type	Southern insert	References
Hypocotyl segments	1–6	Bretagne-Sagnard and Chupeau, 1996
Plastocytes	1–2	Ling and Binding, 1997
Hypocotyl segments	1–8	Drexler <i>et al.</i> , 2003
Anther cultures	1–7	Chen <i>et al.</i> , 2008

1987; Jordan and McHughen, 1988a; McHughen, 1989). Subsequently, PCR analysis of the selectable marker gene and the reporter gene was used to confirm the physical presence of the transgene in the flax genome (Dong and McHughen, 1993a; Mlynarova *et al.*, 1994). Variation in transgene expression within the population of transgenic plants transformed with the same construct and the same transformation experiment has often been encountered (Luck *et al.*, 2000; Qiu *et al.*, 2002; Abbadi *et al.*, 2004; Chen *et al.*, 2008). The factors that have been used to explain this phenomenon in other species include transgene copy number, the position within the genome where transgene is integrated (Matzke and Matzke, 1998), transcriptional gene silencing, or post-transcriptional gene silencing due to the presence of complex integration patterns such as tandem repeats or inverted repeats (Jorgensen *et al.*, 1996; Wang and Waterhouse, 2000) and occurrence of somaclonal variations induced by the tissue culture process. However, the underlying mechanism responsible for the variations in transgene expression observed in flax transformation has not been investigated in detail. Southern analysis of primary transformants was not conducted in some earlier studies due to the technical difficulty (Jordan and McHughen, 1988a; McHughen, 1989). In the few publications where Southern analyses were conducted (Table 7) the correlation between transgene copy number and the level of transgene expression was not established. In a transformation of hypocotyl segments with *A. tumefaciens* using spectinomycin

as the selective agent, it was estimated that between one and six copies of the spectinomycin resistance gene were present in the different transgenic plants analyzed, but the proportion of single copy transformation events was not reported (Bretagne-Sagnard and Chupeau, 1996). Ling and Binding (1997) found that one of four transgenic plants derived from *A. tumefaciens*-mediated transformation of plastocytes had a single insert and that the remaining three plants had two inserts. In a study to evaluate putative seed-specific promoters for *L. usitatissimum*, Drexler *et al.* (2003) reported that most of the transformants had 1–3 copies of the transgene whereas some plants had up to 7 or 8 copies of transgene. In the process of evaluating the anther culture-based high throughput transformation system, approximately 800 transgenic plants from several independent experiments were examined and it was found that insert number ranged from 1 to 7 in different transgenic plants (Chen *et al.*, 2008). Approximately 77% of transgenic plants analyzed had low Southern insert number (1–3) of which about 46% of them had a single Southern insert. Based on Southern analysis, it is estimated that 84% of the plants with single Southern insert were vector backbone free (Chen *et al.*, 2008).

The proof of transmission and stable expression of transgene is the expression of the transgene in the seed progeny (Table 8). In one early study, progeny analysis of 11 transgenic chlorsulfuron-resistant plants derived from flax cv. Norlin using kanamycin as the selectable agent showed that 7 of

Table 8 Inheritance of transgene in transgenic flax

No. of plants	1 locus	2 loci	Homozygous or >2 loci	Chimera	References
11	7 (64%)	1 (9%)	0	3 (27%)	McSheffrey <i>et al.</i> , 1992
22	9 (41%)	2 (9%)	1 (5%)	10 (46%)	Dong and McHughen, 1993b
8	6 (75%)	2 (25%)	0	0	Bretagne-Sagnard and Chupeau, 1996
24	17 (71%)	3 (13%)	2 (8%)	2 (8%)	Chen and Dribnenki, 2003

them (64%) had a single effective transgene locus, 1 of them (9%) had two independent effective transgene loci, and the remaining 3 plants (27%) were chimeric (McSheffrey *et al.*, 1992). However, progeny population analyzed in this study was small (all under 35 seeds). In a subsequent study from the same laboratory using a large progeny population, progeny analysis of 22 transgenic plants derived from the same cultivar using the same selection agent showed that only 9 of them (41%) had a single effective transgene locus and 2 of them (9%) had two independent effective transgene loci. One of them (5%) had multiple transgene loci or transgenes integrated at the same locus of two homologous chromosomes and remaining 10 plants (46%) were chimeras (Dong and McHughen, 1993b). A study from a different laboratory using spectinomycin as the selectable agent showed that 6 of 8 transgenic plants (75%) had a single effective transgene locus and the other 2 plants (25%) had two independent effective transgene loci (Bretagne-Sagnard and Chupeau, 1996). In a more recent study, from a total of 24 transgenic plants derived from transformation using hygromycin as the selective agent, 17 of them (71%) had a single effective transgene locus, 3 of them (13%) had two independent effective transgene loci, 2 of them (8%) had transgenes in the homozygous state, and the remaining 2 were chimeras (Chen and Dribnenki, 2003).

Introduction and expression of a transgene could theoretically lead to diversion of energy that plants would otherwise use to drive other physiological or biochemical processes, and might therefore be capable of impacting on overall yield. However, the expression of various transgenes encoding herbicide tolerance, novel fatty acids or additional disease resistance do not appear to have any obvious phenotypic effect on plant growth and development when the transgenic flax plants are cultivated under growthroom conditions (McHughen, 1989; Qiu *et al.*, 2002; Chen *et al.*, 2008). However, the most convincing results regarding the effect of transgene on plant growth, yield, and quality would be the field evaluation of transgenic lines along with their nontransformed parental line in replicated trials in multiple geographic locations across multiple years. Field test results showed that expression of the mutant *Arabidopsis ALS* gene under the control of the native promoter in flax had

minimal effect on agronomic performance and quality traits including plant height, days to flower, time to maturity, degree of lodging, oil content and quality, 1000-seed weight, and seed yield (McHughen and Rowland, 1991). Based on the stability of transgene expression and comparable agronomic and quality attributes of transgenic line and nontransformed parental line and other check cultivars in field trials, sulfonylurea resistance line 12 115 that expressed a modified acetolactate synthase was registered as CDC Triffid (McHughen *et al.*, 1997). This cultivar was granted unrestricted environmental release in Canada and animal feed use in May 1996 (www.inspection.gc.ca/english/plaveg/pbo/dd9824e.shtml) and human food approval in February 1998 (Health Canada, 1998).

2.6 Regulatory Measures

Because the first transgenic flax cultivar was registered in Canada, the emphasis in this section is placed on regulatory process and measures adopted to assess environmental, feed, and food safety of the unconfined release of transgenic plants in Canada, with some relevant references for other countries, mainly United States. Different from the “process-based” approach taken by other countries, Canada is the only country to adopt a “product-based” approach where the regulatory oversight is triggered by the novelty of the product rather than the methods used in its production. Therefore, genetically engineered plants and genetically engineered food products are evaluated as plants with novel traits (PNTs) and novel foods, respectively. The assessment criteria are familiarity and substantial equivalence of PNTs and their products with their nontransformed counterparts that have an established history of safe use. In Canada, the regulation of PNTs and novel foods is coordinated between the Canadian Food Inspection Agency (CFIA), Health Canada, and Environment Canada. In United States, regulation of agricultural biotechnology products is shared between the Animal and Plant Health Inspection Services (APHIS) of the United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and

the FDA. The regulatory measures adopted in both Canada and United States are in concert with those developed by the Organization for Economic Cooperation and Development (OECD), the World Health Organization (WHO), and the Food and Agriculture Organization (FAO) of the United Nation (FAO/WHO 2000; OECD 2000a). Confined trials of PNTs in Canada can only be conducted after approval from CFIA and according to the guidelines in Regulatory Directive Dir 2000–2007: Guidelines for the environmental release of PNTs within confined field trials (<http://www.inspection.gc.ca/english/plaveg/bio/dir/dir0007e.shtml>). The environmental safety assessment of PNTs includes the molecular characterization of the PNT in comparison with its conventional counterpart and the environmental impact of the whole plant in comparison with its unmodified counterpart according the criteria in Seed Regulations, Part V (<http://www.inspection.gc.ca/english/plaveg/pbo/ptvreve.shtml>), “Regulatory Directive Dir 94-08: Assessment Criteria for determining environmental safety of plants with novel traits” (<http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml>) and its companion document “Regulatory Directive Dir 94-10: The biology of *L. usitatissimum* L” (<http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9410e.shtml>). Livestock feed safety assessment should be conducted according to the guidelines in “Regulatory Directive Dir 95-03: Guidelines for the assessment of livestock feeds from plants with novel traits” (<http://www.inspection.gc.ca/english/anima/feebet/dir95-03e.shtml>). In order to facilitate the submission of supporting data by developers seeking regulatory approval in both countries, Canada and United States harmonize their respective regulatory requirements for the molecular and environmental characterization of PNTs or genetically engineered plants intended for unconfined release. Details can be found in “Canada and United States 2001 Bilateral Agreement on Agricultural Biotechnology Appendix 1a-Molecular Genetic Characterization Data for Transgenic Plants Intended for Unconfined Release” (<http://www.inspection.gc.ca/english/plaveg/bio/int/appenannex1e.pdf>) and “Appendix II-Environmental Characterization Data for Transgenic Plants Intended for

Unconfined Release” (<http://www.inspection.gc.ca/english/plaveg/bio/int/appenannex2e.pdf>). Food safety assessment in Canada should be conducted according to the Guidelines for the Safety Assessment of novel foods, Volume I and Volume II (http://www.hc-sc.gc.ca/food-aliment/mh-dm/ofb-bba/nif-ani/pdf/e_novele.pdf) developed by Health Canada in concert with internationally accepted practice (OECD 2000b). Additional regulation information and related documents for both Canada and United States can be found at www.agbio.com.

3. FUTURE PROSPECTS

3.1 Expected Products

There are excellent prospects for the further improvement of flax through expected future developments in transgenic research. In relation to agronomic traits, the development of insect resistance based around the successful *Bt* technology would seem feasible and desirable. Flax is susceptible to attack by caterpillars from the *Helicoverpa* family, particularly during bud formation, flowering and capsule development. This genus of pests has been successfully controlled in cotton and corn through transgenic expression of the *Bt* insecticidal protein gene derived from *Bacillus thuringiensis* (Pray *et al.*, 2002) and the technology appears to be broadly acceptable to a wide range of plant species. Other promising agronomic traits, such as increased nitrogen use and water use efficiency (Good *et al.*, 2004; Raines, 2006) once clearly proven are likely to have valuable application in flax in improving productivity and in reducing the impact of abiotic stress.

Breeding for improved resistance to diseases is also likely to benefit from transgenic approaches in the future. The principal economic diseases of flax are flax rust (*M. lini*) and flax wilt (*F. oxysporum*). Resistance to various races of flax rust is controlled by five series of closely linked or allelic genes at loci designated K, L, M, N, and P. It is noteworthy that the first ever cloning of a plant gene for rust resistance was the flax *L6* gene (Lawrence *et al.*, 1995). The subsequent cloning of other resistance genes at the L (Ellis *et al.*, 1999),

M (Anderson *et al.*, 1997), N (Dodds *et al.*, 2001a), and P (Dodds *et al.*, 2001b) loci now enables transgenic breeding for rust resistance in flax, including the possibility of pyramiding resistance genes to multiple races through transgenic creation of additional resistance loci. To date, flax wilt has been effectively controlled in conventional breeding programs by early generation selection in heavily infected wilt nurseries. Genetic factors involved in wilt resistance have been located to particular quantitative trait loci (QTL) regions of the flax genome (Spielmeyer *et al.*, 1998) that may be useful in developing marker-assisted breeding strategies and in eventually cloning the wilt resistance genes. In the future, the development of novel transgenic disease resistance mechanisms may prove useful in flax, particularly for controlling pathogens such as *Mycosphaerella linorum* (pasmus), *Polyspora lini* (browning) and *Sclerotinia* that are currently relatively minor but for which no robust resistance mechanisms have yet been identified.

It is likely that most future transgenic research in flax will be directed toward improvements and further diversification of product quality. Flaxseed is already a source of two types of oil, the traditional high linolenic oil, and the high linoleic, low linolenic oil (Linola[®]) developed for the mass edible oil market. Although primarily used as an industrial drying oil, the traditional high linolenic oil has also found a significant and growing place in the specialty food oils market due to the increasing consumer interest in ω -3 oils. As described above, it is possible that linseed varieties will be developed in the future in which much of this ALA has been further converted to longer chain and more unsaturated ω 3 fatty acids, such as EPA and DHA, by transgenic expression of additional fatty acid desaturase and elongase genes (Abbadi *et al.*, 2004). Such highly unsaturated oils are very unstable and would be at considerable risk of oxidation damage during processing. It may be desirable therefore to genetically enhance the levels of fat-soluble antioxidants such as tocopherols and tocotrienols to protect them from breakdown. A gene for γ -tocopherol methyltransferase (γ -TMT), which converts γ -tocopherol to the more biologically active α -tocopherol form, has been cloned from *Arabidopsis* and over expressed in transgenic plants to produce substantially higher α -tocopherol levels (Shintani and DellaPenna,

1998). However, γ - and δ -tocopherols are much better antioxidants *in vitro*, and they are very important for the oxidative stability of cooking oils. Therefore it is important that any attempts at boosting α -tocopherol levels strike a balance between the different forms of tocopherols. Tocotrienols are another group of important antioxidants, mainly present in the seeds of monocots, that are derived from the condensation of homogenistic acid (HGA) and geranylgeranyl diphosphate catalyzed by HGA geranylgeranyl transferase (HGGT) (Collakova and DellaPenna, 2001). Transgenic expression of barley HGGT in *Arabidopsis* was able to increase the total amount of Vitamin E antioxidants up to 15-fold (Cahoon *et al.*, 2003). Such strategies might, therefore, be applied in the future to impart greater inherent stability on linseed oils engineered to contain EPA and DHA.

The development of low linolenic linseed oil (Linola[™]) paved the way for a wider use of flax seed oils in the food industry (Green and Dribnenki, 1994). The virtual elimination of linolenic acid greatly improved the stability of the oil and enabled it to be used in products, such as margarines and salad oils. However, the high content of linoleic acid in Linola oil prevents its use in industrial frying applications, such as in the food service and snack food industries. Such applications require oils with extremely high stability, which has been provided in the past by the more saturated plant and animal fats and oils, such as palm oil and tallow. Recently, the development of high oleic oilseeds has opened up a large market for commercial frying oils that combine high stability with improved nutrition, and it is possible that new high oleic linseed oils could contribute significantly to this food industry sector in the future. Previous attempts to raise oleic acid by mutation breeding have had only limited success, with 47% being the maximum content reported so far (Rowland and Bhatti, 1990). In contrast, transgenic gene silencing technologies targeting Δ 12-desaturase genes (*Fad2*) have proven successful in blocking the conversion of oleic acid to linoleic acid, resulting in very-high oleic acid levels (80–90%) in several oilseeds, including canola, mustard, and cottonseed (Liu *et al.*, 2002; Stoutjesdijk *et al.*, 2002). The cloning of key fatty acid Δ 9- and Δ 15-desaturases from flax (Jain *et al.*, 1999; Vrinten *et al.*, 2005)

and the deposition of the flax $\Delta 12$ -desaturase sequence in GeneBank (Acc No: DQ222824) provides the sequence information needed to design RNA interface (RNAi) silencing constructs to develop high oleic and high stearate linseed oils.

Genetic engineering of fatty acid composition in flax may also be used in the future to develop novel oils with unique industrial uses. There are many unusual fatty acids present in the seed oils of nonfood plants that would be of considerable value as raw materials for industrial chemical production if they could be produced cheaply in high-yielding oilseed crops such as flax. Most recent attention has focused on those C18 fatty acids that are modified at the $\Delta 12$ position by the addition of epoxy or hydroxy groups, or by the formation of triple bonds (acetylenic) or conjugated double bonds. The introduction of these functionalities into C18 fatty acids is catalyzed by a family of divergent forms of the fatty acid $\Delta 12$ -desaturase (FAD2) enzyme. FAD2-like genes encoding $\Delta 12$ -epoxygenases, $\Delta 12$ -hydroxylases, $\Delta 12$ -acetylenases, and $\Delta 12$ -conjugases have all been cloned (Singh *et al.*, 2005), as well as a further variant responsible for the synthesis of dimorphecolic acid (9-OH-18: $\Delta^{10trans, 12trans}$) in *Dimorphotheca sinuate* (Cahoon and Kinney, 2004). The substrates for these enzymes are either linoleic acid, which is present at high levels in the Linola[®] form of linseed, or oleic acid that can be engineered to high levels by transgenic silencing of the endogenous *Fad2* gene. Thus a variety of transgenic flax plants with oils tailored for particular industrial uses could be developed through the use of genetic engineering – oils that could possibly substitute for petroleum-derived chemicals in the future.

In addition, modification of flax fiber quality through genetic engineering approaches that modify the lignin content in fibers might also be possible in the future. For example, it has been shown in tobacco that down-regulation of the cinnamoyl CoA reductase and cinnamoyl alcohol reductase leads to a reduction in lignin content in fibers (Chabannes *et al.*, 2001). A similar approach if extended to flax could lead to a reduction in lignin levels that would potentially result in softer and stronger flax fiber.

3.2 Expected Technology Developments

3.2.1 Improved transformation efficiency

Optimization of any genetic, physiological and environmental factors relating to either DNA delivery or plant regeneration can contribute to improving transformation efficiency. For *A. tumefaciens*-mediated transformation, these may include optimization through choice of the *Agrobacterium* strain and its resident disarmed Ti plasmid, plant genotype and explant type, selectable marker and selection system, tissue culture medium, and culture conditions influencing plant regeneration. Further improvement in transformation efficiency in flax can be expected when these factors that clearly influence DNA delivery and plant regeneration are coordinately optimized. To date, no comprehensive systematic study has so far been conducted to evaluate the effects of these factors on overall transformation efficiency in flax. For example, although two strains of *Agrobacterium* were used in the experiment of Bretagne-Sagnard and Chupeau (1996) the effect of the *Agrobacterium* strain on transformation efficiency was not examined. Similarly, two flax genotypes were used in the study of Jordan and McHughen (1988a), but the effect of genotype on transformation was not reported. Different explant types, including hypocotyl segments, plastocytes, and anther cultures have been used to regenerate transgenic plants in different laboratories (Dong and McHughen, 1993a; Ling and Binding, 1997; Chen and Dribnenki, 2003). In one study, use of anther cultures as the explants had significantly higher transformation efficiency than use of hypocotyl segments as the explants (Chen *et al.*, 2008). The demonstrations of the effect of the preculture period when hypocotyl segments are used as the explants (McHughen *et al.*, 1989) and of the effect of anther age on success of anther culture (Chen and Dribnenki, 2003) are two examples in flax that show the impact of physiological conditions of the explants on transformation efficiency. One of the most critical opportunities for increasing transformation efficiency is in finding ways to promote regeneration of plants from transformed cells. Usually, the percentage of flax explants producing transgenic shoots has been much lower than the percentage of explants

showing transgene (*GUS*) positive cells, indicating that many successfully transformed explants fail to regenerate shoots. Further modification of the media in combination with a well-balanced selection regime may help to improve this aspect of transformation efficiency in the future.

3.2.2 Alternative selectable markers and production of selectable marker-free plants

The function of (positive) selectable markers is to confer the selective advantage to transformed cells over nontransformed cell and thereby facilitate the recovery of the small portion of transformed cells/plants. The selectable marker genes that have mainly been used in flax transformation have been antibiotic resistance genes, i.e., *nptII*, *hph*, and *aadA* (McHughen, 1989; Bretagne-Sagnard and Chupeau, 1996; Chen and Dribnenki, 2003), which have raised some public concerns. Though transgenic flax plants containing the *nptII* gene have been formally approved for commercial release, herbicide resistance genes may have a better chance for public acceptance of the genetically engineered products. The phosphinothricin N-acetyltransferase (*pat*) or bialaphos resistance (*bar*) genes that confer resistance to phosphinothricin have been used successfully to regenerate transgenic flax plants (McHughen and Holm, 1995a, b). However, there are no reports of using either resistance to glyphosate, by expression of a mutated form of *epsp* synthase from petunia (Jordan and McHughen, 1988a) or resistance to sulfonylurea, by expression of a mutated form of *als* gene (McHughen, 1989), as the selectable marker in flax transformation. With some optimization of the transformation protocol it should be feasible to directly use glyphosate or sulfonylurea as the agent to select herbicide tolerant transgenic plants.

Alternative selectable marker systems to antibiotic and herbicide resistance have been developed and successfully employed in some transformation systems. These include phosphomannose isomerase (Joersbo and Okkels, 1996), xylose isomerase (Haldrup *et al.*, 1998), and deoxyglucose-6-phosphatase (Kunze *et al.*, 2001). In these systems, the presence of metabolite analogues (e.g., mannose, xylose, and deoxyglucose) as the

selectable agents favors the growth of transformed cells rather than killing nontransformed cells. Transformed cells expressing the selectable marker genes are able to convert the metabolite analogs into the metabolites that can be used to support cell growth and therefore acquire growth and development advantages on medium containing the metabolite analogs. Therefore, the transformation efficiency could be higher than in antibiotic or herbicide selection schemes where substances released from dead cells could be detrimental to the growth and regeneration of transformed cells into plants (Joersbo and Okkels, 1996). However, these alternative selectable marker systems have not been evaluated in flax. Alternatively, scorable markers can be used to visually differentiate transformed cells and shoot without adding any chemicals to the regeneration medium. These types of scorable markers may include *gfp* gene from jelly fish (*Aequorea victoria*) encoding green fluorescence protein and *ipt* gene encoding for isopentenyl transferase that catalyzes cytokinin synthesis and thereby causes transgenic cell proliferation and differentiation into shoots. The expression of *gfp* enables the identification and isolation of transformed cells at earlier stage of development (Ahlandsberg *et al.*, 1999). Expression of *ipt* in transformed cells results in the regeneration of shoots with “shooty” phenotype, i.e., lack of apical dominance and roots (Endo *et al.*, 2001). Transgenic plants with normal phenotype can be recovered by utilizing the site-specific recombination R/RS system with or without inducible system to remove the *ipt* gene or restrict expression of *ipt* gene (Endo *et al.*, 2002).

In order to simplify the regulatory process and improve public acceptance of genetically engineered products, elimination of selectable marker genes, either antibiotic or herbicide resistance or scorable marker genes, and any foreign genetic elements in final products would be most desirable. Different strategies developed so far include site-specific recombination (Dale and Ow, 1991), homologous recombination (Zubko *et al.*, 2000), transposition (Goldsbrough *et al.*, 1993), and co-transformation of selectable marker gene and gene of interest as separate transgenes to enable segregation of marker-free transgenic plants in progeny generations (Depicker *et al.*, 1985; Daley *et al.*, 1998). To overcome the problem of large size binary vector encountered with two

sets of T-DNA border sequences with a single plasmid in co-transformation, a double right border (DRB) binary vector carrying two sets of right border sequences flanking a selectable marker gene followed by a gene of interest and one set of left border was developed (Lu *et al.*, 2001). Using this type of vector to transform rice, approximately 36–64% of the primary transformants produced selectable marker free progeny (Lu *et al.*, 2001). Recently we have produced over 100 transgenic flax plants using this strategy and evaluation for the presence of marker-free plants in their progeny is in progress (Chen *et al.*, 2008).

3.2.3 Improved transgene expression

The current transformation strategy is to produce sufficient number of transgenic plants in order to select transformation events that have desired level of expression of the gene of interest. This strategy is labor intensive and costly. Of the different strategies that have been proposed to minimize the variation of transgene expression in plants (Butaye *et al.*, 2005), the most feasible approach is to use methods that favor the production of transgenic plants with single copy of transgene insert, since high levels of expression of transgenic traits are reproducibly correlated with single copy of transgene (Hobbs *et al.*, 1993). *Agrobacterium*-mediated transformation generally produces a high frequency of transgenic plants with preferential integration of defined T-DNA's into transcriptionally active regions of chromosome, exclusion of vector DNA, few rearrangements, and stable expression of transgene over generations (Gelvin, 2003). In contrast, microprojectile bombardment and other direct transformation methods often result in the random integration of multiple copies of transgenes into the genome, with possible fragmentation and rearrangements of the gene (Kohli *et al.*, 1998).

All genetic, physiological and environmental factors influencing transformation efficiency can potentially affect transgene copy number. For *A. tumefaciens*-mediated transformation, these factors may include the optimization in selection of *Agrobacterium* strain and its resident disarmed Ti plasmid, plant genotype and explant type, selectable marker and selection scheme, tissue cul-

ture medium, and culture conditions influencing plant regeneration.

The strategy to generate single copy transformation events using cre/lox site-specific recombination system has been developed and successfully used in wheat transformation (Srivastava *et al.*, 1999). Design of vectors with a negative selectable marker located “left” of the left border on the binary plasmid should help to generate more transgenic plants without plasmid backbones since sequences from beyond the left border are incorporated into transgenic plants due to the occasional overriding of left border signal (Martineau *et al.*, 1994).

3.2.4 Seed-specific promoters

Identification of seed-specific promoter is crucial for any transformation projects aiming at modifying seed quality traits. Transgenic flax plants with the unknown seed protein (USP) promoter from *Vicia faba* showed persistent expression between 5 to 40 days after flowering (daf) whereas plants with another seed-specific promoter LeB4 from *Vicia faba* or seed-specific KCS promoter of β -ketoacyl-CoA synthase gene from *Brassica napus* had expression between 11 to 40 or 14–40 daf, respectively (Drexler *et al.*, 2003). The expression levels of transgene under the control of either USP or LeB4 promoter from *V. faba* were 100 to 1000 times higher than that under control of either KCS or napin promoters from *B. napus* (Drexler *et al.*, 2003). In a more recent study, conlinin promoter from flax was found to have the expression level high enough to be useful for seed modification purpose, whereas the *Arabidopsis* FAE and *B. napus* napin promoters had low levels of expression during seed development (Caillot *et al.*, 2006).

3.2.5 Transfer of multigene constructs

Transfer of multiple genes into plants is necessary for the stacking of transgenes specifying different traits and for the introduction of multiple enzymes constituting complex metabolic pathways, such as biosynthesis of LC-PUFAs. This can be achieved by sequential transformation with individual transgenes (Qi *et al.*, 2004), or by recombination through crossing of independently transformed

lines. These two strategies both require an additional generation to determine the expression level of the first transformation and to identify the desirable event for subsequent transformation or crossing. Alternatively, multiple transgenes could be cloned into the same vector under the control of independent promoters. Using this approach a binary vector containing a $\Delta 6$ -desaturase, a $\Delta 6$ -elongase, and a $\Delta 5$ -desaturase each under the control of USP promoter was successfully used to transform flax to produce long-chain $\omega 6$ and $\omega 3$ polyunsaturated fatty acids (Abbadi *et al.*, 2004). More recently, a binary vector containing nine genes under the control of various promoters was used to transform *Brassica juncea* to produce DHA (Wu *et al.*, 2005).

3.3 Risks and Concerns

An increased array of new quality types in crops in the future as a result of the application of gene technology should provide growers with enhanced flexibility in their crop production and marketing decisions. Some new products will probably represent such a significant quality improvement that they become the new standard for the commodity itself, while others will become established only as higher-value specialty types with much smaller markets than the traditional commodity. Capitalizing on the opportunities provided by this expanded range of diversified quality types presents a number of significant challenges for crop production systems and for supply chain management.

3.3.1 Maintaining variety integrity

The need for variety integrity is likely to be more important with the introduction of transgenic (genetically modified, GM) crops, particularly those with new output traits, than is currently the case with traditional crop varieties. Although strict measures are already taken during planting seed production to minimize the risks of cross contamination with other varieties, the consequences of very low admixture levels are generally not significant with traditional varieties. Within quality types of a species the main consequence is the potential for dilution in the overall agronomic performance of superior varieties,

which is probably insignificant with low levels of contamination. However, the consequences of contamination between quality types can be much greater because of the possible failure to meet end product quality specifications. With transgenic crops the consequences of contamination are much more serious, firstly because of the strict requirements for regulatory approval for release of transgenic plants and plant products, and secondly because of the much more significant genetic and compositional differences that transgenic crops will have from their traditional counterparts.

It is, therefore, particularly important to ensure that a GM variety that is being grown either experimentally in prerelease field trials, or commercially for a particular market, where it is approved for release, does not contaminate planting seed or commercial grain of other varieties that are destined for markets where the transgenic trait is not approved for release. This is an important consideration because of the significant international movement of planting seed during multiplication and because of extensive grain trade between countries under different regulatory jurisdictions. The extent of concern for this aspect will be related to the degree of contamination tolerance that is allowable in the market. During the early phases of introduction of transgenic crops regulatory approvals are generally not available in all potential markets, and there is also considerable niche marketing of GM-free products. Both circumstances currently require zero tolerance for GM contamination. As an example of the possible pitfalls, some canola growers in Europe had to destroy crops of a non-GM canola variety when DNA testing revealed that it contained plants carrying the glyphosate resistance transgene that was not approved for release in Europe. The contamination was believed to have occurred during pedigree seed production of the variety in Canada, presumably either by outcrossing with nearby Roundup Ready[®] canola varieties or by physical seed contamination during production, harvest, or subsequent seed handling. This example highlights the difficulty in guaranteeing 100% purity in agricultural systems and underlines the need for introduction of practical minimal tolerance levels in order for conventional and transgenic crops to coexist.

Initial concerns for varietal integrity have mainly centered on ensuring that transgenes that

confer traits that could have agro-ecological consequences, such as herbicide tolerance, do not transfer to or contaminate varieties that do not contain these traits. This is important because of the desire to deploy such genes in a controlled manner to minimize the risk of them transferring to other varieties or related weed species. It is also necessary to avoid the possible unintended development of genotypes that carry resistances to multiple herbicides. The regulatory approval process for release of such GM varieties may require the proponent to develop approved crop management plans that, among other requirements, address the need to contain the transgenic traits within the GM variety. In the longer term this issue will also become particularly important for GM varieties that carry output traits. In some cases, such as where both the conventional and GM varieties are destined for similar food markets, the practical consequences of contamination will be similar to those that exist between conventionally bred quality variants. However, in the case of future GM varieties producing nonfood products there may be serious adverse consequences associated with even a low level of contamination. For example, it will probably be unacceptable for edible flaxseed destined for human consumption to have even low-level contamination from flaxseed that contains, for example, a novel industrial fatty acid that may be potentially toxic to humans, or a pharmaceutical peptide that should be tightly controlled in its human applications. In these cases, it will be extremely important to maintain strict varietal integrity.

Minimizing the risk of gene transfer between industrial and food crops can be achieved more easily in highly self-pollinated crops than in open-pollinated crops. It would therefore seem more advantageous to develop specialty industrial fatty acid products in self-pollinated oilseed crops such as flax or soybean, than in outcrossed crops such as sunflower or rapeseed. However, because this may not always be possible on technical or economic grounds, scientists are already conceiving of genetic methods by which novel varieties can be prevented from intercrossing with other varieties. It may be possible to develop systems that ensure that varieties with particular attributes that require segregation are reproductively isolated from all other varieties in the same way that different plant species are

unable to interbreed. Such systems could be valuable ways to provide certainty that herbicide tolerance genes would not transfer to related weed species, and that industrial product quality traits do not transfer to food-grade varieties.

3.3.2 Identity preserved crop production

Even with the strictest attention to detail in maintaining the integrity of varietal planting seed, there is still potential for cross-contamination of grains during commercial crop production, storage, handling, and processing. For crops that have modified output traits and that therefore need to be channeled toward particular markets or specialty uses, it will be necessary to implement strict procedures for segregation and identity preservation during production and processing in order to capture the added-value associated with the particular output trait. In some markets it may be commercially advantageous to market crop products based on their non-GM status, particularly during the early stages of introduction of GM crops. In this case, the non-GM status can be regarded as a type of output quality trait, and identity preservation may be necessary in order to be able to guarantee this status to the final customer. In contrast, in most circumstances it should not be necessary to maintain segregation and identity preservation for GM varieties that carry only input traits and produce end products that are substantially equivalent to the conventional variety.

Identity preservation is simply a system of crop supply chain management that preserves the identity of the source and nature of the product. It is not a new concept in agriculture since it is already in use for the production of a number of non-GM specialty crop types. Furthermore, the seed industry routinely undertakes identity preservation in the production of varietal planting seed. This is a relatively straightforward process in situations of low volume specialty crops grown either in-house or under contract, where the owner of the product has full control of the production process, takes delivery of all of the final grain, and usually manages its passage through the primary processing stages of the supply chain. In this situation, the implementation of audit trails and quality control monitoring is relatively easy.

However, it is a much harder task for high-volume products that are widely grown and marketed openly by growers because it may be difficult to ensure that all growers comply with all the grain production and handling procedures necessary to ensure product purity. In this situation, quality testing needs to be implemented at all initial grain receival points and be completed prior to any aggregation of grain.

When the product is a grain with particular new quality attributes, there will be incentive throughout the supply chain to maintain the identity preservation in order to achieve the price premiums for the product at each stage. However, when the product does not have any different processing properties or pricing structure, such as for GM grain carrying only input traits, there is greater potential for identity preservation to fall down. This occurred when US processors and retailers had to withdraw a number of corn-based food products from sale after DNA testing revealed that they contained corn carrying the Aventis StarLink[®] insect resistance trait, which had been granted approval by regulatory authorities for nonfood use only. Aventis had introduced a stewardship program to ensure that StarLink[®] corn would only be used for industrial purposes. However, the StarLink[®] trait appears to have been found in other conventional corn varieties developed by StarLink[®]-licensed corn seed producers, thereby escaping the dedicated marketing channels. This incident demonstrates the greater difficulty in implementing fully effective identity preservation and supply segregation in situations where traits are licensed to multiple seed producers for crops that are to be sold on open markets, compared to that within closed-loop contract production and marketing systems.

The need for effective identity preservation, combined with proprietary ownership of many quality trait technologies, should lead to a significant increase in contract production and closed-loop marketing. This type of supply chain management has significant additional costs that will need to be recouped in premiums for the final product to be economically viable. The cost of identity preservation will vary depending on the precise circumstances of the crop and the range of products derived from it, the uses to which they are put, the tolerances and specification set, and the

sophistication of the distribution system. However, initial experience indicates that the cost range is generally in the order of 5–10% of the price of the grain (Buckwell *et al.*, 1998). Identity preservation will only be economically viable for products that have end market price premiums significantly in excess of this amount. This requirement will be a primary factor in determining the commercial success of new product quality types developed by gene technology, as well as in determining the long-term sustainability of the current niche markets for non-GM products that offer no other advantage.

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Sesame

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Sesame (*Sesamum indicum* L. syn. *S. orientale* L.) is one of the oldest cultivated crops known to man. It was a highly prized oilseed in the ancient world because of its resistance to drought, the ease to extract oil from seeds and the high stability of the oil (Langham and Wiemers, 2002). Archaeological remains of sesame dating back to 5500 BC have been found in the Harappa valley in the Indian subcontinent (Bedigian and Harlan, 1986). The origin of the crop has been a major subject of discussion, with proposals for an African or Indian domestication. Based on various lines of evidence including cytogenetics, biochemical composition, nuclear DNA marker comparisons, and cultural history to name a few, Bedigian (2003, 2004) has concluded that this species was first domesticated in the Indian subcontinent. From there, it spread to Africa, the Mediterranean, and the Far East, and into the Americas following trade routes. Today sesame is widely grown in China, Japan, Korea, Turkey, India, the United States, South America, and parts of Africa as an oilseed crop.

1.2 Botanical Description

1.2.1 Taxonomy and cytogenetics

The genus *Sesamum* belongs to the family *Pedaliaceae*, with about 16 genera (Nayar, 1995). A comprehensive taxonomic characterization of the species within this genus has yet to be accomplished. So far, about 35 species have been identified of which 22 are endemic to Africa and only 5 are found in Asia. There are three cytotaxonomic groups with $2n = 26$, $2n = 32$, and $2n = 64$ within the genus. Cultivated sesame along with *Sesamum alatum*, *Sesamum capense*, *Sesamum schenckii* and a few others belong to the first group. There is limited cross compatibility among the species, especially due to the differences in chromosome numbers (Nayar and Mehra, 1970; Nayar, 1995). Owing to the existence of hybridization barriers, it has been difficult to transfer desirable characteristics such as drought, pest, and disease resistance from wild relatives into *S. indicum*.

1.2.2 Growth habit

Sesame is an erect herbaceous annual crop that grows to a height of 0.4–2 m. The plants are

often highly branched, but some varieties are relatively unbranched. Stems are square with grooves. Leaves are hairy on both sides with variable shape (ovate to lanceolate) and size, and may be opposite or alternate. Pale purple to white bell-shaped flowers begin to develop at leaf axils within 6–8 weeks after planting. A single flower is produced at each leaf axil starting from the lower axils and the plant continues blooming until the uppermost flowers on the stem are open (Day, 2000a). Multiple flowering is common in varieties with opposite leaves (Oplinger *et al.*, 1990). Sesame is predominantly self-pollinated, although cross-pollination by insects is common (Pathirana, 1994). The fruit is an oblong capsule, 2–7 cm long, containing 50–100 or more seeds. The seeds are oval and may be white, yellow, red, brown, or black in color. The seeds mature 4–6 weeks after fertilization. Sesame grows indeterminately, producing new leaves, flowers, and capsules at the same time as long as the weather conditions permit. The growth cycle is completed within 70–180 days depending on the variety and growth conditions.

1.2.3 Growth conditions

Sesame is well adapted to high growth temperatures of up to 40 °C and drought. However, it requires adequate moisture during sowing and early growth stage, and at least 300–400 mm of rainfall per season for reasonable yields. Late rainfall in the season prolongs growth and increases seed loss due to shattering. The crop can grow on a range of soil but performs best on well drained, fertile medium textured soils with neutral pH. It has a very low tolerance to salinity and is highly susceptible to waterlogging.

1.3 Economic Importance

India and China are the world's largest producers of sesame, followed by Myanmar, Sudan, Uganda, Nigeria, Pakistan, Ethiopia, and Bangladesh (FAOSTAT, 2005). In 2005, the total world production was about 3.3 million tons grown on 7.4 million hectares. Sesame currently ranks sixth in the world production of edible oil seeds (Table 1) and twelfth for vegetable oil produced (Table 2).

Table 1 World production of major oil seeds in 2005^(a)

Oil crops	Production (million tons)
Soybean	214 347 289
Rapeseed	47 161 378
Cotton seed	41 132 246
Groundnut (in shell)	35 865 389
Sunflower	30 944 162
Sesame	3 325 679
Linseed	2 801 277
Castor bean	1 393 812
Tallowtree	870 000
Safflower	773 427
Mustard	621 023

^(a)Source: <http://faostat.fao.org/>, FAOSTAT (2005). Last updated September 2006

The low ranking of sesame in the vegetable oil market may be attributed to several factors including low seed yield, difficulty to mechanize harvesting, and strong competition from more cheaply produced vegetable oils, such as palm and coconut oil. In addition, there has been limited breeding effort, largely due to the fact that it is mainly produced in resource-poor developing countries that cannot sustain long-term improvement programs for the crop. The establishment of sesame as a major crop has been extremely slow considering its long history. The use of modern plant breeding knowledge and new technologies could benefit research aimed at improving the crop (Ram *et al.*, 1990).

Table 2 World production of vegetable oils in 2003–2004^(a)

Oil	Production (million tons)
Soybean	30 014 000
Palm	28 158 800
Rapeseed	12 566 900
Sunflower	9 949 790
Groundnut	5 900 250
Cottonseed	3 547 180
Coconut	3 325 890
Palm Kernel	3 262 010
Olive	2 982 510
Maize	2 083 850
Rice bran	1 407 700
Sesame	1 067 180
Other crops	5 194 961

^(a)Source: <http://faostat.fao.org/>, FAOSTAT (2005). Last updated September 2006

1.3.1 Uses of sesame

Sesame is grown primarily for its nutritious seeds that are rich in linoleic acid, protein, and calcium as well as vitamin E, and small quantities of vitamins A, B1, and B2. Nearly 70% of the world's sesame seed is processed into oil and meal while the remainder is channeled to food and confectionery industries (Morris, 2002). The oil is mainly used in cooking and salad, and for making margarine. It is also used in cosmetics preparations, pharmaceutical products, paints, soaps, and insecticides (Ashri, 1989, 1998). The meal left after oil extraction contains 35–50% protein and makes a rich feed for poultry and livestock.

Sesame seed is used on bread, buns, cookies, health snacks, and as an additive to breakfast cereal mixes. The seeds may be eaten whole either raw or roasted and salted, or mixed with lemon and honey, but are often ground into paste (Tahini), which may also be sweetened with sugar (Halva). Sesame seed is used as an ingredient in many recipes, added whole or pounded. In Africa, the paste is used as a spread and in preparing soups and sauces.

1.3.2 Economic and nutritional attributes of sesame

Sesame has a relatively superior oil quantity as well as quality in comparison to many major oil crops. The oil content ranges from 34% to 60% but is mostly about 50% of seed weight (Ashri, 1989, 1998). Values of up to 63.2% have been reported in some varieties (Baydar *et al.*, 1999; Uzun *et al.*, 2002). Both genetic and environmental factors influence the oil content in sesame. Late maturing cultivars are reported to have higher oil content than early ones (Yermanos *et al.*, 1972). Uzun *et al.* (2002) observed that indeterminate cultivars accumulated more oil than determinate ones. Variation also occurs between capsules at different positions on the same plant, such that seeds from the basal capsules on the main stem contain more oil than those located toward the apex and on side branches (Mosjididis and Yermanos, 1985; Muthuswamy and Thangavelu, 1993). Black seeded cultivars often have lower oil content than brown or white seeded ones, indicating a possible linkage between oil content

and seed coat color. Kamal-Eldin and Appelqvist (1994a) have attributed the low oil content in black seeded sesame to a high amount of crude fiber in the seed coats. Black seed coats are usually thicker than lighter colored ones.

The *Sesame* genus has limited variability in the seed fatty acid proportions (Kamal-Eldin *et al.*, 1992). The variation in the seed fatty acid composition is mainly with the oleic and linoleic acids among the different cultivars of sesame worldwide (Yermanos *et al.*, 1972; Brar, 1982; Baydar *et al.*, 1999). The oil contains four major fatty acids namely, palmitic, stearic, oleic, and linoleic acids, along with small quantities of vaccenic, linolenic, arachidic, behenic, and eicosenoic acids (Weiss, 1983; Kamal-Eldin *et al.*, 1992; Ashri, 1998; Were *et al.*, 2001). Oleic and linoleic acids occur in nearly equal amounts, constituting about 85% of the total fatty acids.

Cultivars with exceptionally high (>60%) oleic or linoleic acid are rare (Baydar *et al.*, 1999). Uzun *et al.* (2002) found differences in stearic, oleic, and linoleic acids between determinate and indeterminate cultivars. Determinate cultivars generally have higher stearic and oleic acids, and lower linoleic acid compared to indeterminate ones. Capsule position on the plant also affects the relative quantities of the fatty acids; palmitic, stearic, and oleic acids tend to increase up the stem while linoleic acid decreases (Brar, 1977). The fatty acid composition is strongly influenced by environmental factors. Linoleic acid content has been reported to increase under cool growing conditions (Uzun *et al.*, 2002).

The fatty acid composition is a major determinant of edible oil quality. Oils having high oleic acid content, in combination with low quantities of saturated and polyunsaturated fatty acids (PUFAs) are commercially and nutritionally desirable. Saturated fatty acids are associated with high risk of heart disease (Jakobsen *et al.*, 2004). Whereas PUFAs are known to be beneficial for human health, high PUFA quantity in edible oil is undesirable as they are readily oxidized yielding products that are potentially harmful to human health, and which give off-flavors and odors to foods. Sesame oil has a low level of saturated fatty acids ($\leq 15\%$) and approximately equal quantities of mono- and polyunsaturated fatty acids. The oil is nutritionally valuable as a source of linoleic acid that is essential to man.

Despite having a high content of linoleic acid, sesame oil is unusually stable to oxidation compared to other vegetable oils with a similar fatty acid composition. This feature is attributed to antioxidant activities of sesamol and sesaminol together with tocopherols present in the oil (Kamal-Eldin and Appelqvist, 1994b; Suja *et al.*, 2005). A combination of the high stability and a nutritionally acceptable fatty acid composition contributes significantly to the excellent oil quality, making it a high-value edible oil.

Recent studies have shown that sesame oil is beneficial in lowering cholesterol levels and hypertension (Sankar *et al.*, 2004; Frank, 2005), and reducing the incidence of certain cancers (Miyahara *et al.*, 2001). These health enhancing effects of sesame oil are explained by the low level of saturated fatty acids as well as the activity of antioxidants mainly sesamin. Sesamin is known to enhance the availability and functioning of vitamin E (tocopherol) (Ghafoorunissa *et al.*, 2004). An elevated concentration of γ -tocopherol in the blood is associated with reduced risk of heart disease and some cancers, such as those of the upper gut. Thus, sesame oil could be beneficial for enhancing health by improving the vitamin E levels in the body (Frank, 2005).

1.4 Traditional Breeding

1.4.1 Breeding objectives

Although sesame is one of the most important oil crops in the world, it has received less breeding efforts (Ashri, 1987). Its commercial production and extension is constrained by low yields, pests and diseases, shattering of seeds from mature capsules, and lack of uniform maturity of capsules (Langham and Wiemers, 2002). Other breeding challenges include bitter flavor or principles that limit market for whole seed (Oplinger *et al.*, 1990) and drought tolerance since sesame is generally grown in areas with rainfall of less than 600 mm per annum.

The major breeding objectives of sesame were summarized by Ashri (1987), as shown below:

- Improved seed retention in the capsules.
- High seed yield.
- Resistance to diseases and pests.

- Uniformity in plant type and agronomic characteristics including uniform capsule maturity.
- Low amount of bitter principle in seeds.
- High number of capsules/leaf axil.
- Good adaptation to varying environments (e.g., drought resistance).

1.4.2 Breeding strategies and tools

Sesame breeding efforts have in the past utilized naturally occurring variations and induced variations through intra- and inter-specific hybridization or mutations (Lee and Choi, 1985; Pathirana, 1992; Dharmalingam and Ramanathan, 1993; Prabakaran *et al.*, 1995).

Sesame shows great range of natural genetic variation in many of its characteristics (Ashri, 1989) including days to flowering, plant height, branching, corolla color, number of flowers per leaf axil, capsule length and shape, number of carpels, capsule hairiness, seed size and length, early maturity, resistance to drought, diseases and pests, shattering resistance, and oil content. The presence of a number of such easily recognizable simply inherited characters makes it possible to identify crossed progenies and may speed up crop improvement (Weiss, 1983).

Controlled crossing is simple and each cross can produce 50 or more seeds making it easy to breed for improved varieties. Sesame is considered as a self-pollinating crop (Weiss, 1983) although varying amounts of natural outcrossing ranging from less than 10% (Pathirana, 1994) to over 50% (Van Rheenen, 1968; Uzo, 1977) have been reported depending on local conditions and insect populations. Thus traditionally an isolation distance of 180–360 m has been used to maintain seed purity (Weiss, 1983).

1.4.3 Breeding for diseases/pests resistance

Sesame is affected by a number of diseases including angular leaf spot (*Cercospora sesamicola*), white leaf spot (*Cercospora sesame*) (Nyanapah *et al.*, 1995), *Alternaria* leaf spot (*Alternaria sesami*) (Ojiambo *et al.*, 1999), stem rots, powdery mildew (*Oidium* spp.), bacterial diseases (*Pseudomonas sesami*) (Weiss, 1983; Oplinger *et al.*, 1990), and wilts (*Fusarium* spp.).

Although most of these diseases are controlled by cultural practices and fungicides (Oplinger *et al.*, 1990), some of them have been managed through resistance breeding. This is possible since natural genetic variation for resistance exists for some of the diseases. For example, Nyanapah *et al.* (1995) observed variation in resistance to angular leaf spot (*C. sesamicola*) and white leaf spot (*C. sesame*) among Kenyan cultivars. Cultivars SIK 031 and SIK 013 were resistant to white leaf spot, while SIK 031 and SPS 045 were resistant to angular leaf spot (Nyanapah *et al.*, 1995). Breeding programs that incorporate some of these germplasm can produce high yielding cultivars even in areas where these pathogens are a constraint.

Using natural variation, selection breeding has been partially successful in producing varieties that are resistant to bacterial leaf spot (*P. sesami*), *Fusarium* wilt and phyllody (Weiss, 1983). At present there are no totally resistant cultivars to these major diseases. Resistance to *Sclerotinia*, *Fusarium* (El-Shazly *et al.*, 1999) and *Rhizoctonia* (Serry, 1976) exists but these are not yet incorporated into commercial varieties. Phyllody caused by mycoplasma-like-organism (MLO) transmitted by *Orosius albicinctus* is one of the most destructive diseases of sesame in India and Burma (Nakashima and Murata, 1993).

The major damaging pests of sesame include aphids, spider mites, white flies (*Bemisia argentifolii*), jassids, sesame flea beetle (*Alocypha bimaculata*), leafroller caterpillar (*Antigastra cata-launalis*), and army worms (*Cupis unipuncta*) (Langham and Wiemers, 2002). Most of these insect-pests may be controlled by chemical sprays; however, in sesame this is not economical. There are some lines of sesame varying from tolerance/resistance to extreme susceptibility to green peach aphid (*Myzus persicae*) and cabbage lopper (*Pieris rapae*) but resistance/tolerance to these insects are yet to be incorporated into commercial varieties. However, some of the wild species have been found to have resistance to root rot and *Antigastra* spp. *Sesamum radiatum*, *Sesamum laciniatum* and *S. alatum* show resistance to *Antigastra*. *Sesamum malabaricum* is also resistant to this pest. Under the All India Coordinated Research Project on Sesame and Niger, several workers have attempted wide crossing of *S. indicum* with wild *Sesamum* species

and successful interspecific derivatives have been generated (Prabakaran, 1996).

1.4.4 Breeding for resistance to shattering

High harvest losses due to seed shattering in dehiscent varieties continue to limit domestic production of sesame in many countries. According to Langham and Wiemers (2002), as much as 50% of the seeds could be lost due to shattering in certain environments. In most indeterminate sesame cultivars, there is a continuum from mature seed to immature seed on the plants. For these cultivars, early capsules dry, open, and lose seeds before or during harvesting.

Shattering makes harvesting operation difficult and costly, since the cost of manual harvesting constitutes about 70% of the total cost (Khidir, 1972). Production is constrained by shortage of labor during critical harvesting period, since 99% of sesame is harvested manually (Langham and Wiemers, 2002).

Mutants with indehiscent character (shattering resistance) have been developed from mutation breeding using γ -ray irradiation (Çağırhan, 1996, 2001). Unfortunately this so-called *closed capsule* or *idid* genotype is associated with undesirable properties such as semi-sterility, cupped leaves, twisted stem, short capsules, and low yields (Langham and Wiemers, 2002). In addition, plant lines with the indehiscent character are hard to thresh (Day, 2000b). The indehiscence is governed by a single recessive gene and Uzun *et al.* (2003) have developed a molecular marker that is linked to the *closed capsule* mutant trait. This marker allows the easy identification of the indehiscent trait in breeding programs to integrate it into desired genetic backgrounds.

Through intensive breeding program by the Sesaco Company, USA, shattering resistant types free of the undesirable traits have been obtained (Langham and Wiemer, 2002). Shattering resistant mutants that are indehiscent have been extensively used by breeders in the United States to produce high yielding varieties with as low as 5% shattering. Such varieties are suitable for mechanized production of sesame. Mechanical harvesting of sesame reduces the labor cost, and is preferred for increased acreage

(Langham and Wiemer, 2002) and seed yield per acre.

1.4.5 Breeding for increased seed yield

The major limitation to sesame growing/production is the highly variable and low seed yield. The world average is only about 340 kg ha⁻¹ compared to 2250 kg ha⁻¹ obtained under good crop management (Brigham, 1985, 1987). One of the major contributing factors to low yields in sesame is low breeding attention (Pathirana, 1995) and lack of specific research on yield structure as a basis for progress in sesame breeding (Baydar, 2005).

Approaches used for increased seed yield vary greatly from simple plant selection (Kinman and Martin, 1954) to hybrid cultivar development (Quijada and Layrisse, 1995). They have been selecting plants with extra numbers of capsules per axil, extra number of carpels per capsule, and moderate branching habit. The overall strategies in breeding for this trait have largely involved introductions accompanied by mass or single plant selections (Pathirana, 1995; Baydar, 2005), pedigree or hybridization programs to generate new variation and/or combine specific traits, particularly when genetic variation for yield is exhausted within local populations due to continuous selection (Ashri, 1989). While selecting segregating populations, Pathirana (1995) found bulk breeding method to give quick results.

Heterosis has been reported for sesame for several agronomic traits including seed yield (Mishra and Sikarvar, 2001; Sankar and Kumar, 2001; Duhoon, 2004). Several sesame hybrids showing significant heterosis for seed yields of up to 500% have recently been identified (Uzun *et al.*, 2004). This observation is an indication that there is a bright future for hybrid sesame. In addition, the mutation breeding program of IAEA for sesame has also generated fruitful results with the development of higher yielding lines (www.JointFAO-IAEA-mutation.htm; Das *et al.*, 2003; Jain, 2005). Some advanced mutant lines were evaluated in regional and national yield trials, and the mutant-derived variety “Pungsankkae” was officially released in the Republic of Korea by crossing a local variety with the determinate mutant “dr-45” from Israel.

1.4.6 Breeding for high oil content and altered fatty acid composition

Sesame is an important source of edible oil whose demand is increasing due to the high quality of its oil. However, oil content is one of the most variable characteristics in sesame. The seed contains 34–63% oil (Yermanos *et al.*, 1972; Ram *et al.*, 1990; Ashri, 1998; Baydar *et al.*, 1999). Recently, Were *et al.* (2006a) studied the oil content of East African sesame accessions and reported a variation of between 28.7% and 51%. The wide variation in oil content is important and indicates that improvement of this character is feasible.

Variation in fatty acid composition has also been reported. For example, variation in oleic acid ranges from 32.7% to 58.2% (Yermanos *et al.*, 1972) and that of linoleic acid from 27.3% to 59.0% (Yermanos *et al.*, 1972). According to Were *et al.* (2006a) variation in oleic acid content of East African sesame ranges from 31.6% to 42%, while in linoleic acid is 42.9–54.0%. The improvement in oil composition has been achieved through conventional breeding, mainly selection of single plants or mass selection (Weiss, 1983; Pathirana, 1995). Owing to great genetic variation observed in oil content and fatty acid composition, conventional breeding made great strides in producing sesame varieties high in oil content.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional breeding has been successfully used in the past to produce many sesame cultivars. It has been used to produce shattering resistant, drought tolerant, high yielding, disease, and pest resistant cultivars/varieties (Ashri, 1998; Langham and Wiemer, 2002). However, the process of producing cultivars is sometimes slow owing to linkage drag or pleiotropic gene effects (Langham and Wiemer, 2002). For example, the breeding for shattering resistant cultivars took a long time owing to association of the desirable genes with undesirable ones.

Secondly, conventional breeding depends on the genetic relatedness between species and cultivars to allow gene transfer. Interspecific hybridization set an upper limit for gene transfer among species,

which is not the case with modern breeding tools.

Conventional breeding depends largely on the natural genetic variation and hybridization, on induced mutation to create new sources of genetic variation. Modern techniques, such as tissue culture and genetic transformation with transgenes, have more scope and could result in a faster rate of progress for sesame improvement. In addition, adopting transgenic breeding and transforming sesame with novel genes could allow for varieties to be developed that produce specialty oil. There are numerous examples from other crops where transformation with desired genes have radically changed the oil composition and in a much faster fashion than conventional breeding (Drexler *et al.*, 2003).

Owing to these limitations of conventional breeding in sesame, new approaches of molecular biology and cell culture are now increasingly being considered for sesame improvement.

2. DEVELOPMENT OF TRANSGENIC SESAME

A number of traits in sesame, such as low grain yield, sensitivity to certain environmental stresses, and insufficient diversity in oil composition, necessitate genetic improvement. Some of the desirable traits, for example, disease and pest resistance, are present in wild relatives but cannot be introgressed into the crop because of interspecific hybridization barriers. Establishment of an efficient method of gene transfer, which circumvents taxonomic barriers, would speed up the development of new varieties facilitating the use of both conventional breeding and biotechnology.

2.1 Methods Employed

A variety of techniques for cell, tissue, and organ culture including embryo rescue, micropropagation, and regeneration that would be useful in sesame improvement, have been explored (Ashri, 1998). Although much effort is put into developing tissue culture systems for the crop, it has proved to be very problematic to regenerate. Regeneration

through somatic embryogenesis has been achieved from zygotic embryos (Ram *et al.*, 1990) and seedling-derived callus (Mary and Jayabalan, 1997; Xu *et al.*, 1997) but with low efficiency in callus cultures. There are various reports on adventitious shoot regeneration through a callus phase from hypocotyl and cotyledon explants (George *et al.*, 1987; Rao and Vaidyanath, 1997; Taşkin and Turgut, 1997; Kim, 2001), with frequencies ranging between 4.5% and 25.8%. In some cases, shoot formation was not achieved (Batra *et al.*, 1991; Ganesh *et al.*, 1993). A much improved regeneration protocol from seedling cotyledon explants was recently reported (Were *et al.*, 2006b). This study showed the importance of a combined usage of the growth regulators thidiazuron (TDZ) and indole-3-acetic acid (IAA). The protocol gave a regeneration frequency of 63% and 4.4 shoots per regenerating explants.

The availability of an efficient regeneration protocol is a key requirement for developing a transformation method for any given plant species. The prevailing problem of low generation potential portrays one of the major obstacles to the application of genetic transformation to this crop. So far, the use of genetic engineering for the improvement of sesame has been limited by the lack of a transformation system. Taşkin *et al.* (1999) have demonstrated that sesame is susceptible to transformation by *Agrobacterium tumefaciens* but failed to regenerate transgenic plants from callus. Transgenic sesame (*Sesamum schinzianum* ASCH.) was produced via *Agrobacterium*-mediated transfection of a carrot calmodulin gene, *cam-4*, which was specifically expressed upon the contact of carrot cells with oligogalacturonide elicitor (Mitsuma *et al.*, 2004). *A. tumefaciens* 4404 was transformed with the constructed vector, and the crown gall tissues formed in the sesame seedlings were transferred onto appropriate media to obtain redifferentiated plants. Real-time polymerase chain reaction (RT-PCR) followed by Southern blot analysis revealed that *cam-4* gene was appreciably expressed in the transgenic plants. Transformation by *Agrobacterium rhizogenes* has been used for the production of novel compounds in sesame roots (Jin *et al.*, 2005). The antimicrobial compound, 2-isopropenylnaphthazarin-2,3-epoxide, was isolated from hairy roots of *S. indicum* by Ogaswara *et al.* (1993) established by transformation with

A. rhizogenes ATCC 15834. The content of the compound in the roots was over 50 times than that found in the mother plants. Furthermore, two new anthraquinones, 2(4-methyl-1,3-pentadienyl) anthraquinone and 2(4-methyl-3-pentenyl) anthraquinone, were also produced. However, to-date there is no report on regeneration of transgenic plants from transformed sesame roots.

Various approaches to floral transformation have been applied on sesame. The pollen tube pathway of transformation which had been reported to be irreproducible (Shou *et al.*, 2002), was recently used to produce transgenic disease-resistant sesame lines transformed with genes obtained from wild *Sesamum* species (Gao *et al.*, 2004). Were (2006) demonstrated transformation of sesame with reporter (enhanced green fluorescent protein, EGFP) and marker (neomycin phosphotransferase II) genes by *A. tumefaciens* using three different approaches, namely, floral dip, *Agrobacterium* drop suspension, and pollen infiltration. Overall transformation frequencies ranging between 3.4% and 50% were achieved depending on the method used. *Agrobacterium* drop suspension method involving controlled pollination of the flowers after inoculation with the bacteria carrying the genes of interest resulted in the highest number of transformants and gave similar transformation rate to that obtained with the pollen infiltration approach. However, low seed yield was observed in the case of pollen infiltration.

2.2 Donor Gene, Promoters, Selection of Transformed Tissue, and Regeneration of Whole Plant

As much work has not been done on genetic engineering of sesame, not much information is available about the source of transgenes used and the promoters. The available reports have used cauliflower mosaic virus (CaMV) 35S promoter for transformation (Taşkin *et al.*, 1999; Were, 2006).

Incorporation of antibiotic resistance genes in commercial transgenic plants has been a major area of controversy. Keeping in mind the fact that sesame oil as well as seeds are used for edible purposes, it would be worthwhile to go for the recently developed strategies in sesame

bioengineering that result in marker-free plants. Schaart *et al.* (2004) reported a system for production of marker-free transgenic plants that combines an inducible site-specific recombinase for precise elimination of undesired, introduced DNA sequences with a bifunctional selectable marker gene used for initial positive selection of transgenic tissue and subsequent negative selection for fully marker-free plants. Rommens *et al.* (2004) also described the development of a transient selection method to generate marker-free transgenic plants in which replacement of *Agrobacterium* T-DNA (transfer DNA) by a plant DNA (P-DNA) fragment was carried out, thereby achieving a native DNA transformation approach. This method has been derived from the fact that co-transfer of two different DNA molecules from *Agrobacterium* to a single plant cell nucleus is not necessarily followed by the co-integration of both. There are no reports on the activity and stability of inheritance of the gene and adverse effects on growth, yield and quality.

A variety of selection agents including antibiotics and herbicides may be used to screen for transformed tissues and whole plants. Different concentrations of kanamycin, hygromycin and phosphinothricin have been tested by B. A. Were and co-workers to determine the minimum levels required for *in vitro* selection of transformed callus and seedlings. On the basis of their findings the following concentrations may be applied:

	Callus	Seedlings
Kanamycin (mg l ⁻¹)	50.0	75.0
Hygromycin (mg l ⁻¹)	25.0	10.0
Phosphinothricin (mg l ⁻¹)	0.5	5.0

It is worth noting that sesame seedlings confirmed to be transformed with neomycin phosphotransferase II gene, which confers resistance to kanamycin, failed to develop lateral roots upon selection on kanamycin and could hardly get established in the soil. Hygromycin and phosphinothricin appeared to be more efficient than kanamycin but have not been tested on genetically transformed sesame.

For greenhouse selection for herbicide resistance using Basta, a 0.5% (w/v) solution was found to be the minimal concentration that completely kills

wild type sesame seedlings when sprayed with the herbicide two weeks after germination (B. A. Were and co-workers, unpublished results)

2.3 Specific Regulatory Measures Adopted

Since no genetically transformed sesame has been released commercially, there is no information available about any specific regulatory measure for sesame.

3. FUTURE ROAD MAP

For a crop to continue to develop and grow in importance, it is of great importance that new and improved varieties can be provided to the market regularly. This is also true for sesame and that the new varieties can meet the different kind of demands from the growers to the consumers of sesame products. Optimized transformation protocols for sesame, therefore, are important tools as they will allow for the use of genetic engineering to introduce new and improved traits in sesame lines. Such optimized protocols for routine transformation of sesame are not available at this stage. However, based on the results that already have been achieved in the area (see Section 2) there are reasons to believe that a breakthrough in this aspect is not far away. It is therefore important to consider the traits that can be improved in sesame using transgenic methods and what risks and concerns should be envisaged. Much can be learned hopefully from the experiences of the transgenic crops already developed.

In the following sections, a number of breeding objectives and expected products, therefore, have been suggested as potential targets in sesame and for which genetic engineering will be of importance. However, there are possibly many other objectives, for which the same techniques may facilitate improvements, when and if transformation protocols are available for sesame. Therefore, the suggested list of targets to be improved in sesame should not be considered complete.

3.1 Expected Products

In Section 1.4.1, a number of breeding objectives for sesame have been listed. Of those, improved agronomic characteristics, such as resistance to diseases and pests as well as shattering resistance and determinate growth, are important targets for which genetic engineering can facilitate improvements. Another area of significance is improvement of the nutritional qualities of the seed products, such as diversified oil composition, decreased allergenicity or the use of sesame lignans as dietary supplements.

3.1.1 Improved disease resistance

As discussed before, sesame crop bears heavy losses due to various diseases caused by a number of bacterial and fungal pests. Fungal diseases include charcoal rot, *Fusarium* rot and *Alternaria* leaf spot. Bacterial diseases include bacterial blight and seedling blight. Transgenic approach can be used to incorporate resistance in sesame against these diseases.

More recently, transgenic plants have been produced that are resistant to a wide variety of bacterial diseases. These forms of resistance follow a number of strategies, including the use of genes for bacterial toxin tolerance, antimicrobial peptides, and other defense related proteins that tend to act as bactericidal compounds (Tripathi *et al.*, 2004). Li *et al.* (2001) have reported disease resistance, to both a fungal and a bacterial pathogen, conferred by expression of Myp30, an analog of magainin, an antimicrobial peptide, in transgenic tobacco (*Nicotiana tabacum* var. Petit Havana). Another analog MSI-99, when expressed in tobacco via chloroplast transformation conferred both *in vitro* and *in planta* resistance to plant pathogenic bacteria and fungi (De Gray *et al.*, 2001). Similarly, cecropins (antibacterial lytic peptides) are considered as potential candidates to protect plants against bacterial pathogens. It has been used to confer resistance against many bacterial pathogens using transgenic approach in tobacco (Huang *et al.*, 1997), potato (Arce *et al.*, 1999), apple (Norelli *et al.*, 1998), and poplar (Mentag *et al.*, 2003). Transgenic apple expressing attacin, another

antibacterial protein, targeted to the intercellular space, where *Erwinia amylovora* multiplies before infection, has significantly reduced fire blight, even in apple plants with low attacin production levels (Ko *et al.*, 2000). Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Recombinant-DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant (Keen, 1999) or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms (Bent and Yu, 1999; Rommens and Kishmore, 2000). The *bs2* resistance gene of pepper specifically recognizes and confers resistance to strains of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) that contain the corresponding bacterial avirulence gene, *avrBs2* (Tai *et al.*, 1999). Transgenic tomato plants expressing the pepper *bs2* gene suppress the growth of *Xcv*.

The production of enzymes capable of degrading cell walls of invading phytopathogenic fungi is an important component of the defense response of plants. The timing of this natural host defense mechanism has been modified to produce fungus-resistant plants. Transgenic tobacco seedlings constitutively expressing a bean chitinase gene under control of the CaMV 35S promoter showed an increased ability to survive in soil infested with the fungal pathogen *Rhizoctonia solani* and delayed development of disease symptoms (Roby *et al.*, 1990). A similar approach can work for engineering resistance to bacterial and fungal pathogens in sesame.

3.1.2 Improved pest resistance

Sesame has a serious problem of pest infestation. Resistance to these pests belonging to Lepidoptera, Diptera, and Coleoptera can be engineered into this crop through genetic engineering (Estruch *et al.*, 1997; Gatehouse and Gatehouse, 1998; Babu *et al.*, 2003). *Bacillus thuringiensis* (*Bt*) produces one or more insecticidal crystal proteins (ICPs or Cry proteins) during sporulation (Höfte and Whiteley, 1989). The Cry family of *Bt* insecticidal proteins is widely utilized in classic and transgenic approaches to control insect pests.

Also, enzyme inhibitors, proteinase inhibitors, α -amylase inhibitors, lectins, and enzymes have been applied or tried (Gatehouse *et al.*, 1997; Lee *et al.*, 1999; Haq *et al.*, 2004; Sharma *et al.*, 2004; Vila *et al.*, 2005). Similar strategies can be used to transfer these traits into sesame.

3.1.3 Improved seed retention

The shattering is considered as a domestication trait that has been modified years ago in most major crops. The progress from sequencing of the *Arabidopsis* genome, extensive molecular and genetic studies, and the detailed investigations of the seed pod and the mechanism behind seed dispersal have provided new insight into the events leading to pod shattering and release of seeds. Today, a number of genes involved in the transcriptional network controlling seed dispersal in *Arabidopsis* have been identified and characterized (Lewis *et al.*, 2006). The accumulated knowledge is now available and there are some examples in how this has been used in a transgenic approach of improving seed retention in various crops. Expressing the *Arabidopsis* *FRUITFULL* gene in *Brassica juncea* resulted in shattering resistant pods (Ostergaard *et al.*, 2006). In another example, expression in rapeseed of a MADS box gene *MADSB* from mustard, resulted in siliques with a closed valve-replum boundary that prevented seed release (Chandler *et al.*, 2005). Major loci controlling shattering in American wild rice (*Zizania palustris* var. *interior* L.) have been discovered and molecular markers that will allow for more efficient breeding for shattering resistant varieties are being developed in (Kennard *et al.*, 2002). In addition, individual genes for shattering in rice have been mapped to a specific cluster on chromosome 7 from where they now can be isolated (Ji *et al.*, 2006). The authors propose that this cluster can be referred to as a domestication block since several genes involved in crop domestication are closely linked to this region of chromosome 7. A strategy for developing shattering resistance in oilseed rape that were set up by Ulvskov (1996) could perhaps serve as a model approach for how to improve seed retention in sesame. Suppressing the expression of the gene encoding for polygalacturonase responsible for degradation of pectin in the middle lamella in the pod dehiscence zone would lead to

Pods lacking the possibility to open up. By fine tuning the suppression of the genes could lead to sesame capsules that would not shatter prior to harvest but at harvest.

3.1.4 Determinate flowering

The indeterminate growth habit of sesame causes non-uniform ripening of the capsules. As a solution to this problem, breeding for a determinate growth habit in sesame has become an active area of research after the discovery of dt-45, which was the first determinate mutant in sesame (Ashri, 1981). The molecular characterization of this mutant can throw some light on the genes in sesame involved in the determinate flowering trait. Thereafter, this trait can be bred into any sesame germplasm using the transgenic approach or marker-assisted selection.

Further exploration of this area could be aided by the recent advances in the understanding of the developmental transition in plants, from vegetative to reproductive growth (Blazquez *et al.*, 2006). When a plant “decides” to flower, this is influenced by different factors such like day length, light regime, and temperature. In *Arabidopsis* this is known to be regulated through four different pathways that act on a key repressor, the *FLOWERING LOCUS C (FLC)* (Finnegan *et al.*, 2005; He and Amasino, 2005). *FLC* controls several downstream genes that in turn activate genes responsible for the production of flower primordia. The growing understanding of how the complex regulation of the *FLC* is coordinated holds great promises for using genetic engineering and fine tuning the initiation of flowering in sesame. Recently, three genes from Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) and homologs of the *Arabidopsis FLC*, were expressed in *Arabidopsis* and Chinese cabbage (Kim *et al.*, 2006a). The flowering time was significantly delayed in *Arabidopsis* as well as in the transgenic Chinese cabbage, which clearly shows the potential in modifying the onset of flowering. Other genes have been identified that potentially could be used to control the termination of flowering, for example, *TERMINAL FLOWER1* and *2 (TFL1, TFL2)* (Larsson *et al.*, 1998; Foucher *et al.*, 2003; Kotake *et al.*, 2003). Out of the accumulated knowledge about how the transition

from juvenile to adult vegetative development or from vegetative to reproductive growth is controlled, a model has emerged that involves small interfering RNA (siRNA) that works in *trans* (Baurle and Dean, 2006). If this holds true it could open up for using transgenic RNA silencing techniques to develop sesame varieties with determinate flowering. Interestingly, the mobile trigger for flowering known since long as “florigen” (Colasanti and Sundaresan, 2000), was recently identified to be a protein (*Flowering locus T* or *FT*) that is produced in the leaves and moves in the phloem to the shoot apex where it induces flowering (Abe *et al.*, 2005; Wigge *et al.*, 2005; Corbesier *et al.*, 2007; Tamaki *et al.*, 2007).

3.1.5 Herbicide tolerance

Early season weeds significantly reduce sesame yields. After planting, sesame is not highly competitive with weeds (Sharma, 1985). Annual weeds create a shade canopy and intercept sunlight above the crop. Even moderate infestation of annual weeds reduces sesame seed yields up to 135% (Grichar *et al.*, 2001a). As much as 10 000 acres of sesame have been discarded in some years because of weed infestations. Sesame typically grows 0.9–1.8 m tall, which creates a shade canopy and, due to its height advantage, suppresses late-season weeds (Smith *et al.*, 2000). Tillage, manual weeding or herbicide application is practised to control weeds in sesame. Recently several soil-applied herbicides as well as various postemergence herbicides were tested for sesame tolerance (Grichar *et al.*, 2001a, b). Due to yield reductions from damage inflicted by all broad leaf herbicides, they recommended soil-applied herbicides for broad leaf weed control. Introducing herbicide tolerance through the transgenic approach could be an efficient way of controlling weeds in sesame cultivation and subsequently increasing the seed yields. Herbicide tolerance would be specifically important in the early phase of establishing the sesame plants and particularly for controlling broad leaf weeds.

3.1.6 Diversified oil composition

Sesame is known to be very rich in genetic variability. However, while there is quite a bit

of variability in the oil content among cultivars ranging from 34% to 60% (Ashri, 1998; Were *et al.*, 2006a), most of the current varieties have a low diversity in the oil composition. The general oil composition is 9–11% palmitic, 5–7% stearic, 36–45% oleic, and 38–52% linoleic fatty acid (Kamal-Eldin, 1993; Namiki, 1995; Were *et al.*, 2006a). Using genetic engineering to create a more diverse fatty acid composition in the oil should make sesame more attractive in the competition with other oilseed crops, such as soybean and groundnuts. There are numerous examples today where radical changes have been introduced in the oil composition of many oil crops (for a review, see Thelen and Ohlrogge, 2002). Introduction of new cultivars with a wide diversity in the oil composition will not only improve sesame oil in its present areas of usage but also allow it to diversify out into new areas of usage, thereby increasing its market value. For example, a drawback that may hamper an increased usage of sesame oil is its low level of nutritionally important omega (ω)3 fatty acids. Sesame has high levels of ω 6 fatty acid (linoleic) and insignificant content of ω 3 fatty acids (1–2% α -linolenic). An unbalanced ω 6/ ω 3 fatty acid ratio in the western diet has been recognized as a factor behind many recent health problems like heart disease, arthritis, and inflammatory and autoimmune diseases (Simopoulos, 1999). The relative proportions of ω 6 to ω 3 fatty acids in food has been suggested to be a more important health factor than high intake of the long chain ω 3 fatty acids alone (Horrobin *et al.*, 2002). Therefore, raising the levels of ω 3 fatty acids in sesame will help to increase the usefulness of sesame oil in aiding in the battle of this dietary problem.

Changing the fat quality in a crop with respect to the ω 6/ ω 3 balance has been recently demonstrated. A total content of upto 40% of ω 3 fatty acids was reported from rapeseed transformed with Δ 15 and Δ 6 desaturase genes (Ursin, 2003). The total content of ω 3 fatty acids was 50% and the ratio of ω 6/ ω 3 was 1:2. Expression of a Δ 6 desaturase in soybean resulted in a production of upto 35% of total ω 3 fatty acids (Sato *et al.*, 2003). The aim of their research was to create oil crops as a source from which unique fatty acids can be extracted in masses.

Another example of expanding the area of usage for sesame oil would be to target the enzyme responsible for high levels of linoleic fatty acids,

i.e., the Δ 12 desaturase. The coding gene for this enzyme in sesame was recently identified and characterized (Jin *et al.*, 2001). Down-regulating the expression of the sesame Δ 12 desaturase gene using RNAi interference would constitute an efficient approach to decrease the activity of the same enzyme. The RNAi-mediated silencing are facilitated by publicly available vectors optimized for generating the hairpin RNA molecules that triggers gene silencing (Miki and Shimamoto, 2004; Helliwell and Waterhouse, 2005). The oil produced in the resulting transgenic sesame will be highly enriched in oleic acid. Such oil constitutes an excellent choice as cooking oil. A similar approach was recently successfully tried in cotton (Liu *et al.*, 2002). Silencing of the Δ 12 desaturase by post-transcriptional gene silencing raised the levels of oleic fatty acids from 13% to 78%. The resulting high oleic cotton oil has excellent properties as cooking oil.

3.1.7 Decreased allergenicity

It was recently reported that sesame had quickly risen to become one of the top food allergens (Gangur *et al.*, 2005). Food products containing sesame in any form are, therefore, now required to be labelled as potential allergenic in Europe and Canada. About 65% of all plant food allergens are caused by the presence of proteins that belong to only four different protein families (Jenkins *et al.*, 2005). A prominent sesame allergen, a 14 kDa 2S albumin precursor, belongs to one of the four families, i.e., the prolamins (Wolff *et al.*, 2003). Jenkins *et al.* (2005) pointed out that conserved structures or epitopes, among the potent proteins are important for their allergenic properties. In the example of the 14 kDa 2S albumin precursor it was shown that the allergic reaction coincided with a distinct structure of the proteins chain corresponding to residues 24–94 (Wolff *et al.*, 2003). Recently candidates for a new class of major allergens in sesame were found to be oleosins (Leduc *et al.*, 2006). The authors suggested that a reason for the previous lack of detecting oleosin as potent allergens by SPT (skin prick test), could be that their epitopes may be masked by the proteins tertiary structure (Leduc *et al.*, 2006).

An attractive strategy to reduce the allergenicity in a food crop like sesame could be to remove

or alter only the epitopes responsible. Shewry *et al.* (2001) suggested using protein engineering to design homologous and non-allergenic proteins with similar functions to the original ones and express these in the plant of choice. The allergenic epitope in the major apple allergen, *Mal d 1*, was removed by changing a serine to a proline in the protein structure suggesting the success in the above approach (Son *et al.*, 1999).

3.1.8 Antioxidant lignans as a dietary supplement

It was reported that sesame seeds contain lignans comprising 0.5–1.0% of seed dry weight. Sesame lignans are known to have antioxidant activities including elevating plasma γ -tocopherol, enhancing vitamin E bioactivity, and inhibiting proliferation of human cancer cells (Coonev *et al.*, 2001; Miyahara *et al.*, 2001). Although sesame lignans have long been of interest as a dietary supplement, most of the enzymes involved in the biosynthetic pathways leading to sesame lignans have not been identified owing to the difficulty of protein purification. Based on radio- and stable-isotopically labeled precursor administration experiments in sesame seeds, it was proposed that tyrosine or phenylalanine is first converted into coniferyl alcohol by the phenylpropanoid pathway. Two coniferyl alcohols are subsequently coupled to pinoresinol, and then pinoresinol is converted into “oxygen-inserted” lignans, sesamol, or sesamin via piperitol containing a single methylenedioxy bridge (Jiao *et al.*, 1998; Kato *et al.*, 1998). As an initial approach to identify genes involved in the biosynthesis of antioxidant lignans, 3328 expressed sequence tags (ESTs) were obtained from a complementary DNA (cDNA) library of 5–25-day-old immature seeds. Preliminary identification of EST candidates corresponding to each enzymatic reaction in the biosynthetic pathway of sesame lignans was carried out (Suh *et al.*, 2003).

In the phenylpropanoid pathway for the formation of coniferyl alcohol from phenylalanine or tyrosine, caffeic acid *O*-methyltransferase (COMT), which was abundant in sesame developing seed ESTs, was proposed as a catalyst for *O*-methylation leading to coniferyl alcohol. Three ESTs encoding dirigent protein were detected

in developing sesame seed ESTs, suggesting that a dirigent protein might be involved in the reaction of bimolecular phenoxy radicals for the formation of pinoresinol from coniferyl alcohol in developing sesame seeds. According to the previous observation, that a methylenedioxy bridge formation for the synthesis of piperitol was catalysed via an O_2 /NADPH-requiring cytochrome P450-dependent enzyme (Jiao *et al.*, 1998), seven sesame EST candidates possibly involved in methylenedioxy bridge formation and oxygen insertion were identified (Suh *et al.*, 2003). Although only a few genes involved in the biosynthesis of sesame lignans are available to date, identification of functional activity of the genes and elucidation of the pathways involved in sesame lignan production will provide useful information in industrial applications of sesame lignans as a dietary supplement.

3.2 Addressing Risks and Concerns

Concerns about genetically modified (GM) crops include transgene flow to compatible wild species and unintended ecological consequences of potential transgene introgression (Snow, 2002; Ellstrand, 2003). However, there has been little empirical documentation of establishment and distribution of transgenic plants in wild populations. The first global evidence of transgene escape into natural weedy populations was only recently reported for herbicide (glyphosate) resistant *Brassica* (Warwick *et al.*, 2003, 2004). Reichman *et al.* (2006) have reported glyphosate-resistant creeping bentgrass (*Agrostis stolonifera* L.) plants expressing *CP4 EPSPS* transgenes outside of cultivation area in central Oregon in US.

Sesame is usually considered to be a self-pollinated crop (Kinman and Martin, 1954) although the amount of cross-pollination that occurs is considerable. Honeybees are the primary pollinators in sesame (Langman, 1944). However, sesame has a large diversity in the African and Asian continents with many wild species. Also, other *Pedaliaceae* members, such as *Ceratotheca sesamoides*, share close proximity to *Sesamum*. This plant has edible uses; its leaves are used as valuable leaf vegetables play in daily life in African countries, particularly during times of shortage and famine. All these factors pose a potent risk in

development of transgenic sesame in the countries where wild sesame also exists. Also, sesame being a source of edible oil and seeds, the food safety and biosafety concerns would be major. Efficient management strategies would have to be in place for management of *Bt* or herbicide tolerant sesame, whenever developed in future.

3.3 Expected Technologies

3.3.1 Improved transformation efficiency

Sesame can boast of very few reports on efficient and reproducible system of regeneration from somatic tissues. The low potential for shoot morphogenesis of this species has affected the success achieved in optimization of a genetic transformation method. Establishment of an efficacious protocol for *Agrobacterium*-mediated genetic transformation is the first step in the development of transgenic sesame resistant to any disease or shattering.

3.3.2 Identification of specific promoters

The metabolic engineering of complex traits often requires more than one promoter for the introduction of multiple genes. In the examples mentioned above with diversified oil composition or decreased allergenicity, seed-specific promoters are required for developing transgenic sesame plants. At present, the napin storage protein promoter from *Brassica napus* and the rice glutelin promoter are utilized extensively for the expression of introduced genes in dicotyledonous and monocotyledonous transgenic seeds, respectively (Poirier *et al.*, 1999; Rezzonico *et al.*, 2004; Paine *et al.*, 2005). Recently the promoter of the seed-specific microsomal $\Delta 12$ desaturase gene (*SeFAD2*), which functions by inserting a double bond between carbons 12 and 13 of oleic acid at both the *sn*-1 and *sn*-2 positions of phosphatidylcholine, was isolated from *S. indicum*. When the β -glucuronidase (GUS) reporter gene under the sesame seed-specific *SeFAD2* promoter was introduced into *Arabidopsis* plants, GUS expression was restricted to the mid- and late-developmental phases of the developing seeds,

which is consistent with the timing of storage lipid accumulation. Deletion analysis of the *SeFAD2* promoter revealed that –660 to –180 promoter region functions as a negative *cis*-element in the seed-specific expression of the *SeFAD2* gene. And the –179 to –53 region of the *SeFAD2* promoter was found to harbour positive *cis*-elements, which are associated with the quantitative regulation of *SeFAD2* gene expression during seed development. Eight potential *cis*-elements—the (CA)₂ element, E-box, CCAAT box, G-box, ABRE motif, G-box-like element, RY repeat element, and P-box—all of which have been implicated in the regulation of gene expression in developing seeds, were represented in the –179 to –53 region of the *SeFAD2* promoter (Kim *et al.*, 2006b). The results suggested that the *SeFAD2* promoter might be useful for the modification of the seed phenotypes of transgenic plants.

Promoters controlling gene expression in a tissue-dependent manner and according to the developmental stage of the plant are desired over constitutive promoters. The transgenes driven by these type of promoters will only be expressed in tissues where the transgene product is desired, leaving the rest of the tissues in the plant unmodified by transgene expression. In sesame, seed-specific promoters will be useful in fatty acid profile engineering. Root-specific promoters can play an important role in developing bioengineered sesame resistant to wilt. Dehiscence zone-specific promoters will be extremely valuable in developing shattering-resistant transgenic sesame.

3.4 Intellectual Property Right (IPR), Public Perceptions, Industrial Perspectives, Political and Economic Consequences

Till date, there are no IPRs for any genetically engineered sesame variety or process. However, certain shattering-resistant varieties which have been developed by Sesaco in the United States are protected by patents (US Patent No. 6 100 452). Sesame is used in traditional foodstuffs of many Asian and African countries. The concerns of these native people also need to be kept in mind while developing biotechnological approaches in sesame, one of the ancient oilcrops of the world.

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Safflower

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1. INTRODUCTION

Health is one of the major concerns in today's fast-paced life. With the increase in awareness, more and more people are switching toward healthy food habits. A lot of attention is being paid to dietary habits including the oil in which food is cooked. Oils which are unsaturated, high in nutrition, have good shelf life, and do not turn rancid are preferred. Among the oil crops, few qualify these criteria and safflower (*Carthamus tinctorius* L.) happens to be one of these (Dajue and Mündel, 1996; Weiss, 2000). The current review focuses on the biology, breeding, and biotechnological progress in transgenic research of the genus *Carthamus*.

1.1 History, Origin, and Diversification

Safflower is believed to have originated in Mediterranean region (Garnatje *et al.*, 2006). The domestication of the plant has been indicated in 2000 BC in Egypt, for the orange dye obtained from the florets. However, later it was cultivated for oil. Revenue papyrus of Ptolemy II, 259–258 BC, is also indicative of safflower being cultivated as an oil crop. According to Pliny (1st century AD), in Egypt, safflower was used as a milder substitute for castor oil. In 1000 AD, Mesua, an inhabitant of Baghdad, in his work on contemporary Arab

and Greek medicine, named the plant as *cnicus*. He noted the existence of both wild and cultivated varieties. Safflower was probably introduced to China during AD 200–300 as a source of dye and cooking oil. Western expansion of the Arabs in 5th and 6th centuries promoted spread of safflower cultivation along Maghreb and into Europe via the Iberian Peninsula. From Irano-Afghanistan area, it was introduced into southern Russia. From China it was introduced to Japan. Safflower became a major crop of the United States after World War II. For a detailed account on these aspects, readers are referred to Weiss (2000).

1.2 Habit, Habitat, Taxonomy, Cytogenetics, and Genetic Resources

Safflower is commonly known as “kusum” in India and Pakistan, “honghua” in China, and as “false saffron” in various other countries. It is a branched, thistlelike, medium sized (30–150 cm), herbaceous annual, with numerous spines on leaves and bracts (Dajue and Mündel, 1996; Weiss, 2000). It has a tap root system, which penetrates deep into the soil. The life cycle of plant constitutes a slow-growing rosette stage followed by an active vertical growth-preceding flowering. The elongation starts with the increase in day length and temperature. During this period, plant elongates quickly, produces number of branches,

and subsequently each branch is terminated by an inflorescence, which is a capitulum. Safflower is a self-pollinated crop. However, certain degree of outcrossing has also been reported (Claassen, 1950). The main pollinators of safflower are bees (*Apis mellifera*). After 4–5 weeks of flowering, each capitulum produces 15–30 or more achenes from which oil is extracted (Dajue and Mündel, 1996; Weiss, 2000).

Safflower is a crop of warm temperate region. It can be grown as an autumn sown crop in the northern hemisphere and under irrigation in the dry tropical regions (Weiss, 2000). Traditionally, it is a crop grown in the Mediterranean to Pacific Ocean belt at latitudes between 20°S and 40°N (Dajue and Mündel, 1996; Weiss, 2000). Although safflower is drought tolerant due to its deep penetrating tap root system, it grows best in well drained, deep, fertile, and sandy loam soils. Safflower is used as a support crop in drought prone areas and can help prevent dry land salinity. However, it cannot withstand waterlogging (Dajue and Mündel, 1996).

Carthamus, belonging to the family Asteraceae is composed of 15 species. It is distributed from Spain, North Africa, West Asia to India (Vilatersana *et al.*, 2005). Among its species, only *C. tinctorius* exists under cultivation. *C. tinctorius* is diploid with 12 pairs of chromosomes ($2n = 24$). It is understood to have originated from a cross between self-compatible as well as self-

incompatible *Carthamus oxycanthus* ($2n = 24$) and self-incompatible *Carthamus persicus* ($2n = 24$; Ashri and Knowles, 1960), which in turn have originated from *Carthamus palaestinus* ($2n = 24$), a self-compatible species (Figure 1; Imrie and Knowles, 1970; Khidir and Knowles, 1970). All the four species have been given genome formula BB and are reported to intercross in all combinations and produce fertile hybrids. In addition, there are other safflower species such as *Carthamus arborescens*, *Carthamus rhiphaeus*, and *Carthamus nitidus*, which have $2n = 24$ but do not qualify for BB genome (Ashri and Knowles, 1960; López-González, 1990). Some *Carthamus* species produce purple, blue, and pink flowers. These are *C. boissieri*, *C. dentatus* having genome formula A_1A_1 , *C. glaucus* and its subspecies having genome formula AA or AA/A_3A_3 , *C. leucocaulos* with genome formula A_2A_2 , and *C. tenuis* (Dajue and Mündel, 1996). A few species of *Carthamus* have 11, 22, and 32 pairs of chromosomes. These are *C. divaricatus* ($2n = 22$), *C. lanatus* ($2n = 44$) having genome formula $A_1A_1B_1B_1$, *C. creticus* having genome formula $A_1A_1B_1B_1A_2A_2$, and *C. turkestanicus* ($2n = 64$) having genome formula $A_1A_1B_1B_1AA$, whereas *C. glaucus* has $2n = 20$. Knowles (1958) divided the species of *Carthamus* into four sections based on the chromosome number with those having $n = 12$ in section I, $n = 10$ in section II, $n = 22$ in section III representing only *C. lanatus*, and $n = 32$ in section IV containing

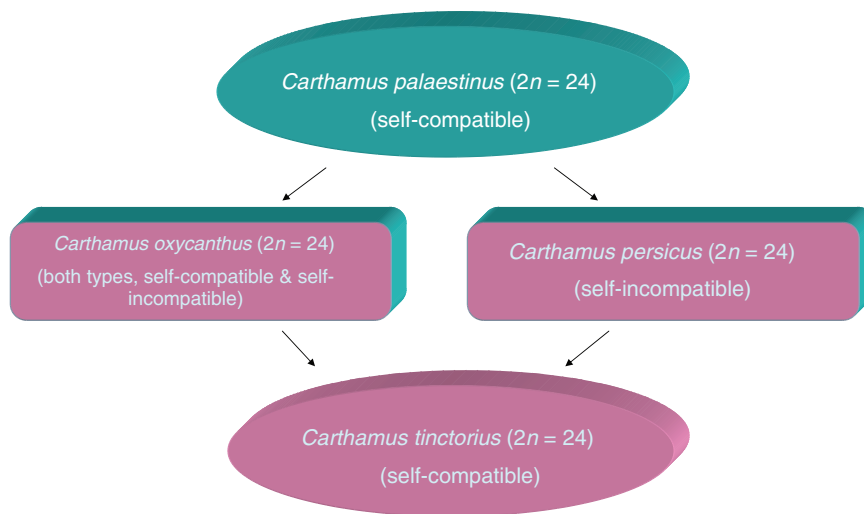


Figure 1 Proposed origin of *Carthamus tinctorius* [Reproduced from Dajue and Mündel (1996)]

two species *C. creticus* and *C. turkestanicus*. Estilai (1977) added one more section V to this to represent *C. divaricatus*, the only species having $n = 11$. On the contrary, on the basis of morphological characters, Hanelt (1963) established five sections under the genus *Carthamus*. These sections were *Thamnacanthus* ($n = 12$), *Carthamus* ($n = 12$), *Odontagnathius* ($n = 10$), *Lepidopappus* ($n = 10$ and 12), and last section *Atractylis* ($n = 11, 22$, and 32). The species under *Thamnacanthus* were put in a separate genus *Phonus* (López-González, 1990; Vilatersana *et al.*, 2000). As the classification of the genus *Carthamus* was considered ambiguous, Vilatersana *et al.* (2005) used random amplified polymorphic DNA (RAPD) markers to resolve the taxonomic problems related to the genus *Carthamus*. Based on their study, Vilatersana *et al.* (2005) reduced the number of sections from five to two. These were *Carthamus* and *Atractylis*. The other two sections *Lepidopappus* and *Odontagnathius* were also included in the latter section. The status of *Thamnacanthus* of Hanelt (1963), species of which were already put in a different genus *Phonus*, was not changed (Vilatersana *et al.*, 2005).

The centers of origin and producing areas of safflower are rich in its genetic resources. Knowles (1969) coined the term “centers of similarity” to identify seven regions that have remarkably similar safflower types. These centers are not necessarily the centers of origin but are the regions having abundance of safflower. Ashri (1973) added three more centers of similarity to this list. Patel *et al.* (1989) confirmed the fact that factors other than geographic isolation contribute to the genetic diversity of *C. tinctorius*. About 25 179 accessions of safflower germplasm have been collected and conserved in 22 gene banks of 15 countries throughout the world (Zhang and Johnson, 1999).

1.3 Economic Importance

Carthamus is valued for both its oil and dye, carthamin. The use of carthamin as dye is since times immemorial. It was used to anoint Egyptian mummies 4000 years ago. *Carthamus* as a source of dye and oilseed has been mentioned in scriptures of different cultures. The medicinal value of safflower was also long realized as evident from *Materia Medica* (Weiss, 2000).

C. tinctorius was originally domesticated for its dye, carthamin, extracted from its petals. This was used for coloring rice, bread, pickle, cheese, sausages, etc. and for dyeing clothes and carpets (Weiss, 2000). The tubular florets are commonly used as an adulterant or cheaper substitute for saffron; hence the name bastard saffron was given to the plant. The soot from charred safflower plants was used as local eye cosmetic, kohl in Egypt. Less thorny varieties of safflower are used as vegetable or as fodder for livestock (Weiss, 2000).

Currently, safflower is grown mainly for oil and in some parts of the world for birdseed. It produces two types of oils, one rich in monounsaturated fatty acid (oleic acid, MUFA) and the other rich in polyunsaturated fatty acid (linoleic acid, PUFA). Both contain chiefly palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3). Depending on the genotype, the percentages of these constituents vary (Singh and Mehta, 1992; Friedt, 1994). The genetic analyses of safflower has revealed that the linoleic acid and oleic acid levels in seed oil are controlled by a single gene (Horowitz and Winter, 1957; Knowles and Mutwakil, 1963; Friedt, 1994) having three alleles *OL*, *ol*, *ol^l* (Knowles, 1965). Another gene, “*st*”, determines the level of stearic acid. The substitution of “*st st*” instead of “*St St*” in high linoleic acid (*Ol Ol*) or high oleic acid (*ol ol*) varieties, leads to an increase in the stearic acid content and a corresponding decrease in the content of linoleic acid or oleic acid or both (Ladd and Knowles, 1971).

Out of the two different varieties of safflower, the one rich in linoleic acid yield oil that is good for heart patients. The oil extracted is used as salad oil and in margarine. The oil extracted from varieties with high oleic acid is thermostable and is used for cooking purposes. The oil is also a rich source of vitamin E, tocopherol (Stobart *et al.*, 1997). Since high oleic acid oil is considered drying or semi-drying and has nonyellowing property upon drying, it is used in paints and varnishes. After the oil is extracted, seed cake is used as a cattle feed (Weiss, 2000). High content of linoleic acid in safflower oil can control arteriosclerosis, reduce serum cholesterol, and thus cure hypertension, high blood fat, coronary heart disease, and obesity. It can also control blood pressure, enhance physique, improve microcirculation, and recover nerve function (Dajue and Mündel, 1996). High

oleic sunflower and safflower seeds have also been used for the production of environment friendly, effective lubricants containing estolides. These lubricants can be used as hydraulic fluid in heavy equipment or as crankcase fluid (Durham and Wood, 2002). The crop, especially the high oleic acid varieties hold tremendous potential as a pollutant reducing diesel fuel additive as the oil is free of sulfur and biodegradable. This could help in reducing emissions, which would help in reducing acid rain, green house effect, and surface pollution (Bergman and Flynn, 2001).

Safflower petals are of high medicinal and therapeutic value. Safflower petals possess immunomodulating and blastogenic properties (Wakabayashi *et al.*, 1997). *Carthamus* petals are rich source of gamma-linolenic acid, which has high antifungal and antiviral properties (Srinivas *et al.*, 1999). It is useful in treatment of skin infections such as atopic eczema.

Carthamus ranks second in the world's total oilseed production and approximately 726 121 ha land is dedicated to safflower production (Camas and Esendal, 2006). According to an FAO estimate, in 2005, yield was approximately 776 327 metric tons. The chief safflower producers are India, the United States, and Mexico (Table 1,

<http://www.fao.org/es/ess/top/commodity.html?lang=en&item=280&year=2005>)

In India, it is cultivated along the Deccan peninsula in Maharashtra, Karnataka, Andhra Pradesh, Madhya Pradesh, Orissa, and Bihar (Patil *et al.*, 2003). Most of the produce is consumed domestically, owing to its increasing acceptance and improvement in the fatty acid profile. Thus, in coming years, more area is expected to be brought under its cultivation.

1.4 Traditional Breeding

In breeding, the genotypes that are stable in different environmental conditions are highly desirable. Thus, the prerequisite of any breeding program is evaluation of the germplasm and selection of stable genotypes. Ehdaie *et al.* (1977) from Iran evaluated 33 local and introduced genotypes of safflower at seven locations to understand the genotype X environment interaction. Although the selected local varieties did not exhibit significant genotype-environment interactions, the introduced varieties were significantly affected. Among the 12 introduced genotypes, only one exhibited stability and high yield in diverse environmental conditions. It highlighted the importance of locally adapted genotypes as starting material in breeding. Similarly, Ashri *et al.* (1975), evaluated germplasm from Indian subcontinent, Turkey, and Egypt at different locations in Utah, Israel, and Washington, for two strongly correlated characters, the length of the growing period from planting to flowering and plant height. These were strongly influenced by source countries and test locations. However, interestingly there was no significant correlation of these characters with the yield and oil content. This observation was utilized for selection of varieties, which had shorter growing period and were high yielding. Similarly, Ranga Rao and Ramachandram (1977) carried out an analysis of 215 entries from India and the United States for association of components of yield and oil. While a strong positive correlation of seeds per capitula and seed weight with yield per plant was observed, seed size and oil content exhibited a negative correlation due to hull thickness. Moreover, they did not observe any effect of plant height, days to flowering, and total crop

Table 1 Annual production of safflower in different countries^(a)

S. No.	Country	Production (t)
1	Mexico	2 12 765
2	India	2 10 000
3	United States of America	91 000
4	Australia	60 000
5	Argentina	51 000
6	Kazakhstan	40 000
7	Ethiopia	38 000
8	China	32 000
9	Kyrgyzstan	20 000
10	Uzbekistan	10 000
11	Tanzania	5 000
12	Tajikistan	3 000
13	Canada	2 000
14	Hungary	650
15	Iran	500
16	Russian Federation	200
17	Turkey	150
18	Pakistan	40
19	Spain	17
20	Palestine	5

^(a)Source: FAO (2005)

growth on yield and oil content. Patil *et al.* (1994) studied the efficiency of selection for desirable traits in F₂, F₃, and F₄ generations. Single plant selections from F₂ progenies were carried out based on number of capitula per plant, number of seeds per capitulum, test weight, and seed yield. Among various selection classes, number of capitula per plant and yield per plant were observed to be the important parameters in individual plant selection in early segregating generations. It was observed that selection based on these characters had potential of genetically improving the productivity of cultivated varieties of safflower. Likewise, Ramachandram and Goud (1981) studied the inheritance of seed yield and oil content in a diallel cross between F₁ and F₂. The combining analysis revealed the predominance of *gca* variance for plant height, total capitula, seed weight, seed number, and seed yield in F₁ and F₂ generations, and for days to flowering and oil content in F₁.

For breeding, it is important to know the existing variability and the interspecific relationship within the existing divergence. To understand the diversity, allozyme patterns of alcohol dehydrogenase, governed by two gene loci *Adh1* and *Adh2* were studied in 1553 cultivated varieties of safflower by Efron *et al.* (1973). The study helped in understanding the interspecific relationships in the genus *Carthamus* and the origin of polyploid species, namely, *C. lanatus*, *C. baeticus*, and *C. turkestanicus*.

One of the major challenges for safflower research and development is development of cytoplasmic male sterile lines to be used for production of hybrids of commercial importance. This need has emerged primarily due to lack of suitable well-functioning male sterility systems, though structural and genic male sterility systems in safflower do exist (Heaton and Knowles, 1982; Hill, 1989; Weisker, 1996). However, these cannot be utilized to a large extent at a commercial scale for want of desirable hybrids. Among all techniques, chemical male sterility is believed to be more cost effective and a practical approach. The utilization of chemicals for achieving male sterility in safflower has stemmed from series of reports where the hormonal regulation of flowering has been emphasized (Baydar and Ülgen, 1998; Baydar, 2000). It was found that gibberellic acid can induce male sterility in plants. Baydar and

Gökmen (2003) obtained reduced pollen viability from 81.6% to 6.7% in safflower after three foliar sprays with 100 ppm GA₃ after 75, 82, and 89 days.

The yield of safflower is adversely affected by a number of pathogens and pests. Breeding for disease/pest resistance has been one of the major thrust areas of research in safflower. It requires screening of large number of genotypes for selection of donor(s) possessing resistance genes, which is then bred with the cultivated varieties. Ashri (1971) screened about 2000 accessions of *C. tinctorius* and nine wild species for selecting genotypes resistant against safflower fly, *Acanthophilus helianthi*. Only two closely related species, *Carthamus flavus* and *C. palaestinus* showed fly-free accessions and, therefore, these were projected to be the potential donors for safflower fly resistance gene. One of the key barriers in breeding is incompatibility between the donor plant and the cultivated variety, which leads to no or sterile hybrids. Therefore, it is imperative to study the breeding behavior of the cultivated varieties along with closely, distantly, and unrelated wild species for proper gene tapping. Ashri (1974) studied natural hybridization of the cultivated safflower, *C. tinctorius* and unrelated wild species *C. tenuis*. It was found that the two formed sterile hybrids. However, it was supposed that continuous pollination might have produced viable seeds and introgression somewhere in the wild must have taken place. Similarly, drought hardiness and resistance to *Alternaria* leaf blight have been partly incorporated into cultivated types by crossing with the resistant accessions or species and followed by repeated backcrossing and selection (Mündel and Huang, 2003).

Certain agronomic traits such as size of capitulum and length of involucre bracts are expected to be directly related to yield. Thus, cultivars with large heads and smaller outer involucre bracts are the desired ones. Ashri *et al.* (1975) evaluated 900 lines from over 20 countries for these characters. Considerable variability within the regional pools was observed. However, the correlation of these two characters with yield was observed to be inconsistent (Ashri *et al.*, 1975). Selection of the desired trait depends largely on the selection criteria adopted. In addition to yield components, there are other factors, which indirectly affect the yield. In many

instances, it has been observed that a single trait-governing yield affects one or more other traits either positively or negatively. Thus, most of the workers use path analysis to define the best selection criteria. The number of capitula/plant, number of seeds/capitulum, weight of seed, and capitulum diameter are considered important yield components (Patil and Jadhav, 1977; Chaudhary, 1990; Prasad *et al.*, 1993; Prasad and Agrawal, 1994; Pascual-Villalobos and Alburquerque, 1996; Omid Tabrizi, 2000). Likewise, total biomass, plant height, leaf number, number of primary branches, capitula/plant and seeds/capitulum 1000-seed weight, seed weight/capitulum, distance between ground level and the first fertile branch, number of days to the beginning of branching and flowering duration have also been found to have a significant positive effect on the seed yield (Chaudhary, 1990; Bidgoli *et al.*, 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Genetic transformation is an edge-cutting technology that allows the transfer of useful genetic information, within and across the taxonomic boundaries, resulting in new and novel genotypes. The same can be assiduous and time consuming through conventional means. For instance, it takes 5–10 years of repeated back crossing and selfing to introgression of a desirable trait from a landrace or a closely related species into the recipient parent, through conventional breeding methods. Moreover, if the donor parent is evolutionarily a distant relative, it takes even longer. Furthermore, such wide crosses do not take place easily because of the pre and postfertilization barriers (Jauhar, 2001). In addition, in few instances, due to linkage drag, many undesirable genes also get incorporated along with the gene of interest. However, genetic engineering overrides all such coercions and offer precise incorporation of the desired gene. Although incorporation of a gene is a single-step process, the development of a transformed or a transgenic plant involves several distinct steps, namely, gene selection, insertion, integration, expression and demonstration, and elucidation of its inheritance (Webb and Morris, 1992).

2. DEVELOPMENT OF TRANSGENIC SAFFLOWER

2.1 Transgenes

The expression of a foreign gene in an alien genetic background is hampered because of the gene's inability to use host's regulatory sequences for transcription. Thus, for expression of a foreign gene, the plasmid construct is designed in a manner that along with the "gene insert", 5' and 3' regulatory elements, capable of transcribing the inserted gene in an alien environment, are also ligated. Since most of the foreign genes are bacterial in origin, bacterial promoters such as Nos (nopaline synthase), Mas (mannopine synthase), and Ocs (octopine synthase) are commonly used. Besides bacterial promoters, CaMV 35S promoter from cauliflower mosaic virus is also used. CaMV 35S exhibits high expression of dicot, monocot, as well as bacterial "gene inserts" in the transformed plant cells (Webb and Morris, 1992).

All the transformation studies that have been carried out in safflower used *Agrobacterium tumefaciens*. Ying *et al.* (1992) used disarmed strain LBA4404 (Hoekema *et al.*, 1983) containing a binary plasmid pBI121 (Jefferson *et al.*, 1987), which had a *GUS* reporter gene driven by CaMV 35S promoter and a neomycin phosphotransferase gene (*nptII*) under control of Nos. Orlikowska *et al.* (1995), in addition to LBA4404/pBI121, used another strain EHA105/p35SGUSInt (Cardi *et al.*, 1992; Li *et al.*, 1992). Similarly, Sankara Rao and Rohini (1999) and Rohini and Sankara Rao (2000) used the bacterial strain, LBA4404 harboring a binary vector pKIWI105, which had *uidA* reporter gene driven by CaMV 35S promoter and a neomycin phosphotransferase II (*nptII*) gene driven by the Nos promoter. The reporter gene of pKIWI105 had a modified *uidA*, which lacked the bacterial ribosome binding site and showed no expression in *Agrobacterium* but showed good activity in plant cells (Janssen and Gardner, 1989). Sujatha (2006) used a construct, pCambia1305.1 harboring β -glucouronidase (*gus*) as a reporter gene and hygromycin phosphotransferase (*hpt*) as a selectable marker.

In a novel technique, Nykiforuk *et al.* (2006) produced transgenic oilseeds producing human insulin by targeting protein to oilbodies as

oleosinfusions. *Arabidopsis thaliana* plants were transformed with a binary expression vector pSBS4405. The construct had a fusion protein, oleosin + human insulin (OB-hIN), comprising recombinant human mini-insulin with an N-terminal trypsin cleavable propeptide (Klip27-mini-insulin; Kjeldsen *et al.*, 2001) fused to the C-terminus of the *A. thaliana* 18-kDa oleosin-Klip8 polypeptide (Nykiforuk *et al.*, 2006). Using a similar approach, genetically transformed safflower, capable of synthesizing insulin in its seeds was also developed at SemBioSys Genetics, Inc. As in *Arabidopsis*, a cleavage site was included to permit purification of the target protein after extraction of the oil (US Patent 2005/0039235 A1; Moloney *et al.*, 2005).

In another report, Kneusel *et al.* (1994) identified roughly 12% of brefeldin A (BFA) esterase sequence and cloned it in pUC18. One of the seven clones was sequenced and found to express full size esterase protein of 372 amino acid residues. The BFA esterase was isolated from *Bacillus subtilis*. This enzyme can hydrolyse Brefeldin A, a phytotoxin from *Alternaria carthami*, which causes devastating leaf blight disease of safflower. This investigation has opened the avenues for development of transgenic safflower resistant to *Alternaria* blight.

2.2 Transformation Methods Employed

Gene insertion into a plant can be brought about either by direct gene transfer method or *Agrobacterium*-mediated transformation. For safflower, *Agrobacterium*-mediated transformation has mostly been used. It requires co-culture of explants with the bacterium (Ying *et al.*, 1992; Orlikowska *et al.*, 1995; Sankara Rao and Rohini, 1999; Sujatha, 2006). For instance, Sankara Rao and Rohini (1999) grew *Agrobacterium* overnight in LB medium containing (50 mg l⁻¹) kanamycin. Thereafter, the bacterial cells were pelleted and resuspended in (100 ml) Winan's AB medium. The cotyledons and pricked cotyledonary nodes were incubated for 10 min in AB medium with suspended *Agrobacterium*. The explants were taken out and blot dried on sterile blotting paper and co-cultured for 2 days on regeneration medium. Thereafter, the explants were washed with regeneration medium supplemented

with (500 µg ml⁻¹) cefotaxime and after blot drying incubated on selection/regeneration medium.

In addition, Rohini and Sankara Rao (2000) developed an *Agrobacterium*-mediated *in planta* transformation technique where the *in vitro* regeneration step was completely omitted. DNA was injected directly into the plant. This method has also been used for other crops, namely, rice (Junhi and Guhung, 1995), cauliflower (Eimert *et al.*, 1992), sunflower (Schoneberg *et al.*, 1994; Sankara Rao and Rohini, 1999), etc. Rohini and Sankara Rao (2000) used 2-day-old seedlings for *in planta* transformation. For *vir* gene expression, acetosyringone (100 mM) and extract of wounded tobacco leaves (1 gm l⁻¹) were included in the medium used for suspension of *Agrobacterium*. Out of the two, tobacco leaf extract helped in improvement of the transformation efficiency while acetosyringone did not have a significant effect. Acetosyringone has been reported to increase the receptivity of the plant material and the infection ability of *Agrobacterium* and has been shown to increase the transformation frequency in safflower (Orlikowska *et al.*, 1995). For infection, 2-day-old seedlings from which one cotyledon was removed and the exposed cotyledonary nodes were pricked were incubated in *Agrobacterium* containing medium for 10 min. This was followed by co-cultivation on MS basal medium for 24 h. Thereafter, the seedlings were washed with cefotaxime (500 µg ml⁻¹) for 1 h and planted in sterile soilrite. Sujatha (2006), in addition to *Agrobacterium*-mediated transformation, had attempted direct gene transfer through particle gun bombardment. However, better results were observed by *Agrobacterium*-mediated transformation.

A group at SemBioSys Genetics, Inc. also used *Agrobacterium*-mediated transformation of leaf and stem tissues of safflower for packing important proteins in the oil storing bodies, oleosomes (Moloney *et al.*, 2005; US Patent 2005/0039235 A1; Nykiforuk *et al.*, 2006).

2.3 Selection of Transformed Tissue

The key to success of any transformation protocol is selection of the transformed cells. Although, the tumorigenic genes present in *Agrobacterium* can

be used as selection marker, this usually entails abnormal shoots and development of crown galls, which is disruptive of normal growth of the transgenic plants. The replacement of tumorigenic gene with the single dominant gene that provides resistance to a selective agent allows selection of the transformants and at the same time does not disrupt plant regeneration. A number of chemicals, mostly antibiotics, have been used as selection agents. However, a number of factors govern their efficacy. A selection agent should be toxic to growth of normal cells. Only the transformed cells, which have selectable marker gene, should be able to grow on the selection medium. The time and dose of application are also critical for elimination of untransformed cells and selection of transformed cells. The concentration of the selection agent in the medium should not be suboptimal as there may be some background resistance to the selection agent in the untransformed cells. This would lead to selection of untransformed cells as well. Similarly, time of application is critical to limit the number of nontransformed cells which would survive through cross-protection from transformed cells (Webb and Morris, 1992). Regeneration ability of the transformed cells is also affected by the kind and dosage of the selection agent used. Selection agents, such as antibiotics, severely affect the differentiation response. In the cultivar “Centennial”, exposure of the co-cultivated explants to kanamycin or gentamicin, drastically reduced the regeneration response (Orlikowska *et al.*, 1995). However, the transformed shoots could be obtained only when kanamycin selection was done after 1 or 2 days of co-cultivation. The transformation efficiency was monitored through the activity of β -glucuronidase (GUS). Antibiotics, such as kanamycin and hygromycin are the most commonly used selection agents. The genes that impart resistance against them are *nptII* and *hptII*. Sujatha (2006) used three selection agents, namely, hygromycin ($0\text{--}100\text{ mg l}^{-1}$), kanamycin ($25\text{--}500\text{ mg l}^{-1}$), and phosphinothricin ($0\text{--}10\text{ mg l}^{-1}$) for safflower. Out of these, kanamycin even at 500 mg l^{-1} was not effective, while hygromycin proved inhibitory at the lowest concentration 1 mg l^{-1} . Ying *et al.* (1992) selected transformed shoots of safflower on medium containing 500 mg l^{-1} carbencillin and 50 mg l^{-1} kanamycin.

Since Rohini and Sankara Rao (2000) followed an *in planta* method of transformation, they did not have any selection procedure. Initially, selection on kanamycin was attempted, however, the survival percentage reduced drastically. The transformed plants were screened using polymerase chain reaction (PCR) and Southern analysis, discussed under Section 2.5.

2.4 Regeneration of the Whole Plants

The success in obtaining a transgenic plant depends essentially on (i) the competence of the cell or tissue to take up and integrate foreign DNA into its genome and (ii) the ability of the transformed tissue to regenerate into plantlets (Potrykus, 1990). Thus, a tissue for which an *in vitro* regeneration protocol has been standardized or the one having a high natural regeneration potential, such as shoot apical meristem, is used for transformation. For safflower, a number of regeneration protocols either through somatic embryogenesis (Mandal *et al.*, 1995, 2001; Mandal and Dutta Gupta, 2003) or organogenesis (George and Rao, 1982; Tejavathi and Anwar, 1984; Baker and Dyer, 1996; Nikam and Shitole, 1999; Walia *et al.*, 2005; Radhika *et al.*, 2006) have been reported. However, regeneration response in safflower is highly variable and is dependent on number of variables, namely, genotype, culture media, culture conditions, etc. Moreover, rooting of the *in vitro* regenerated shoots and field transfer too pose major limitations (Baker and Dyer, 1996; Walia *et al.*, 2005; Sujatha, 2006). Despite that, a number of transformation protocols have been developed with at least one of them reaching commercial scale.

The explants that have been used in transformation of safflower are cotyledons (Ying *et al.*, 1992; Orlikowska *et al.*, 1995), leaves (Orlikowska *et al.*, 1995), and apical meristems (Orlikowska *et al.*, 1995). The regeneration of transgenics from explant other than apical meristem is mostly via a callus phase, which is genotype dependent and gives rise to sectorial chimeras (Ying *et al.*, 1992; Orlikowska *et al.*, 1995). On the other hand, regeneration from apical shoot meristem is direct, genotype independent, and yields both homohistonts and chimeric transgenics (Orlikowska *et al.*, 1995). The time

at which the explant is exposed to the selection agent is also critical step for the conversion of a transformed cell into a transgenic plant. Orlikowska *et al.* (1995) observed regeneration only in those explants, which were transferred to selection medium after 1 or 2 day(s) of co-cultivation with *Agrobacterium*. The efficiency of regeneration response was markedly decreased by co-cultivation. One important aspect of the protocol developed was that the transformation frequency equaled the regeneration frequency in terms of the number of explants producing shoots following co-cultivation. The number of shoots per responding explant in transformed tissues was less but the shoot quality was good when compared to the untransformed explants where innumerable shoots that were often hyperhydric were observed (Sujatha, 2006).

2.5 Activity and Stability of Inheritance of the Gene, Adverse Effects on Growth, Yield, and Quality

The entry of any transgene in a plant cell is followed by its integration into the plant genome. The transgene along with reporter gene need to express in plant cell and this forms the basis for the transient assays. The coding sequence of bacterial enzymes can be assayed to study the transient expression in the transformed cells. These assays help in ascertaining the transformation efficiencies of the technique and help in developing stable integration systems. Some of the reporter genes commonly used are nopaline synthase (*nos*), chloramphenicol acetyltransferase (*cat*), bacterial or firefly luciferase (*luc*), *nptII*, and *gus*. The activity of the gene product can be assayed chemically, histochemically, or by using antibody techniques (Webb and Morris, 1992). For instance, Sankara Rao and Rohini (1999) studied GUS expression in *in vitro* regenerated shoots of safflower using a histochemical assay. As the GUS activity was confined to certain sectors, selection of GUS expressing shoots and calli was necessary. The activity of *nptII* was studied in total protein content of GUS positive shoots and calli by nondenaturing polyacrylamide gel electrophoresis (PAGE) assay. Transformation of safflower with *nptII* was confirmed when the total proteins of the GUS positive putative

transformants showed phosphorylated kanamycin at the expected position on the autoradiogram. The GUS protein was assayed by Western blot analysis using an anti-GUS antibody and a 74 kDa protein with the molecular weight similar to that of GUS protein was detected. Similarly, Rohini and Sankara Rao (2000) tested the transient expression of *uidA* gene in putative transformants from A-1 and A-300 varieties of safflower, after 4 days of postcultivation with *Agrobacterium* by Western blot analysis to identify transformed explants. At least four out of the 10 seedlings tested showed GUS gene expression. The immunostaining of the extracted immobilized proteins was done with GUS antibody. The integration and expression of the *uidA* gene in the putative transformants was confirmed by a 74 kDa protein band in Western blot analysis. Likewise, transient GUS expression was also checked by Orlikowska *et al.* (1995) and Sujatha (2006).

Enzymatic assay for the marker/reporter gene though indicate status of the cell as being transformed, yet is not an ample proof of the same, as some cells may have an inherent resistance to these drugs or might have acquired resistance during the process. Thus, assays such as PCR, Southern, Northern, and Western blot are used to confirm the integration of foreign DNA into plant genomic DNA and its expression (Schrammeijer *et al.*, 1990; Ying *et al.*, 1992; Knittel *et al.*, 1994; Schoneberg *et al.*, 1994; Orlikowska *et al.*, 1995).

PCR assays are done employing the primers for the incorporated gene. Thus, only those regions, which have the gene insert, get amplified. Orlikowska *et al.* (1995) confirmed stable integration of genes in four shoots out of six for *NPTII* gene and three out of six for *GUS* gene. Rohini and Sankara Rao (2000) carried out PCR analysis for checking the presence of 514 bp *uidA* and 800bp *nptII* genes. The PCR analysis of T₀ plants revealed genotypic variation in the transformation ability of varieties A-300 and A-1. Out of the two, A-1 was more efficient. The overall transformation frequency of A-1 was 5.3% compared to 1.3% for A-300. Out of ten plants of A-300, three plants contained *uidA* marker gene and five plants contained *nptII* gene. While, of the 24 A-1 plants analyzed, 14 contained *uidA* marker gene and 11 *nptII* gene. The absence of residual bacteria was confirmed when

amplification of *virC* primers was not detected in the DNA.

Southern hybridization is another confirmatory test for stable integration. It involves preparation of probe by radioactive or nonradioactive labeling of the inserted sequence, isolated by digesting the recombinant plasmid. The probe is hybridized with the isolated, restricted recombinant genomic DNA. The region where the probe hybridizes gives a signal, confirming the stable integration of the gene into the plant cell (Ying *et al.*, 1992; Orlikowska *et al.*, 1995). Sankara Rao and Rohini (1999) carried out dot blot analysis of the putative transformants. The membrane was hybridized with a $-^{32}\text{P}$ random prime labeled 2.1 kb fragment of the *uidA* gene. The *uidA* probe gave a strong signal after hybridizing with total genomic DNA of the transformants, indicating the presence of *uidA* gene. The study concluded stable integration of *uidA* and *nptII* genes in the transgenic shoots. However, the recovery of transgenic plants was a limitation as rooting of shoots was difficult. Similarly, Rohini and Sankara Rao (2000), used pUC-GUS121 from *Escherichia coli* strain XL-1 Blue for probe preparation. The plasmid carried a *uidA* gene with the CaMV 35S promoter and the Nos terminator. Digestion of the plasmid with *EcoRI* and *BamHI* restriction enzymes was reported to release a 2.1 kb fragment comprising the *uidA* coding region along with the Nos terminator. Southern analysis of the T₀ plants confirmed stable integration. The transgenes were inherited to the T₁ generation also. Four T₀ plants of A-1 yielded transformed progeny. However, the inheritance could not be confirmed due to small sample size (Rohini and Sankara Rao, 2000).

2.6 Specific Regulatory Measures Adopted

The risk assessment aims to identify and evaluate the potential adverse effects that a genetically modified organism (GMO) may have on the conservation and sustainable use of biodiversity in the receiving environments. The field trials and release of transgenic plants is monitored and regulated by global regulatory framework. These agencies ensure effective risk management that includes efficient monitoring systems, research programs, technical training, and improved domestic coordination amongst government agencies and services. The major issue

in risk assessment of transgenics is the possibility of gene escape to wild or weedy relatives which could have serious ecological and environmental bearings, such as aggressive weediness of the weedy relatives, possible loss of diversity, disturbance in ecological relationships with natural communities, unexpected phenotypic effects, etc. (Snow, 2002). Development of regulatory framework should involve quantification of gene flow from the developed transgenic to natural stands, understanding of population genetic structure of the recipient species, and estimate of spatial distribution of probable areas of gene flow (Bradford *et al.*, 2005).

The consideration should be given to both benefits and risks involved in the technology. Each country has different regulatory policy and stringency of these policies varies with the country. For instance, in the United States, the release of a new GMO requires scrutiny by four different federal bodies, namely, NIH (National Institute of Health), APHIS (Animal and Plant Inspection Health Service), FDA (Food and Drug Administration), and EPA (Environment Protection Agency). Each regulatory body has a different role to play. They monitor the development of GMO in laboratory, its trial, and finally its release in the market (Falk *et al.*, 2002).

APHIS had issued four release permits in the year 2005 to SemBioSys Genetics, Inc. for value added proteins in safflower. In one of the trials, SemBioSys Genetics, Inc. has conducted a field trial for its product, Immunosphere under the permit APHIS # 05-320-01r. The transgene in the developed product was evaluated by APHIS for the risk of the new plant pest introduction, impact on nontarget organism or on the threatened or endangered species, and was concluded by them that the DNA inserted into plant does not have an inherent plant pathogenic properties and shall not pose any rise. APHIS had set guidelines for quarantine and disposal of the transgenic material during and after the field trial. The guidelines clearly mentioned:

- Dedicated use of equipments for planting and harvesting to prevent any inadvertent transfer of transgenic material to other areas.
- A fallow 15.24 m (50 ft) boundary around the trial plot.
- Physical containment of the transgenic field trial from the sexually compatible species. The

site shall not have any compatible plant at least within 3.128 km miles periphery. Isolation of transgenic plants by at least 8.045 km miles from the conventional seed production site. Any accidental or unauthorized release of the transgenic material has to be reported to APHIS.

- Weekly monitoring of the test sites for weed, disease, and insects.
- Monitoring of the test site for safflower volunteers for at least two growing seasons after harvest (http://www.aphis.usda.gov/brs/aphisdocs/05_32001r.ndd.pdf).

Likewise, another permit was issued by APHIS for genetically modified safflower expressing one of the IgG binding domains present in native ProteinA expressed as fusion to oleosin (http://www.aphis.usda.gov/brs/aphisdocs/05_02502r.ndd.pdf).

3. FUTURE ROAD MAP

3.1 Expected Products

The edible oils yielded by members of Asteraceae are polyunsaturated, used as a cooking medium and are rich in linoleic acid. Though the oil is nutritionally rich, it is thermally unstable and turns rancid on exposure to air. Thus, in these oilseeds, incorporation of genes conferring high thermo stability and monounsaturations is highly desirable (Weiss, 2000).

The knowledge of seeds as protein reservoirs (Murphy, 1993) led Abenes *et al.* (1997) to test the *Arabidopsis* oleosin-GUS reporter fusion protein in *C. tinctorius*, *Brassica napus*, *Helianthus annuus*, *Ricinus communis*, and *Linum usitatissimum*. Though the expression in *C. tinctorius* was very low, the ingenious technique was later utilized by a Canadian Company, SemBioSys Genetics, Inc. for developing transgenic proteins of therapeutic as well as nutritional value (Seon *et al.*, 2002; Nykiforuk *et al.*, 2006; <http://www.sembiosys.com>).

SemBioSys Genetics, Inc. is the only company that has been able to grow transgenic safflower, which can produce insulin in seeds at commercial scale in Canada, the United States, Mexico, and Chile (<http://www.sembiosys.com>). Moloney *et al.* (2005; US Patent 2005/0039235 A1) describes

that to cater to the therapeutic protein demand of quarter of the earth population only 1000 ha of land is required, which would produce 2 t of the therapeutic proteins annually. In addition to insulin, SemBioSys Genetics Inc. is also involved in development of other pharmaceutical and nonpharmaceutical products in transgenic safflower, namely, Apo AI, Immunosphere, docosahexaenoic acid (DHA), gamma linoleic acid (GLA), etc. Apo AI is the major lipoprotein associated with high-density lipoprotein, which removes excess of cholesterol from arteries by reversing cholesterol transport. The anticipated product requirement to attain desirable results is 5–6 g of ApoAI per person. The company intends to achieve commercial levels of Apo AI expression in both *Arabidopsis* and safflower. They proposed to initiate clinical trials in 2007 and the product shall be launched by 2012 (<http://www.sembiosys.com>). Their another product, Immunosphere is an immunostimulatory protein-based feed additive, which imparts disease resistance to shrimp. This product is envisaged to cater to the problem of disease in shrimps which in turn is affecting US \$18 billion shrimp industry, across the world (<http://www.sembiosys.ca/Main.aspx?id=18>). In addition, SemBioSys Genetics, Inc. is developing a genetically engineered safflower, which could produce essential long chain polyunsaturated omega-3 fatty acid DHA. It is essential for the normal brain development in infants and is used in infant formulas. The company is expected to commercialize its product in 2009–2010 (<http://www.sembiosys.ca/Main.aspx?id=19>). Over and above, SemBioSys Genetics Inc. is engaged in developing GLA-rich transgenic safflower. GLA is an essential fatty acid used in topical, medical, food, and nutrition markets. The GLA-rich seeds shall be commercially produced by Arcadia Biosciences Inc. (<http://www.sembiosys.ca/Main.aspx?id=20>).

Nykiforuk *et al.* (2006) developed method for easy separation of proteins from other seed components using a simple technique of liquid–liquid phase separation. This separation process is highly cost effective compared to the number of chromatography steps. In an Information Systems for Biotechnology (ISB) news report, the company claimed that they were able to produce 1.2% insulin of the total

seed protein and this was envisaged to transform economics and scale of insulin production. Safflower-produced insulin could reduce capital costs compared to existing insulin manufacturing by 70 % and product costs by 40 %.

3.2 Addressing Risks and Concerns

Transgenic research has outpaced the understanding of its effect on the environmental factors, largely due to technology push revolution by the private laboratories. Their main objective is earning rich dividends by improving plant health and productivity. The gap in knowledge of their effect on environment remains unabridged (Batie and Ervin, 2001). There has been a growing concern over the food safety of the genetically modified crops. Based on the work done by WHO and Food and Agriculture Organization, Organization for Economic Cooperation and Development (OECD), an intergovernmental organization with members from 29 countries, proposed a concept of substantial equivalence. The concept recognizes the safety assessment of the product/organism derived through recombinant DNA technique in light of its similarities and differences with the analogous conventional product as a standard. It analyzes how the modified organism is different from the parent (Mayers *et al.*, 2002). It relies on the existing history of safe food use of a conventional food product and helps in safety assessment of GMO under conditions of similar exposure, consumption pattern, and processing practices.

The major environment risks are: (a) threat to genetic diversity and (b) transfer of transgenes, especially genes for resistance, to the wild and semi-domesticated relatives of safflower thus creating superweeds (Hall and McPherson, 2006). Two biosafety risks have been identified in field grown transgenic safflower. These are gene flow to cultivated safflower and to its wild relatives (Anonymous, 2006). The outcrossing in safflower and its wild relatives varies from 100 % to 0 % depending upon the environmental conditions, genotype, and the pollinator activity. However, the average is between 15 % and 20 % (Claassen, 1950). Though it is self-pollinated, insect pollination also takes place. However, the pollinators do not travel more than 10 km (Anonymous, 2006). Therefore, in addition to studying the distance pollen travels,

viability of the carried pollen under different conditions needs to be examined. McPherson *et al.* (2004) highlighted the relevance of these concerns especially with respect to genetically modified safflower because of compatible hybridization between cultivated safflower and its six wild relatives. The cultivated safflower belongs to the BB genome and all others members of this genome (*C. tinctorius*, *C. persicus*, *C. oxycantha*, and *C. palaestinus*) cross readily. The gene flow between the members has high probability if grown in vicinity (Knowles, 1980; Johnson, 2006). In addition safflower is used as bird seed and may also be carried away. This would mean entering of transgene into food chain through birds. Since safflower is grown as a minor crop in most of the countries, physical isolation would serve as one of the methods for confining crop-to-crop gene flow. Temporal confinement by putting up a barrier crop in the area surrounding transgenic cultivations is another possibility that can be explored (Anonymous, 2006). The outcrossing can be prevented by spraying the crop with an insect repellent or an insecticide. In addition, modeling of pollen movement by studying the behavioral patterns and the distances covered by the pollinators can also serve as an important tool. Studies are needed to understand gene flow, pollen viability, pollinator dynamics, and pollen/seed movement/dispersal (Anonymous, 2006). More information should be gathered on the outcrossing frequency, distance to the crop, feral safflower, and its weedy relatives. Moreover, these data should be of studies carried out for several years.

3.3 Expected Improvement in Transgenic Technology

The progress in transgenic research of safflower has been very slow, with only few reports on demonstration of development of transgenic safflower using antibiotic-resistance genes. So far, there has been only one successful report from a Canadian Company, SemBioSys Genetics, Inc., which is planning to launch its pharmaceutical and nonpharmaceutical products, in near future, using oleosin-fusion protein technology. The snail-paced progress has been observed to be primarily due to nonamenability of the plant material to the transformation procedures, inability of

the transformed tissue/sectors to regenerate and survival of the transformed plants. To overcome these shortcomings, concerted efforts are required to identify the candidate genes for carrying out value addition to the safflower oil for therapeutic, nutritional and industrial use, improvement in the current transformation techniques, improvement in the turn over rate of transformed plants by improving the overall efficiency of the regeneration and optimization of the survival conditions of the regenerated transformed plants. The future research areas in safflower would entail development of varieties/lines with modified fatty acid profile, thin hull, nonspiny, disease and stress resistance, oils with nutritional fortification, etc.

3.4 Specific Details for Intellectual Property Right (IPR)

Moloney *et al.* (2005) had obtained a US patent on the methods of insulin production in plants (US 2005/0039235A1). In the patent, they have provided a method for expression of human insulin gene in seeds. SemBioSys Genetics, Inc. has been able to achieve significant patent protection for oilbody/protein production.

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Common Bean

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1. INTRODUCTION

1.1 History, Origin, Botanical Description, and Taxonomy

The *Phaseolus* genus consists of more than 30 species, but only five (*P. acutifolius*, *P. coccineus*, *P. lunatus*, *P. polyanthus*, and *P. vulgaris*) have been domesticated (Debouck, 1999). The common bean (*P. vulgaris* L.) is the most widely cultivated species, occupying more than 85% of the total area cropped with *Phaseolus* throughout the world (Singh, 2001).

The diversity detected in *Phaseolus* species in relation to common bean is organized into primary, secondary, tertiary, and quaternary gene pools (Singh, 2001). The primary gene pool includes the modern and old cultivars, improved lines, and wild types of *P. vulgaris*. Within the primary gene pool, there is no intercrossing problems and normally includes the most cultivated species of the genus (*P. vulgaris*). The secondary gene pool consists of the species *P. coccineus*, *P. costaricensis*, and *P. polyanthus* (Broughton *et al.*, 2003). These three species intercross easily *inter se* and also cross with *P. vulgaris* without requiring embryo rescue, especially when the common bean is used as the female parent (Singh *et al.*, 1991). However, in some crosses partial sterility may occur in certain individuals, especially when the *P. vulgaris* is used as the male parent. The tertiary

gene pool consists of *P. acutifolius* and *Phaseolus parvifolius*, which are species that produce fertile progeny without requiring embryo rescue when intercrossed. Embryo rescue is required, however, when they are crossed to *P. vulgaris*. Normally, one or two backcrosses to *P. vulgaris* are needed to restore fertility. Again, the use of *P. vulgaris* as female, both in the cross and in the backcross with *P. acutifolius* increases the chance of obtaining fertile individuals in the segregant population. Fertile progeny is not obtained from crosses between the quaternary gene pool and *P. vulgaris*, even using the embryo rescue technique. This germplasm includes the *Phaseolus angustissimus* and *P. lunatus* species, which to date have not been proved to share genes and alleles with *P. vulgaris*.

There are approximately 30 300 *P. vulgaris* accessions in the germplasm bank at CIAT (International Center for Tropical Agriculture), of which approximately 29 000 are cultivated common bean accessions and 1300 are wild type. This bank also contains further 1000 accessions of secondary germplasm and more than 350 accessions of tertiary germplasm (Broughton *et al.*, 2003). There are thousands of other accessions of the primary, secondary, tertiary, and quaternary gene pools distributed in germplasm banks throughout the world. However, most of this variability has not yet been used directly in the common bean breeding programs (Miklas, 2000). The introgression and pyramiding of favorable

alleles between and within this germplasm could increase the genetic base of the segregant populations, maximize genetic gain from selection, and increase the stability of disease resistance incorporated in the new cultivars.

Although the common bean (*P. vulgaris*) dispersion process is not fully understood, there is a general agreement that its origin is in the American continent. Dispersion seemed to be noncentric, having started from Mesoamerica or South America, or independently from both regions. The agreement among specialists was reached after most evidence confirmed the theory (Singh, 1989; Singh *et al.*, 1991). The common bean dispersion process continued. It was taken to the old world by the Europeans after the discovery of the United States in the 15th century. The study by Vavilov (1951) supported the hypothesis of origin in the United States for the *Phaseolus* genus. The author showed that the center of genetic diversity of the common bean species *P. vulgaris*, *P. coccineus*, *P. lunatus*, and *P. acutifolius* is located in Mexico and Central America, because it is in this area that the largest diversity of forms of these species was found. A secondary center of genetic diversity was identified in a mountain area in Peru. Although the center of diversity and center of origin do not have the same meaning, the discovery by Vavilov was a strong indication in favor of the American origin of the common bean (Vieira *et al.*, 1999).

Morphological evidence showed that the wild-type bean, ancestor of the common bean, is extensively distributed in the United States, from West Mexico to Northeast Argentina, covering an almost continuous range of approximately 7000 km of mountain areas. Along this area, morphological differences that might affect the adaptation of the wild-type bean to contrasting environmental conditions were observed (Kami *et al.*, 1995). There are botanical differences among the wild beans found in Mexico and Central America (*P. vulgaris* var. *mexicanus*) and those in South America (*P. vulgaris* var. *aborigineus*). However, there are several traits (climbing plants, with a large number of small and dehiscent pods, with small, hard seeds that are difficult to germinate) that are common to the wild types but not to the cultivated forms (*P. vulgaris* var. *vulgaris*). This species underwent extensive modification in its characteristics during the domestication

process, and the current cultivars present a small number of flowers, pods, and seeds, which is a consequence of the smaller proportion of flowers that reach maturity compared to the wild types. In addition, modern cultivars have more restricted plant growth and more compact form, making them shorter and more erect; larger leaves; more robust stem; larger flowers, seeds and pods; fewer seeds per pod; seeds more permeable to water, that allows uniform germination and shorter cooking time; suppression of the seed dispersion mechanism; decrease in the percentage of fiber content in the pods and neutrality to photoperiod.

The wild beans cross easily with the modern *P. vulgaris* cultivars, producing fertile F₁ and F₂ generations, indicating that the wild and cultivated forms belong to the same species and no reproductive mechanism has emerged to isolate them. According to Singh (2001), the genetic differences between the two forms seem to affect only a small proportion of loci, specifically the occurrence of the *D1-1* gene in the Middle American and *D1-2* in Andean wild and cultivated populations.

Studies on the domestication and organization processes of the common bean genetic variability was first based on morphological traits and biochemical markers. In the late 1980s, after the discovery of polymerase chain reaction (PCR) methodology, a number of molecular marker technologies have been developed. The direct analysis of DNA variation allowed a more precise estimation of the genetic variation that can be accessed at the nuclear and/or organelle genomes, enabling the investigation of different aspects of the genetic structure of crops species. The characterization of genetic diversity among common bean accessions from divergent gene pools using molecular markers, has been shown to be effective to elucidate mechanisms of origin and evolution of common beans, to investigate the effects of domestication process in the reduction of the genetic diversity that has characterized common bean gene pools, and to allow an efficient management and effective exploitation of the germplasm (Gepts, 2004). These studies have been performed using molecular markers that access specific genetic polymorphisms at a known or anonymous DNA sequence site, including random amplified polymorphic DNA (RAPD) markers that showed to be effective to identify

ances within the Mesoamerican gene pool (Beebe *et al.*, 2001), the use of amplified fragment length polymorphism (AFLP) markers that showed to be effective to assess the level and direction of gene flow between wild and domesticated bean populations (Papa and Gepts, 2003) and the analysis of variability at specific translated genomic regions that allowed to conclude about the level of diversity between wild and domesticated common beans (McClellan *et al.*, 2004). More recently, using microsatellite markers (simple sequence repeat, SSR), new findings where the intrapopulation diversity of the Andean gene pool was higher than within the Mesoamerican gene pool groups was reported by Blair *et al.* (2006a), opening new perspectives for the exploration of the genetic variability at the Andean gene pool.

Over the last years, several research groups have made efforts in the development of microsatellite markers for common bean, those are considered the most informative class of markers. Microsatellites, that present the advantages to be co-dominant, multiallelic, and PCR-based, were initially derived from GenBank sequences by Yu *et al.* (1999, 2000), followed by Guerra-Sanz (2004) and Blair *et al.* (2003). Methods of microsatellite discovery based on enriched genomic libraries were developed by Gaitán-Solís *et al.* (2002), Yaish and Pérez de la Vega (2003), and Buso *et al.* (2006). The development of SSR markers derived from bacterial artificial chromosome (BAC) was conducted in common beans by Caixeta *et al.* (2005).

Johns *et al.* (1997) studied the genetic variability in the common bean domestication centers using RAPD markers and morphological traits. The authors reported that their RAPD markers were capable to screen the genotypes into well-defined groups, which corresponded to the Mesoamerican and Andean domestication centers, whereas the morphological markers were less efficient in identifying the origin of the germplasm. The indication is that these data of diverse nature are representing different portions of the total variability because they probably suffered distinct evolutionary pressures. According to Gepts (1991) this apparent paradox was related to the different type of gene actions that control these markers. Some common bean morphological traits are controlled by genes with large phenotypic effect and are thus subject to human selection. On

the other hand, biochemical and molecular traits rarely have a marked effect on the phenotype and are less likely to suffer selection. Consequently, the chance of perpetuating mutations in biochemical and molecular traits is smaller than the chance of maintaining mutations that cause large morphological alterations, which affect the total variability accessed by each one of these markers.

1.2 Economic Importance

Common bean is an important source of protein in the diet of more than 300 million people throughout the world. It is especially important in tropical and subtropical developing countries, with significant regional variations concerning taste and grain type preference. The grains have high protein content, high quantity of complex carbohydrates, fiber, oligosaccharides, and phytochemicals, and are also an important source of iron, phosphorus, magnesium, manganese, and to a lesser extent zinc, copper, and calcium (Broughton *et al.*, 2003).

The common bean grain quality traits include low dietary fiber content (6%) compared to other products such as wheat (10.5%), maize (9%) (Juliano, 1993), soybean meal (20.6%), oat flakes (6.5%) (Cozzi and Lajolo, 1991), but a greater content than brown rice (4%) (Juliano, 1993). A quantity equivalent to one cup of common bean supplies at least half of the daily requirement of folic acid, which is especially important during pregnancy. It also supplies 25–30% of the recommended daily requirements of iron, 25% of magnesium and copper, and 15% of potassium and zinc.

The composition of the common bean (*P. vulgaris* L.) grains provides several health benefits for human nutrition. The grains are indicated in the dietary treatment of several diseases such as heart diseases, diabetes mellitus, obesity, and cancer (Geil and Anderson, 1994). Beans are a good source of food fiber, especially soluble fiber (Kutos *et al.*, 2003). The consumption of soluble fiber-rich foodstuffs has efficiently decreased total cholesterol serum levels and, consequently, reduced cardiovascular diseases in the population (Glore *et al.*, 1994).

There are 119 producing countries in the world if all genera and species included as

common beans in the FAO statistics (FAO, 2004) are considered. The common bean (*P. vulgaris*) is the most widely cultivated species of the *Phaseolus* genus. FAOSTAT places world common bean production at around 22 million tons cropped in approximately 35 millions ha. Only 10 countries account for approximately 70% of the world production; India is the largest producer with 14.31%, followed by Brazil with 13.39%, Nigeria (10.4%), Myanmar (7.58%), and Mexico (5.25%). Only 15% of the total common bean production worldwide is exported, with five countries accounting for 80% of the market: China 21%; Myanmar 27%; the United States 10%; Canada 9%, and Argentina 13% (FAO, 2004).

1.3 Traditional Breeding of Common Bean

1.3.1 Objectives in common bean breeding

A sustainable plant breeding program must envisage the prebreeding, breeding, and postbreeding stages of cultivar development. Extra effort can be directed to a specific stage requiring greater progress at any point of time. The breeding and postbreeding stages normally receive most attention in a common bean cultivar development program. On the other hand, the prebreeding stage has been neglected, in part due to the need for interdisciplinary work, better qualified human resources, high costs, and long-term returns. Strong prebreeding programs can contribute to the synthesis of new promising broad genetic base populations, to the identification of potentially useful genes and alleles. It would also enhance knowledge of the germplasm of the species and help establishing nuclear collections to ensure continuous gains in the plant breeding programs.

The objective of most breeding programs is to increase yield and guarantee yield stability at a production cost that maximizes the economic returns to the farmers. Under favorable conditions, yield increases through breeding are achieved by accumulating alleles that maximize biomass production and foster efficient assimilate distribution. In unfavorable environments, stable yield and reduced production costs can be obtained through breeding for tolerance to biotic and abiotic stresses that cause yield losses. Regarding the biotic stresses, diseases cause the most losses

and, therefore, always deserve special attention in the major breeding programs. In addition to improving the level of resistance to the most common disease pathogens that cause anthracnose, common bacterial blight (CBB), rust, angular spot, and common mosaic, a breeding program is also expected to develop stable resistance to pathogens of potentially dangerous new diseases, for example, *Curtobacterium* blight, scab (caused by *Colletotrichum truncatum*), Asian rust and soil diseases, especially white mould, root rot, and wilts. There is the challenge of obtaining resistance to the *Bean golden mosaic virus* (BGMV) and to the *Bean golden yellow mosaic virus* (BGYMV).

The fact that common bean cropping has become an important agricultural business changed the relative importance of the cultivar traits to the farmers. Growers currently seek cultivars with erect plant canopy (with pods that do not touch the soil, with short guides and closed branching) to allow mechanical harvesting, with low loss indices, and high grain quality. Cultivars must also allow good plant aeration in the field and decreased disease incidence. Earliness has become an important trait for growers, since it allows larger flexibility in the cropping system management, greater water and electricity economy in irrigated crops, and better chance of escaping seasonal droughts, insects, and diseases, and also gives a faster return on invested capital. The ever more frequent water shortage problems associated with the greater likelihood of paying for irrigation water indicate that drought tolerance has become an essential trait for future common bean cultivars. Movement of common bean cropping areas to higher temperature regions and the emerging problem of global warming have prompted the breeding programs to work on the development of high temperature tolerant cultivars. There are large common bean areas cropped under drought and high temperature conditions, where the subsistence family agricultural system predominates and the common bean plays a fundamental role in the food safety of millions of people. Genotypes adapted to these conditions must be developed.

The consumer demand for common bean grain showing higher nutritional, organoleptic, and functional quality has made this area of research a priority for some genetic breeding programs in the world. The most common agents

that affect mineral bioavailability are phytates, tannins, fibers, polysaccharides, and oxalates. The polyphenols (tannins) and phytic acid are part of the antinutritional compounds found in the common bean, and may be involved in the development of the hard-to-cook effect (Hohllberg and Stanley, 1987), which increases cooking time, decreases palatability, and reduces protein digestibility (Reyes-Moreno and Paredes-López, 1993). Polyphenol compounds, which consist of polyphenol acids and their derived substances, affect the nutritional quality of foodstuffs and their biochemical and physiological properties. These polyphenol substances are also partially associated with the changes that occur in the grains during storage, and play an important role in the development of postharvest grain hardening (Bhatti, 1990; Shomer *et al.*, 1990). Mineral availability, especially iron, is affected by the presence of tannin and dietary fibers. Commercial acceptance and consumption of common bean grains are influenced by an irreversible darkening of the grain tegument that depends on the cultivar and occurs gradually after harvesting. Prolonged storage periods, especially at high temperatures and relative humidity, considerably increase the cooking time and darkening of the grain. These characteristics are also influenced by the environment, as shown in the study carried out by Michaels and Stanley (1991) with 20 common bean cultivars in three different locations. Breeding for grain quality has long been reported (Meiners and Litzenberger, 1975; Ghaderi *et al.*, 1984). Current research has shown the possibility of decreasing the common bean antinutritional factors, such as phytates and phytic acid, and has identified the presence of wide variability within the species (Coelho and Lajolo, 1993; Kigel, 1999).

1.3.2 Common bean breeding: tools and strategies

The increase in yield potential of the common bean (*P. vulgaris*) crop has been gradual and low, in spite of the broad variability present for most traits, including grain yield (Nienhuis and Singh, 1988). One of the main reasons for this low increase in yield potential has been the consumer market requirement for different commercial grain types according to regions.

This requirement has reduced the germplasm resources used and limited the genetic variability available in the breeding programs. Also, the tendency of breeding new cultivars with erect stem with fewer ramifications has added to the difficulty of increasing the yield potential of modern cultivars. The use of genetically similar genotypes has become more evident after studies by Singh (1988, 1989), who grouped the common bean germplasm into 12 genetic pools and later further grouped them into six races (Singh *et al.*, 1991), indicating that breeders have traditionally carried out hybridization involving materials predominantly from a single gene pool. Singh (1988) observed that the variability for grain yield and its primary components and other agricultural traits was greater among than within gene pools. Similar result was also obtained by Abreu *et al.* (1999) assessing several agriculturally important morphological traits in common bean.

The genetic diversity studies of wild common bean ancestors measured by DNA markers suggest a considerable index of diversity spread within and among the gene pools (reviewed by Blair *et al.*, 2006a). In contrast, the genetic diversity of cultivated common bean is thought to be smaller than that of wild common bean due to a genetic bottleneck that occurred during crop domestication, dispersal from center of origin, and selection for specific traits (Gepts, 2004; Papa *et al.*, 2005). The introgression of valuable genetic variants from wild ancestors of common bean by means of applying marker-assisted backcrossing or genetic transformation strategies can help in broadening the genetic base of the elite breeding gene pool, increasing the chances of genetic gains in the breeding programs.

The use of genotypes from different gene pools as parents in crosses to obtain segregant populations increases the probability of obtaining genetic combinations with greater yield and production stability potentials. Singh (1988) reported that crosses between cultivars stemming from gene pools from the same center of origin and presenting similar growth habit and seed size showed greater frequency of desirable recombinants. This is probably due to the fact that crosses between very divergent parents can lead to low adaptation of the segregant population and, consequently, difficulties in the selection process. Theoretically, crosses between cultivars

from different gene pools but from the same center of origin should contribute to increase gain from selection, especially when adapted parents are used. More recently, an advanced backcross population derived from a cultivated Andean genotype and a Mesoamerican wild accession of common bean was developed and the results showed that this strategy was advantageous, since no fertility barriers were observed between the wild and cultivated gene pools, the derived lines themselves were close to commercial type, and new diversity for plant vigor and tolerance mechanisms were incorporated into the cultivated background (Blair *et al.*, 2006b). These findings opened new perspectives for the introduction of allelic variants from nonadapted genotypes into cultivars with modern traits, such as seed types and plant architecture.

Kelly *et al.* (1997) proposed a hierarchical pyramidal system with three levels of germplasm improvement for common bean. Germplasm movement is from the base to the top as gene accumulation for yield in specific genotypes occurs. Therefore, at the pyramid base the breeder works with several limiting factors using the full genetic variability present in the poorly adapted, wild type, interracial, and interspecific germplasm; at the intermediate level the number of limiting factors is restricted, but the breeder still uses considerable levels of genetic diversity; and at the top of the pyramid, elite germplasm breeding is carried out, involving agriculturally and economically acceptable materials within each commercial grain type.

A breeding program for common bean should be structured to meet the demand of growers and consumers of the new cultivars. The breeder should make use of the genetic diversity of the available germplasm, of the knowledge of the problems of the growing regions and of the knowledge of problems that might occur with the establishment of the crop in the different production systems and growing seasons. The selection criteria should emphasize well-defined regional demands such as preference for grain type, which includes characteristics such as size, color, shape, shininess, and cooking quality, and allow association of desirable traits during the development of inbred lines superior to the cultivars traditionally in use. The intent of increasing production to supply the external market has required breeding of special common bean types with large grains,

aiming at increasing product acceptance in the international market with higher aggregated value and differentiated price.

Common bean germplasm shows high levels of variability in grain type and size. However, the market requirements for grain type, as already commented, and the need for resistance to disease have limited selection progress through restrictions to germplasm utilization and to the number of progeny advanced, therefore increasing the genetic vulnerability (Smale, 1997; Abreu *et al.*, 1999) of the crop. Recurrent selection is a breeding method that has been used in common bean and has enabled the development of superior lines, without reducing the genetic variability. Because it involves a cyclic process of recombination and selection, yield alleles accumulate in segregant populations while maintaining high variability for other traits offering the breeder ways of exploiting immediately this variability by obtaining new inbred lines at each selection cycle. Recurrent selection in inbred species involves the stages of base population formation, endogamous family assessment and selection followed by recombination of the superior families. Ramalho *et al.* (1988) suggested that breeders using this breeding method should be aware of the following points: (1) parents used to form the base population should be divergent but should also express good phenotypes for the greatest number of traits of interest; (2) recombination will be efficient if the best families to be intercrossed are identified. In this case, recombination certainly will contribute to increase the frequency of favorable alleles in the population; and (3) selection of individual plants is not efficient, mainly if the trait has low heritability. Selection should be based on endogamous families assessed in replicated experiments. The general rule is to carry out a recombination cycle at every two generations of assessment; the first assessment in a single location, because of the small quantity of seeds, and the second in at least two locations. Therefore, superior family selection in each assessment cycle can be based on the joint analysis of the generations and locations.

The common bean haploid genome is considered one of the smallest among the legumes (Zheng *et al.*, 1991), estimated at 450–650 Mbp (mega base pair) (Bennett and Leitch, 1995) and has 11 chromosomes. According to Vallejos *et al.* (1992), in common bean the mean ratio

of the physical distance compared to the map distance is 530 kbp cM⁻¹. As the common bean genome has approximately 637 Mbp, it is estimated that the total size of the map of this species would be around 1200 cM. Several reports in the literature have demonstrated the efficient use of DNA markers to construct informative linkage maps with high coverage for common beans (for more details, see review by Miklas and Singh, 2007). In the early 1990s, genetic maps for common bean based mainly on co-dominant restriction fragment length polymorphism (RFLP) markers (Chase *et al.*, 1991; Vallejos *et al.*, 1992; Nodari *et al.*, 1993a), dominant RAPD markers (Adam-Blondon *et al.*, 1994) and combination of different classes of markers (Freyre *et al.*, 1998) were constructed. In recent years, due to the increasing number of microsatellite that became available, these markers have been extensively mapped in common beans (Yu *et al.*, 2000; Blair *et al.*, 2003, 2006a). The availability of high-density linkage maps based on a set of co-dominant multiallelic microsatellite markers will lead to the construction of an integrate linkage map for common bean allowing more precise positioning of markers, widely distributed across the genome, minimizing the occurrence of large interval without markers. These maps will expand the prospects of making comparative analysis, turning more real the advancement in quantitative trait loci (QTLs) detection, establishment of synteny and validations across different mapping populations, increasing the potential use of these linkage maps among the research groups. In addition, these co-dominants mapped makers could be placed on the corresponding BAC clones, that are already available for beans (Kami *et al.*, 2006), allowing us to probe insights into the aspects of the genome structures and organization of the *P. vulgaris* genome, to make an assessment of the correspondence between the physical and genetic distances and also to define physical intervals of desirable genome sequences in order to sequence and clone them.

Following the construction of linkage maps, genomic regions involved in the control of simple as well as complex heritable traits are being identified. QTL for important traits of interest have been detected in the genus *Phaseolus* using a variety of molecular marker classes and several types of segregating populations (Frei *et al.*, 2005;

Beebe *et al.*, 2006; Blair *et al.*, 2006b; Miklas *et al.*, 2006). A wide range of quantitatively inherited traits, such as domestication syndromes related traits, complex disease resistance traits, phenological traits, plant architecture, seed weight and yield components, where generally a few major genes control large proportion of the total variation, have been identified (Koinange *et al.*, 1996; Park *et al.*, 2001; Tar'an *et al.*, 2002; Blair *et al.*, 2006b). The experimental approaches used have been showed to be effective to successfully identify genomic regions that have a significant effect on the expression of QTLs and provide information about the number and the magnitude of the effects of the traits.

However, it should be pointed out that the identification of an important QTL in a particular genetic background and in a specific environmental condition may not be assumed to all genetic backgrounds and locations. When the genotypes are assessed in more than one location, year and/or cropping season, forming different environments, there is a possibility to identify and estimate the genotype by environment interaction (G × E) effects. There are several studies on common bean (Ramalho *et al.*, 1988; Takeda *et al.*, 1991; Melo *et al.*, 1997; Tar'an *et al.*, 2003) that report strong G × E effects for the main traits used in breeding in this species. Melo *et al.* (2002, 2004) and Teixeira *et al.* (2005) studied the QTL × environment interaction and ascertained that this interaction was significant for all the analyzed traits (flowering time, reaction to powdery mildew, angular spot, seed size, plant architecture, and grain yield), and the correlation among the means of the families in the different environments was mostly low, indicating that in the common bean the phenotypic expressions, and therefore that of the QTLs, depended strictly on the environment and on its specific interaction with each family of the population. Since in the common bean crop, G × E interaction is highly significant due to the diversity of cropping locations and seasons (environments) used, most QTLs are expected to suffer the effect of this interaction, and be expressed only under specific conditions. However, some stable QTLs that maintained their significant effects across more than one environment and were consistent with QTLs from previous studies in common beans, were reported (Melo *et al.*, 2002, 2004; Teixeira *et al.*, 2005) for days to flowering

and seed weight traits (Park *et al.*, 2000; Blair *et al.*, 2006b).

Most of the traits of agricultural importance are controlled by multiple genes, with small individual effects, subjected to a pronounced environmental effect, and dependent of the genetic background. Significant progress in map construction and QTL identification has been reported for common beans in recent years. A large set of microsatellite markers has been made available and, in addition, the growing pool of expressed sequence tag (EST) sequencing for *P. vulgaris* genome (Ramírez *et al.*, 2005) is making available molecular markers based on expressed sequences that could facilitate the direct establishment of probable relationships between candidate genes and specific QTLs through the development of a transcriptional map, opening the interesting opportunity to test these markers in association mapping experiments. To date, the molecular data generated could not be yet effectively used into the breeding programs. Progress on mapping experiments based on genetically more informative markers with a broad and regular genome coverage will support to look for useful homologies of QTL regions across independent crosses also helping to better elucidate aspects of the architecture of the important QTLs and also contributing to increase the map information mainly for traits that do not segregate within a single population (Brondani *et al.*, 2006). Furthermore, informative markers associated to QTLs controlling traits of interest will allow to explore, in an efficient and effective way, the allelic variation at these QTLs opening new perspectives for expanding the gene pool of cultivated common beans and identify potentially and useful source of genetic diversity to be introduced into the *P. vulgaris* cultigen.

As expected by breeders, the use of these markers will help particularly in the selection process to identify lines with desirable phenotypes, which are expressed in most of the environments, thus contributing to the increase in the efficiency of the common bean breeding programs.

1.3.3 Successes in common bean breeding

Common bean production takes place with several problems of biotic and abiotic origins. Common biotic problems are diseases caused by fungi,

bacteria, and virus, and damage from insect and nematode attack. The most common abiotic problems are low soil fertility, especially deficiencies in nitrogen, phosphorus and zinc, and toxicity from aluminum and manganese. Drought is also one of the most generalized abiotic problems affecting common bean yield in all producing regions of the world, especially in the Brazilian northeast, the central and northern Mexican highlands, East Africa, and the intermountain regions of the United States, where it causes production losses (Singh, 2001). Breeding programs have worked to increase tolerance to biotic and abiotic stresses to indirectly increase common bean yield potential and reduce sensitivity to environmental adversity. However, there is another line of work to directly increase the common bean productive capacity by increasing its productive potential in optimum management conditions, that is, under conditions of low environmental stress.

Yield increase in most crops can be attributed to the genetic gains obtained through improved cultivars, to better cropping technologies and agricultural practices, and also to the improvement of cropping environments. It is a combination of these factors that facilitate maximum yield per unit of area. Frequently, improved cultivars have been the main factor for yield increase and have provided the stimulus to adopt better agronomic and agrichemical practices, leading to an additional increase in yield (Singh, 1992).

1.3.4 Limitations of conventional breeding and potential of genetic engineering

Some of the challenges of common bean breeding are more difficult to overcome due to restrictions imposed by limited genetic variability within the species and also due to difficulties in trait assessment. One typical example of limitation is the low genetic variability for resistance to the common BGMV in Brazil, for sources of resistance is almost nonexistent. The BGMV and BGYMV, which occur in the main common bean producing areas in Latin America, are transmitted by the whitefly *Bemisia tabaci* Gen., and have been especially important in the dry growing season (Morales and Anderson, 2001; Morales, 2006). Annual crop reductions in the range of 90 000–280 000 tons, amount sufficient to feed

6000–20 000 adults, are estimated. In addition to the economic problems caused by yield reduction, this virus also brings other social consequences, since it precludes common bean cropping in family based agricultural systems. Approximately 180 000 ha are currently not apt for common bean cropping in the dry season in Brazil, due to the occurrence of BGMV. These areas can be returned to the productive process after cultivars with adequate resistance level are available. Although there have been advances in scientific knowledge, technological results with direct application for growers have not been achieved, suggesting that further research and adjustments of procedures are required in several areas. Immunity reaction to both the viruses has not yet been found in *Phaseolus* spp. accessions evaluated by national and international research institutions. Tolerance to the disease is controlled by polygenes and, therefore, difficult to be efficiently transferred to commercial cultivars (Pessoni *et al.*, 1997; Park *et al.*, 2001). However, literature reports on the genetic control of tolerance to BGMV are sometimes conflicting, with some authors also suggesting monogenic (Blair and Beaver, 1993) or oligogenic control (Blair *et al.*, 1993). There are also reports of cultivars that usually show certain degree of tolerance to the disease, but become susceptible and suffer severe yield losses when submitted to high infestations of the insect vector. This fact is aggravated by the failure of natural control measures and by the high cost of chemical control.

Regarding the implementation of marker-assisted selection (MAS) to supplement the common bean breeding programs for disease resistance, in the last decade, with the advantage and accessibility of the molecular marker technology, a number of markers for traits related to the major disease resistance with significant impact in the common bean agriculture have been successfully identified, as extensively reviewed by Miklas and Singh (2007). A list of markers linked to specific genes conditioning resistance to several pathogens causing bean diseases has been released for CBB, anthracnose, angular leaf spot, BGYMV, and bean rust (Kelly *et al.*, 2003). Markers linked to these major genes can be used to trace the presence of target genes through MAS procedures, where the genotype carrying the target allele is, by indirect way, selected for

the desirable phenotypes. Once important genes are tagged with a marker, selection in successive backcross generations are performed in order to select individuals in the progeny that possess a marker allele from the donor parent, improving the efficiency and facilitating introgression of the resistance genes into elite common bean genotypes (Oliveira *et al.*, 2002). However, due to the constant appearance of new races of a pathogen, there is a necessity to constantly incorporate new resistance genes into bean lines and cultivars. This problem has been partially overcome by the pyramiding of multiple genes for prevalent pathogen races into selected cultivars with MAS strategy, in order to achieve durable resistance. Lines resistant to angular leaf spot carrying resistance genes derived from selected cultivars were developed by Oliveira *et al.* (2005). In addition, advanced Mesoamerican bean lines with “Carioca-type” grains (Ragagnin *et al.*, 2005) and black and red bean cultivars (Costa *et al.*, 2006) have been developed with multiple disease resistance genes also through marker-assisted strategies. However, although significant progress has been made, some limitations for the extensive use of MAS as routine in the common bean breeding program still remain. As early pointed out by Kelly and Miklas (1998), the main limitations of success of MAS are: (1) the genomic sequence related to the marker is not sufficiently close to the resistant gene allowing that it segregates independently during the recombination; (2) most of the markers tightly linked to the target genes are not universal in all genetic backgrounds, making their utilization restricted to the population where the genes were mapped; (3) new races of the pathogens evolve constantly in different environments and, in addition, the pathogens rapidly overcome the resistance of the gene identified; (4) new cultivars with advantageous agronomic attributes are currently being released and the MAS process should be individually performed for each of the accession, taking time for the introduction of the desirable allele.

Drought tolerance in common bean, as in others crops, has dramatic consequences on production, mainly in developing countries, reducing the grain yield in more than 1.5 million ha of cultivated area in the world (Teran and Singh, 2002). Drought tolerance is considered as an example of a trait difficult, expensive, and inaccurate to assess,

because of the complexity of the environmental control. Water shortage is the climatic factor that contributes the most to harvest frustrations. Low crop yield due to water shortage in greater or lesser intensity is frequently reported in most of the common bean cropping regions. Flowering is the most vulnerable stage, followed by the grain filling period. In Brazil, common bean grown from January to April is highly constrained by water shortage, which leads to low and unstable yield. Considering that 44% of the Brazilian production is in this period, it is recommended that new cultivars adapted for sowing from January to April should be developed. As a part of the Generation Challenge Program initiative, phenotyping sites are being structured in Brazil in order to characterize genetic materials at the phenotypic level, testing for adaptability for drought tolerance and the effects on the productivity. The availability of these sites will allow several studies on drought phenotyping in common bean.

Regarding the potential of molecular markers to improve drought tolerance in common bean, there is available today a large set of drought tolerance QTLs and candidate genes previously identified for model crops, such as rice (Vinod *et al.*, 2006). These can be used as a starting point to search for homologous genes in other species, like *P. vulgaris*. Once candidate genes are identified, one of the ways to evaluate their association with the trait of interest is using the approach of association mapping studies, where the informative markers potentially identified as associated to drought tolerance traits could be used in MAS. Projects are being conducted in maize, rice, and beans, also as a part of the Generation Challenge Program initiative. All strategies discussed until now are related to common bean genetic variability to drought tolerance. However, other genes, alleles, promoters, etc., related to drought tolerance, are constantly being identified in other species, however, crossing barriers prevent their direct transfer by hybridization. Therefore, genetic engineering could be a powerful tool to obtain common bean cultivars more tolerant to drought.

2. BEAN GENETIC ENGINEERING

Several methods have been developed for inserting genetic information into plant cells, such as

Agrobacterium-mediated system, direct DNA uptake into protoplasts and particle bombardment. Due to the advances in the methodologies for gene delivery and plant tissue culture, it was assumed that plant transformation would become a routine for most important crops. Unfortunately, legumes, in particular common bean (*P. vulgaris*), became one of the greatest challenges to transformation efforts. Indeed, the ability to genetically engineer common bean is still not trivial (Aragão and Rech, 2001; Svetleva *et al.*, 2003; Popelka *et al.*, 2004; Veltcheva *et al.*, 2005).

Early efforts to transform common beans demonstrated its susceptibility to *Agrobacterium*, and some transgenic tissues or organs, such as calli, leaves, meristems, cotyledon, and hypocotyl have been achieved (Lippincott *et al.*, 1968; McClean *et al.*, 1991; Franklin *et al.*, 1993; Becker *et al.*, 1994; Lewis and Bliss, 1994; Brasileiro *et al.*, 1996; Nagl *et al.*, 1997; Karakaya and Ozcan, 2001). Mariotti *et al.* (1989) reported production of transgenic bean plants through utilization of the *Agrobacterium* system. However, there was no molecular evidence for genetic transformation or progeny analysis. Transient gene expression using either electroporation or polyethylene glycol (PEG)-mediated protoplast transformation was demonstrated (Crepý *et al.*, 1986; Bustos, 1991; Leon *et al.*, 1991; Giovinazzo *et al.*, 1993). Dillen *et al.* (1995) demonstrated the applicability of electroporation of intact tissue to introduce and express the *gus* gene in bean embryonic axes. A gene transfer system has been recently developed by Liu *et al.* (2005) using sonication and vacuum infiltration-assisted *Agrobacterium*-mediated transformation for Kidney beans, without any tissue culture step.

During the last two decades, efforts to achieve an efficient methodology for bean transformation were obstructed due to the lack of an efficient tissue culture system to regenerate bean plants from transformed cells. Numerous attempts have been made to regenerate bean plants from several types of isolated cells and tissues. Although no satisfactory results have been achieved, some methodologies have described shoot organogenesis (through multiple shoot induction) of the apical and axillary meristems from bean embryonic axis (McClean and Grafton, 1989; Malik and Saxena, 1992; Mohamed *et al.*, 1992, 1993). Cruz de Carvalho *et al.* (2000) employed the transverse thin

cell layer (tTCL) method to optimize the frequency of shoot regeneration without an intermediate callus stage. The same authors also showed that addition of 10 μM silver nitrate (AgNO_3) to the medium with benzylaminopurine (BAP) enhanced the number of shoots that developed per explant and increased shoot elongation.

2.1 Transformation Methods

The particle bombardment method was initially proposed by the group of John Sanford (Cornell University) with the objective to introduce genetic material into plant genome (Klein *et al.*, 1987; Sanford *et al.*, 1987). Since 1980s, the universality of application of particle bombardment has been evaluated, demonstrating to be an effective and simple process for the introduction and expression of genes in to bacteria, protozoa, fungi, algae, insects, mammals, plants, and isolated organelles, such as chloroplasts and mitochondria (Daniell *et al.*, 1991; Carrer *et al.*, 1993; Klein and Fitzpatrick-McElligott, 1993; Sanford *et al.*, 1993; Vainstein *et al.*, 1994; Bogo *et al.*, 1996; Rech *et al.*, 1996). The basis of the particle bombardment method is the acceleration of DNA-coated microprojectiles (particles of tungsten or gold) at high speed (about 1500 km h^{-1}) toward living cells. After penetration in the cell, the DNA dissociates from the projectiles and integrates into the chromosomes. Several devices (particle guns) have been constructed in order to accelerate these microprojectiles. All these systems are based on the generation of a shock wave with enough energy to move the microprojectiles. The shock wave can be generated through: a chemical explosion (Sanford *et al.*, 1987), discharge of gas helium (Sanford *et al.*, 1991; Finer *et al.*, 1992; Takeuchi *et al.*, 1992), vaporization of a drop of water through the electric discharge with high voltage and low capacitance (McCabe *et al.*, 1988) or low voltage and high capacitance (Rech *et al.*, 1991), discharge of compressed air (Morikawa *et al.*, 1989).

Particle bombardment allowed the bombardment of intact plant cells facilitating possible transformation of plants for which a regeneration system was not available. The status of *Phaseolus* tissue culture was well reviewed by Nagl *et al.* (1997), Svetleva *et al.* (2003), Popelka *et al.* (2004) and Veltcheva *et al.* (2005).

Although several authors have claimed the achievement of *P. vulgaris* regeneration, so far, only cytokinin-induced shoot organogenesis was obtained. Multiple shoots are formed in the peripheral regions of the apical meristem (Aragão and Rech, 1997). Induction of shoot formation in bean meristems can be achieved by culturing the mature embryos in the presence of cytokinins such as kinetin, zeatin, and BAP (Kartha *et al.*, 1981; Martins and Sondahl, 1984; McClean and Grafton, 1989; Franklin *et al.*, 1991; Malik and Saxena, 1992; Mohamed *et al.*, 1992, 1993; Araújo and Rech, 1997; Delgado-Sánchez *et al.*, 2006). Compounds such as thidiazuron (TDZ) and N-(2-chloro-pyridyl)-N'-phenylurea (CPPU) that possess cytokininlike effects have also been tried (Mohamed *et al.*, 1992).

Consequently, the apical region of embryonic axes became an obvious target for the development of a system based on the bombardment of meristematic cells. Early experiments showed the applicability of particle bombardment for introduction and transient expression of genes in apical meristematic cells (Genga *et al.*, 1991; Araújo *et al.*, 1992, 1993; Russel *et al.*, 1993). However, how deep the particle could penetrate in order to reach the cells that could generate transgenic plants has yet to be determined. The different parts of meristem have been divided into layers (L1, L2, and L3). The layer L1 is the most external and forms the epidermis of the differentiated regions. The layers L2 and L3 divide preferentially in the anticlinal and periclinal planes to form the organs. Several studies have demonstrated that the differentiated *de novo* shoots are originated from the subepidermal layers (L2 and L3) of the apical meristem. However, the L1 layer could participate in their formation (McClean and Grafton, 1989; Franklin *et al.*, 1991; Malik and Saxena, 1992; Mohamed *et al.*, 1992). The shoots are formed in the peripheral regions of the apical meristem (Aragão and Rech, 1997). The bombardment of *P. vulgaris* meristematic cells showed that it was possible to efficiently reach these layers, demonstrating that it would be possible to achieve transgenic plants (Aragão *et al.*, 1993; Figure 1).

Russel *et al.* (1993) were able to achieve transgenic navy bean (cv. Seafarer) plants using an electrical particle acceleration device. It was

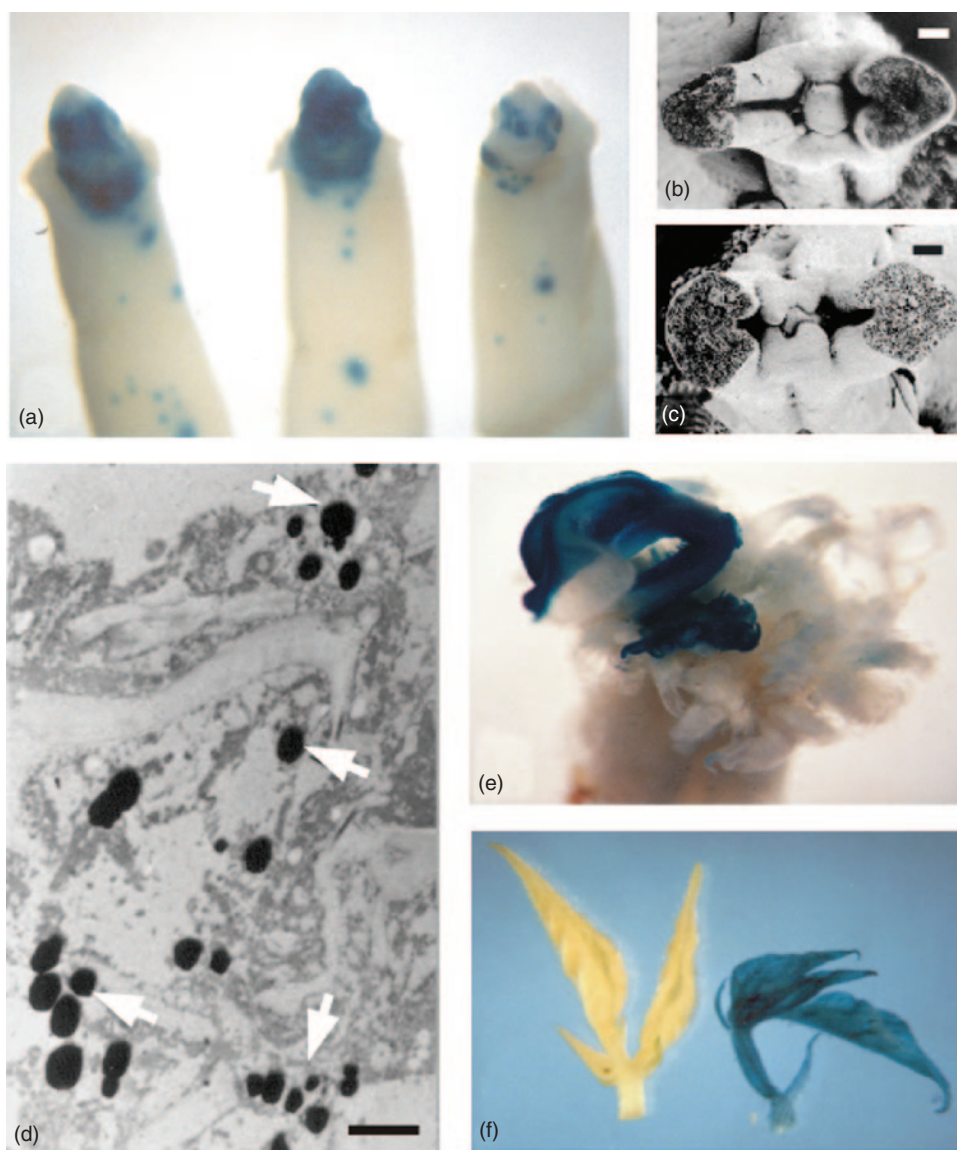


Figure 1 Bean transformation via microparticle bombardment. (a) Embryonic axes expressing the *gus* gene 24 h after bombardment. (b–c) Scanning electron micrograph showing the morphology of the shoot apical meristem regions of two varieties presenting the apical dome exposed (b) and partially covered by primordia leaves (c). (d) Transmission electron micrograph showing tungsten microparticles (arrows) penetrating up to the third cell layer from the meristematic apical region. (e) Transgenic shoot developing from a multiple shooting apical meristem. (f) Leaves from transgenic plantlets (right) and control (left). Bars represent 100 μm in b and c, and 2 μm in d

the first report of *P. vulgaris* transformation, presenting molecular evidences of transgenic progeny. However, the frequency of transgenic plants obtained was much lower (0.03%) and variety limited. In addition, the tissue culture protocol described was time consuming, involving several

temperature treatments and medium transfers of the bombarded embryos before recovery of transgenic shoots. Kim and Minamikawa (1996) achieved transformation bombarding embryonic axes, obtaining stable transformed bean plants (cv. Goldstar).

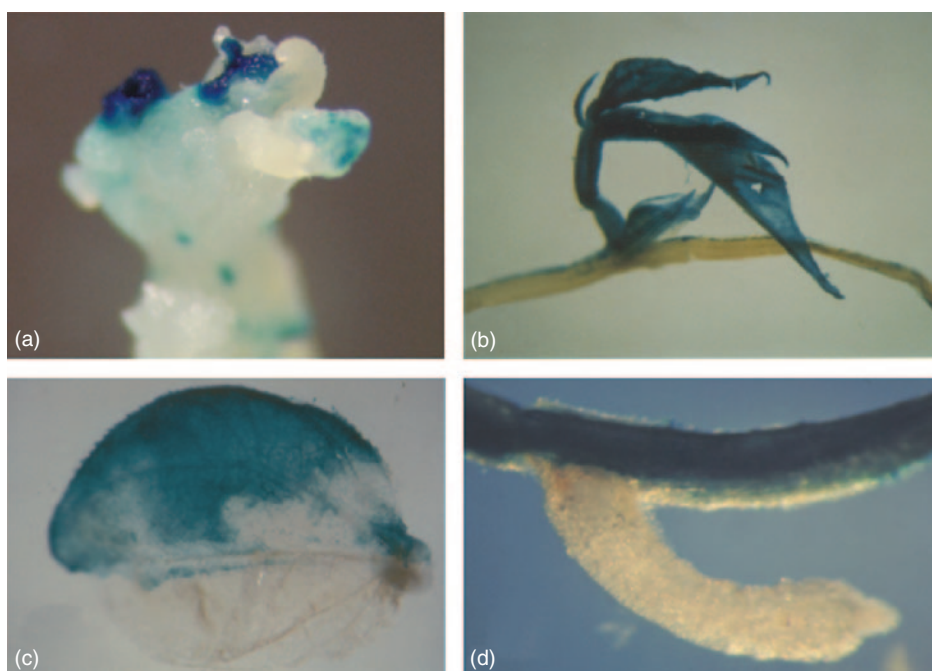


Figure 2 Occurrence of chimerism during the process of obtaining transgenic shoots from particle-bombarded apical meristematic region (a). Chimerical transgenic branch (b), leaf (c), and root (d) presenting *gus* gene expression

A reproducible system to achieve transgenic bean plants routinely was developed. The bean transformation system was also based upon the development of a tissue culture protocol of multiple shoot induction, shoot elongation and rooting (Figure 1). The average frequency of transformation (the total number of putative transgenic plants divided by the total number of bombarded embryonic axes) was 0.9% (Aragão *et al.*, 1996; Araújo and Rech, 1997). In addition, they have been able to transform several varieties of *P. vulgaris*, including those that were considered recalcitrant to transformation in previous studies. Molecular analysis and progeny test of several generations of transgenic lines revealed the presence of a small number of integrated copies of the foreign genes and segregation in a Mendelian fashion in most of them. This is extremely important in order to accelerate the introduction of these plants in a breeding program as well as the production of transgenic commercial varieties.

The shoots regenerated from the bombarded meristematic apical region may include all, some,

or none of the cells that received the transgenes. Consequently, undesirable periclinal, mericlinal, or sectorial chimeras can be produced in the adventitious shoots (Figure 2). Chimeric organs have been observed in transgenic common bean plants, such as leaves and roots (Figure 2). This limitation could probably be removed by using a more appropriated selection agent. It is difficult to have an efficient selection for transformed cells because only a few cells from the meristem are transformed and generally cannot be reached by the selective agents. Although antibiotics and herbicides, such as kanamycin and glufosinate ammonium (GA) have been used (Aragão *et al.*, 1996, 2002), this selection is not efficient. Recently, this group has developed a novel system for selecting transformed meristematic cells based on the use of imazapyr, a herbicidal molecule capable of systemically translocating and concentrating in the apical meristematic region of the plants. This selectable marker coupled with an improved multiple particle bombardment protocol, resulted in a significant increase in the

recovery of fertile, transgenic material compared with standard soybean transformation protocols (Aragão *et al.*, 2000). This selection system is being successfully used to introduce genes into dry bean and chimerical plants have not been observed.

Genetically modified common bean have also been obtained by using linearized vectors (Vianna *et al.*, 2004; Bonfim *et al.*, 2007). Although the frequency of transformation is lower (0.2–0.6%), the use of linear vectors is important to eliminate undesirable antibiotic selective genes.

Particle bombardment has been considered a universal method to introduce macromolecules into any living cell, not limited by genotype or variety (Sanford, 1990). However, the morphology of the explants utilized during bombardment may greatly influence the successful recuperation achievement of transgenic bean plants (Aragão and Rech, 1997). In some cultivars, the embryonic axes revealed the apical meristematic region partially exposed, whereas only the central region could be visualized (Figure 1). The number of meristematic cells, which could be reached by the microparticle coated-DNA, will be drastically reduced. Consequently, the efficiency of transformation could also be reduced. Several studies have shown that *de novo* shoot differentiation in embryos of bean grown on cytokinins appeared in the peripheral layers of the meristematic ring (McClellan and Grafton, 1989; Franklin *et al.*, 1991; Malik and Saxena, 1992; Araújo and Rech, 1997). Thus, based on these concepts, cultivars with a nonexposed apical meristematic region are not suitable for transformation using particle bombardment, considering that removal of the leaf primordia is not practical.

Although the common bean is susceptible to *Agrobacterium*, only recently stable transformation mediated by *Agrobacterium* was demonstrated. Liu *et al.* (2005) reported the development of an *Agrobacterium*-mediated transformation system based on sonication and vacuum infiltration of *Agrobacterium tumefaciens* (LBA4404) into germinated kidney bean embryos (var. Green Light). Inoculated germinating seeds of kidney bean were sown in soil pots and from a total of 525 surviving plants, 16 were transformed (Liu *et al.*, 2005). The transgenes were detected up to the second generation.

2.2 Introduction of Useful Traits

The methionine-rich 2S albumin gene from Brazil nut (*be2s1* gene) was the first agronomically important gene expressed in common bean tissues (Aragão *et al.*, 1992). Mature embryos were transformed in order to have transient expression of a methionine-rich albumin gene from Brazil nut (*be2s1* gene), which could be detected by Western blot and enzyme-linked immunosorbent assay (ELISA), 24 h later (Aragão *et al.*, 1992). Further, stable genetically engineered common bean lines containing the *be2s1* gene were obtained aiming to improve the methionine content in the seeds. The transgene was stable and correctly expressed in homozygous R₂–R₆ generations. In two of the five transgenic lines, the methionine content was significantly increased by 14% and 23% over the nontransgenic plants (Aragão *et al.*, 1999). However, 2S albumin from Brazil nut was identified as an allergen (Nordlee *et al.*, 1996; Koppelman *et al.*, 2005). Indeed, transgenic soybean containing the Brazil nut 2S albumin was allergenic to patients. Consequently, the development of a transgenic common bean variety with improved methionine content was aborted.

Russel *et al.* (1993) introduced the *bar* gene that confers resistance to the herbicide phosphinothricin and the coat protein gene from bean BGMV in an attempt to achieve virus-resistant plants. The introduced *bar* gene showed to confer strong resistance in transgenic bean plants to the herbicide in the greenhouse. However, bean plants containing the BGMV coat protein gene did not show resistance to the virus (D. Maxwell, personal communication).

The 5' regulatory sequences from several genes have been transient and stably studied in bean tissues transformed using the particle bombardment. Regulatory regions from the seeds specific promoter from Brazil nut 2S gene were studied in bean embryos (Grossi de Sá *et al.*, 1994; Vincentz *et al.*, 1997). Transgenic bean plants were produced containing the β -glucuronidase gene (*gus*) under the control of the canavalin gene promoter (Con A) from jack bean. Either the organ and maturation stage-specific promoter regulation was studied in seeds of transgenic plants (Kim and Minamikawa, 1996, 1997).

Transgenic bean lines containing the *bar* gene, which encodes phosphinothricin acetyl

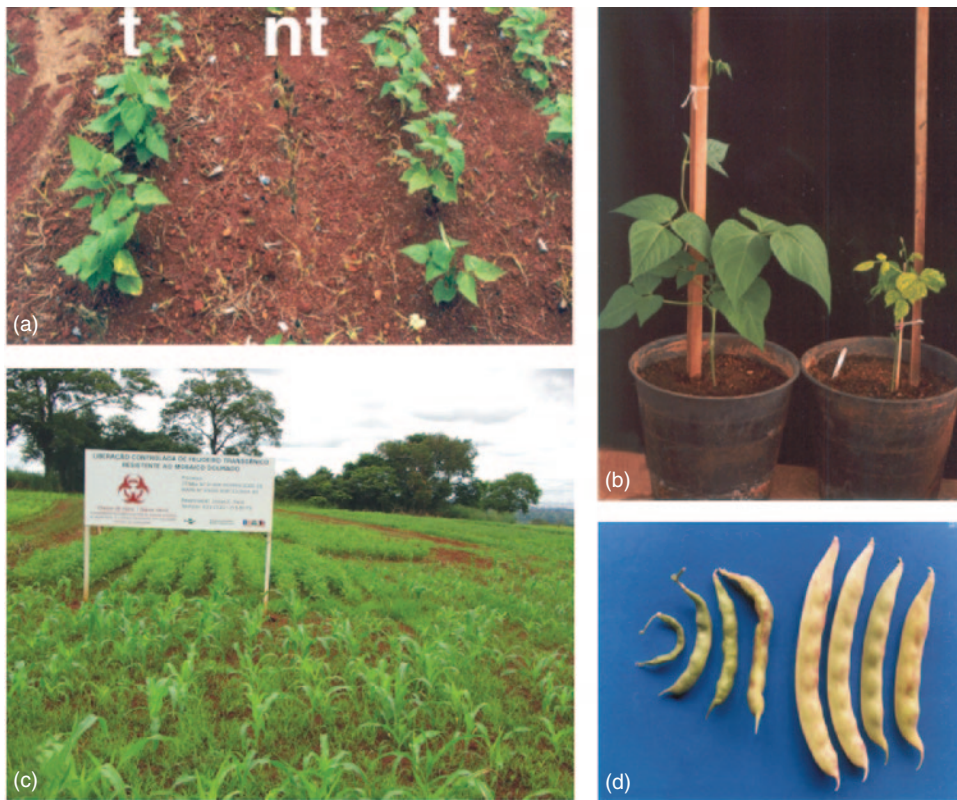


Figure 3 Field trials with genetically modified common bean plants. (a) Transgenic lines (t) tolerant to 400 g ha⁻¹ of glufosinate ammonium compared to a nontransgenic line (nt) treated with the herbicide. (b) Transgenic plants resistant to the BGMV (left). (c) Lines resistant to BGMV being tested in the field. (d) Effect of virus infection on pods of nontransgenic plants (right) and transgenic (left) plants, both inoculated

transferase, and resistant plants to the herbicide GA were generated (Russel *et al.*, 1993; Aragão *et al.*, 2002) and tested in field (Aragão *et al.*, 2002; Figure 3). Two transgenic events were tolerant to 500 g ha⁻¹ of GA, under green house conditions, with no visible symptoms and developmental growth. Field evaluation has shown that the plants tolerated up to 400 g ha⁻¹ of GA (Aragão *et al.*, 2002; Figure 3).

In order to obtain common bean plants resistant to BGMV, the genes *Rep-TrAP-REN* and *BCI* from the virus were cloned in antisense orientation under the control of the CaMV 35S promoter and used to transform bean. Two transgenic lines were obtained both of which had delayed and attenuated viral symptoms (Aragão *et al.*, 1998). Using the strategy of transdominance, transgenic bean lines were obtained with a vector contained

the mutated *rep* (*ACI*) gene from the BGMV. The mutated *rep* gene codes for a mutated AC1 (REP) protein with amino acid codon change in the putative NTP-binding motif (D262R). One line exhibited resistance to the virus. However, the resistance was studied during several generations and depended on the inoculation level (Faria *et al.*, 2006). More recently, the concept of using RNA interference (RNAi) construct to silence the *ACI* viral gene has been explored to generate highly resistant transgenic common bean plants (Bonfim *et al.*, 2007). Eighteen transgenic common bean lines were obtained with an intron-hairpin construction to induce post-transcriptional gene silencing against the *ACI* gene and one line exhibited high resistance (approximately 93% of the plants were free of symptoms) upon inoculation at high pressure (more than 300 viruliferous whiteflies per

plant during the whole plant life cycle) and at a very early stage of plant development.

Transgenic kidney bean plants were obtained expressing a group 3 LEA (late embryogenesis abundant) protein gene (*ME-leaN4*) from *Brassica napus* (Liu *et al.*, 2005). Plants showed enhanced growth ability under salt and water stress. The increased tolerance was also reflected by delayed development of damage symptoms caused by drought stress. In addition, transgenic lines that presented high level of *ME-leaN4* gene expression showed higher stress tolerance than lines with lower expression level (Liu *et al.*, 2005).

2.3 Field Evaluation

Studies on the behavior of transgenic common bean plants under field conditions have been conducted. The first field trial was carried out from November 2000 to February 2001 in Distrito Federal, Brazil to evaluate the resistance of the T₂ generation of an event tolerant to glufosinate ammonium (Aragão *et al.*, 2002; Figure 3). Since then, several transgenic lines resistant to the BGMV have been evaluated in the field at Embrapa Rice and Bean (Goiás, Brazil) (Figure 3). In these studies, it is appraised the interactions of the transgenic plants with microorganisms, insects, and others plants from of the agricultural and natural environment. Moreover, the stability of foreign genes expression, gene flow, and factors related to their interaction with the complex physiology of these plants exposed to natural stress in tropical are being evaluated. Furthermore, food biosafety analysis is being carried out by the Biosafety Network from Embrapa to determine differences in nutritional and antinutritional compounds as well as verify presence of toxic molecules.

3. FUTURE PROSPECTS

Several species of the genera *Phaseolus*, such as *P. coccineus*, *P. acutifolius*, and *P. angularis* (Nagl *et al.*, 1997), have been regenerated in the *strictu sensu*, e.g., plants regenerated *de novo* from undifferentiated cells originated from differentiated cells. The extrapolation of these technologies to *P. vulgaris* would be a breakthrough toward

the development of a transformation system to accelerate the generation of new transgenic commercial varieties. The ideal system seems to be the regeneration of fertile mature plants through somatic embryogenesis or organogenesis, preferentially from mature embryonic axes, which has been considered the most adequate tissue for transformation either by *Agrobacterium*, electroporation and particle bombardment. The improvement in the bean transformation technology is essential for obtaining a larger number of transgenic lines, increasing the probability to select transformation events presenting the desirable phenotype. Depending on the introduced trait, only one of 30–40 independent transgenic plants obtained has the desirable transgene expression without undesirable characteristics, such as multiple transgene loci and nontransference of the foreign genes to the first generation.

Since the first report of transformation of *P. vulgaris* in 1993, a few groups have transformed bean to introduce useful traits. Difficulties that still exist in obtaining transgenic plants might account for this fact. Nevertheless, during the last two decades, efforts to achieve efficient methodologies for regeneration and transformation of common bean plants advanced. In parallel, the availability of characterized genes (coding and regulatory sequences) has increased as a direct result of several structural and functional genomics projects in plants.

An increased understanding of common bean genome associated with contributions from biotechnology will provide an opportunity for breeders to accelerate the development of new varieties with valuable agricultural traits. Common bean has a relatively small genome of about 630 Mb with 11 haploid chromosomes and genomics studies are in progress because of its social and economic value (Vallejos *et al.*, 1992; Blair *et al.*, 2003; Broughton *et al.*, 2003; Nodari *et al.*, 1993b; Pedrosa *et al.*, 2003). The Phaseomics project (<http://www.phaseolus.net>) was started in 2001 by an international consortium of laboratories with an aim of developing high yielding bean varieties that have high protein quality and are stress and disease resistant. They plan to initially sequence large scale ESTs from different tissues of *P. vulgaris*, which will be followed by sequencing, and analysis of the bean genome. At this moment, about 47 300 nucleotide sequences from

genus *Phaseolus* are released (about 26 200 from *P. vulgaris*) and these numbers are increasing rapidly. These facts generated an excellent scenario for introduction of useful traits as well as study gene function in *P. vulgaris* plants. There is a considerable interest in the introduction of genes for several useful traits in common bean, such as virus, insect, bacteria, and fungi resistance, environmental stress tolerance, and improve nutritional properties. In addition, manipulation of plant architecture and phenological characteristics might facilitate management, increasing yield, quality, and diversity. Several traits might be manipulated by genetic engineering, such as plant life cycle and the transition from vegetative to reproductive growth, controlling flowering time. Indeed, several genes controlling phenotypical traits have already been cloned and characterized in distinct organisms. This information can be used to either express heterologous genes or suppress the expression of homologous genes in common bean plants. Biotechnological tools complement those from classical breeding and have the potential to accelerate the generation of new varieties containing genes from agronomic traits, which are difficult to be found in the primary or secondary gene pool.

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Cowpea

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1. INTRODUCTION

Legumes provide high-quality protein food for people, protein-rich fodder for livestock, and fixed nitrogen for the soil. In Africa, all three are in short supply. And in Africa, where it took its origin as an agricultural crop, cowpea (*Vigna unguiculata* var. *unguiculata*) (L.) Walp.) is the most important legume, at least in terms of economics (Langyintuo *et al.*, 2003). Cowpea is also known as blackeye pea, southern pea, frijole, lubia, feijao caupi, and niébè. One of its distinguishing features is its adaptation to the hot, low-and-erratic rainfall climates of the Sahelian and Sudanian zones in Africa. Historical records of the domestication of cowpea are sparse, but it may have been domesticated and spread as a crop together with sorghum and pearl millet (Steele, 1976). Today, over much of its range in Africa it is still widely grown in the traditional way, intercropped with sorghum, millet, maize, or cassava. Cowpea grown in monoculture may be increasing as farmers begin to realize its potential as a source of cash income. It is mainly grown by low-resource farmers who prize it for its ability to yield well on poor soil with a minimum of moisture—as little as 300 mm annually. In addition to providing food and fodder

it is a cash crop sold by women growers in local markets and by increasing numbers of commercial farmers in the ancient and substantial regional trade system. For Africa, which includes about 80% of the cowpea growing area of the world (Langyintuo *et al.*, 2003), there are potential—but almost completely undeveloped—export markets in South America and Europe.

There are good reasons for the economic importance of cowpea; one of them being the excellent nutrition it offers. At different places and times in Africa the grain, the green pods, the dried leaves, and hay all command good market prices. One factor driving demand is the high-quality protein it offers. On average, the grain contains about 23–25% protein by weight. Dried cowpea foliage is likewise protein rich, offering on a dry weight basis levels similar to the grain (Ohler *et al.*, 1996). In many parts of Africa, fresh tender green cowpea leaves picked before flowering are the first part of the crop harvested. These leaves provide needed protein during the period Africans call the “hungry time” (“lokotchin yinwa” is the name for it in the Hausa language). This is the time when the harvest of the previous year has been sold or consumed and food is scarce before the next harvest comes in.

In parts of East Africa, there is a substantial commercial market for dried leaves, and in the Sahel, cowpea hay sometimes commands very high prices. In Senegal in recent years green pods have become very popular as a cash crop; women selling basins of green pods dot the roadsides when the pods are maturing in the fields. In addition to protein, cowpea grain is an excellent source of bulk carbohydrate (CHO); indeed, in this regard it is nearly as good as cereals, containing roughly 60% CHO by weight, principally starch. Cowpea grain also offers key vitamins including thiamin, riboflavin, ascorbic acid, niacin, and folic acid. It is low in fat, containing about 1% by weight, and it represents a fair source of fiber at about 6%. It is relatively low in sulfur amino acids but high in lysine and other essential amino acids, making it a good complement to the mainly cereal diets. Thanks to the nutrition it offers, cowpea has been considered by the US National Aeronautics and Space Administration as a possible space station crop (Ohler and Mitchell, 1992).

Cowpea provides excellent ground cover and so helps to preserve precious moisture in the semi-arid zones where it thrives. Thanks to its ability to fix nitrogen (Bado *et al.*, 2006), it adds substantially to soil fertility as well.

Investment in cowpea research and extension has been modest-to-poor at best. This is partly because it has always been mainly a crop of impoverished regions, with sub-Saharan West Africa and northeastern Brazil being by far the major cowpea-growing areas of the world. African governments, which might be expected to invest in cowpea improvement in their own self-interest, have largely neglected it. This neglect was easy in the case of cowpea—widely known as “poor man’s meat”—because cowpea is regarded as a crop of poor people and is mostly grown by women farmers who have little political influence. Several international agencies have helped to advance the cause of cowpea research, particularly the International Institute of Tropical Agriculture (IITA), which has the mandate among the International Agriculture Research Centers (IARCs) to work on cowpea, and; the United States Agency for Agricultural Development (USAID)-supported Bean/Cowpea Collaborative Research Support Program (CRSP), which has worked on cowpea improvement since 1980. Charitable donors have also played an important part, among the most prominent being

the Rockefeller Foundation and, more recently, the Kirkhouse Trust. An informal group of scientists called the Network for the Genetic Improvement of Cowpea for Africa (NGICA) was formed in 2001 to push all aspects of cowpea improvement. Among NGICA’s achievements was the promotion of the use of the tools of biotechnology for the genetic improvement of cowpea. Toward that end NGICA also helped convince the new African Agriculture Technology Foundation—an African public–private partnership designed to facilitate access by African farmers to technology from technology providers through royalty-free licenses and agreements—to incorporate *Bt*-cowpea as one of its projects.

1.1 History, Origin, and Distribution

Cowpea was domesticated in Africa, presumably in the northeastern part of the continent in present-day Ethiopia. The progenitor of the modern cultivated *V. u. unguiculata* is probably the wild annual form, *V. unguiculata* var. *spontanea*. In support of the idea that the crop originated in northeastern Africa, Steele (1976) noted that the variability of the wild relative *V. unguiculata* spp. *dekindtiana*—which has also been considered as a possible progenitor of cultivated cowpea—is greater in that part of Africa than in West Africa. Pasquet and Baudoin (2001) likewise support a Horn of Africa origin based on ethnobotanical, linguistic, as well as phytogeographical considerations. Still, some scientists have considered West Africa a possible site of origin because of the high variability of *V. u. dekindtiana* in this region (Faris, 1965). Lack of archeological records for cowpea cultivation hinders efforts to establish its site of origin unequivocally. Like its New World relative, common bean, cowpea may prove to have two or more sites of origin. The current consensus seems to be that domesticated cowpea originated in the northeastern region of sub-Saharan Africa (cf. Smartt, 1985) and spread westward and southward from there. This Horn of Africa origin is also supported by recent studies using molecular markers (Ba *et al.*, 2004).

1.2 Botanical Description

The taxonomy of domesticated cowpea (*V. unguiculata* var. *unguiculata*) has a history of

revisions, changes, and modifications that leave the nonexpert perplexed. The pantropical genus *Vigna* forms part of the subfamily Papilionoideae under the family Fabaceae (Leguminosae). Cowpea belongs to the subgenus *Vigna*, section Catiang. It is genetically isolated from other *Vigna*, which includes only one other distinctly African species, bambara groundnut (*V. subterranea*). There are several Asian *Vigna* crop species such as urdbean (*V. mungo*), mothbean (*V. aconitifolia*), and mungbean (*V. radiata*). Morphological, ethnographical, molecular and other criteria led Pasquet (1999) to a classification of *V. unguiculata* that recognizes 11 subspecies, 10 of which are perennial and one of which (cowpea) is annual. Annual cowpea has two forms, the cultivated *V. unguiculata unguiculata* var. *unguiculata* and the wild/weedy form *V. u. u.* var. *spontanea*, both of which are inbreeding. *V. u. u. spontanea* is typically found only near the borders of cultivated cowpea fields and within them.

The 10 perennial *V. unguiculata* subspecies include (i) some that are exclusively outcrossing: subspecies *baoulensis* (A. Chev.) Pasquet, ssp. *burundensis* Pasquet, ssp. *letouzeyi* Pasquet, ssp. *aduensis* Pasquet, and ssp. *pawekiae* Pasquet, and (ii) others that are both outbreeding as well as inbreeding: ssp. *dekindtiana* (Harms) Verdc., ssp. *stenophylla* (E. Mey) Verdc., ssp. *tenuis* (E. Mey) Marechal, Mascherpa, and Stainier, ssp. *alba* (G. Don) Pasquet, and ssp. *pubescens* (R. Wilczek) Pasquet. Pasquet (personal communication to LLM) points out that the number of subspecies is likely to change as additional living material becomes available for study and as new molecular characterization tools are applied.

Originally only three, then later four cowpea cultigroups were recognized (Baudoin and Marechal, 1985). A fifth has recently been added (Pasquet, 1998). Smartt (1985) accounted for the emergence of two of the cultigroups on the basis of selection practiced in Asia after cowpea reached that continent, probably via India, about 2000 years ago. The cultigroups are: (1) Unguiculata, the African cowpea treated here, (2) Biflora, an erect woody perennial grown for fodder and seed, (3) Sesquipedalis, grown for its long, succulent pods in the Far East, (4) Textilis, cultivated in northern Nigeria and Niger; it has long peduncles, and is grown for the textile fibers it provides, (5) Melanophthalmus, originally from West Africa, is

able to flower quickly under inductive conditions; the seeds have thin and often-wrinkled testa (Pasquet, 1998).

The growth habit of cowpea ranges from indeterminate to determinate. As regards plant architecture, there is great variability. Plants range from erect, semi-erect, and prostrate (spreading, creeping) to climbing. One of the key features of cowpea is its long tap root, which enables the plant to obtain moisture at depths that cannot be reached by most plants.

The cowpea genome is estimated to be 613 Mb distributed amongst 22 chromosomes. In this regard, it closely resembles the model leguminous plant, *Medicago truncatula* (also estimated at 613 Mb) (Arumuganathan and Earle, 1991). The sequencing and analysis of whole genomes of model plants, such as *Arabidopsis thaliana*, have paved the way for orphan crops like cowpea, where far fewer resources are available for research (Mahalakshmi and Ortiz, 2001). Fortunately, the Kirkhouse Trust, a Scottish Charity established by Sir Ed Southern, has recently committed resources to enable detailed molecular characterization and genetic improvement of cowpea. Thanks to Kirkhouse, the cowpea genome is now being sequenced. The sequencing approach is known as Gene Spacing under the trade name GeneThresher^R (<http://cowpeagenomics.med.virginia.edu>). It exploits a fundamental difference between active (transcribed) and inactive DNA, namely that inactive DNA sequences are methylated and active ones are nonmethylated. By cloning genomic DNA into a methylation-sensitive bacterial host the sequences that are not methylated are the only recombinant clones recovered. By this means only the active DNA is selected for sequencing. In a preliminary study conducted by the University of Virginia and Orion Genomics in 2004 using GeneThresher^R, Michael Timko and colleagues (Timko, 2006) achieved a fourfold enrichment of the original 600+ Mb of DNA resulting in only 150 Mb of active DNA to be sequenced. Sequencing of the 150 Mb is expected to result in at least 95% of the entire transcribed sequences of cowpea being described. Application of this new technique should accelerate the development of genetically improved cowpea varieties and strengthen the molecular breeding efforts currently underway to introgress desirable traits using markers.

Complementary to this are efforts underway by the Generation Challenge Program involving

the IITA and collaborators. They are developing expressed sequenced tags (ESTs) for simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) in cowpea to be used in marker-assisted breeding. The IITA project may pool its efforts with those of the International Livestock Research Institute (ILRI) project on the development of germplasm for livestock nutrition using genomics (Crouch and Ortiz, 2004; <http://www.generationcp.org>). Feltus *et al.* (2006) recently used the conserved-intron scanning primers (CISPs) approach to study conserved sequences and comparative genomics in orphan crops. Unfortunately cowpea was not one of the crops chosen for study.

Cytological studies help us understand the underlying genetic factors that govern interspecific compatibility. Adetula *et al.* (2005) as well as others have attempted to make wide crosses between domesticated and wild *Vigna*, especially *Vigna vexillata*, in the hope of accessing new sources of genes for insect resistance and other needed traits. *V. vexillata*, like *V. unguiculata*, has a diploid genome with 22 chromosomes. Adetula and colleagues identified satellite DNA on chromosome 2 and demonstrated this chromosome to be highly variable, thereby lending itself to serve as a marker for breeding. More recently, however, Adetula (2006) found that in 20% of the cells under study, the mitotic chromosome number of *V. unguiculata* ssp. *dekintiana* var. *pubescens* (TVNu110-3A) was actually 23, confirming a previous study (Adetula, 1999) where the meiotic chromosome number had been determined. Polytenic chromosomes occur in highly specialized tissues of some angiosperms and are indicative of high metabolic activity (Carvalheira, 2000). In plants, they are found in only a few species and appear to be limited to the ovaries and immature seed tissues. Within cowpea the polytenic chromosomes observed in anther tapetal cells were reported to be 3.5 times larger than those found in mitotic cells (Guerra and Carvalheira, 1994). *In situ* hybridization using both radioactive and nonradioactive labeling has been used to identify, visualize, and localize these chromosomes. Fluorescent *in situ* hybridization (FISH) was used in *V. unguiculata* to detect and localize DNA in anther tapetal cells (Guerra, 2001). Furthermore, utilization of polytenic chromosomes helped in the identification of the 45S ribosomal DNA sites in both *Phaseolus coccineus*

and *V. unguiculata*. This led, more importantly, to the identification of an even larger number of 10 ribosomal DNA sites in cowpea and to the suggestion that perhaps this increase in sites in *Vigna* over *Phaseolus* could have arisen by gene conversion (Galasso *et al.*, 1995, 1999).

1.3 Economic Importance

Cowpea is today grown throughout the world, with the most intense production in the northern savannahs of sub-Saharan Africa, with Nigeria and Niger the leading producers. According to Langyintuo *et al.* (2003), some 10 million hectares are under cowpea cultivation worldwide, with the sub-Saharan Africa cowpea belt producing about two-thirds of the annual world yield. Total annual grain production is about 3.7 million tons. The second largest production area after Africa is Brazil, where the crop is well suited to the relatively low rainfall and poor soils in the northeastern part of the country. Cowpea is also grown in marginal areas of eastern and southern Africa, especially in Sudan, Somalia, Mozambique, Botswana, and southern Zimbabwe. Cowpea is mostly grown as an intercrop with cereals, but little of that harvest reaches regional markets. The most important export market for cowpea in West Africa is Nigeria, simultaneously the world's largest cowpea consumer as well as producer. There is significant cowpea production in the Mediterranean, South Asia, and in the southern and southwestern United States.

Total world harvested area of cowpea in the 1990s was 9 738 000 ha, 7 804 000 of which was produced in only 12 nations in West and Central Africa. The greatest production (1 691 000 tons) was in Nigeria, followed by its neighbor to the north, Niger, with 359 000 tons (Langyintuo *et al.*, 2003). The importance of cowpea as a nutritional complement to the mostly cereal and root-and-tuber-based diets of West and Central Africa has resulted in an ancient cowpea trade system. Demand for cowpea is particularly high in Nigeria. Its dense population and large oil revenues create enormous effective cowpea demand by both urban and rural consumers. Cowpea markets in West Africa link the humid coastal zones with the semi-arid interior. In essence, cowpea produced in the drier regions (northern Nigeria, Niger, Burkina

Faso, northern Ghana, northern Cameroon, etc.) flows southward toward or into Nigeria, the most populous of the African nations. This pattern of cowpea trade toward the Gulf of Guinea has been given the name “Nigerian Grain Shed”. A second, smaller trading geography is found to the west. The Senegalese Grain Shed occupies Senegal, Mauritania, and parts of Mali.

In West and Central Africa, grain marketing is organized through formal and informal channels. Formal market places are subject to government control. Informal markets are not officially recognized but still operate well, though outside of government control. An informal market may be nothing more than a group of women or men who meet to carry out a transaction in a village or at a roadside. Farmers who are constrained by liquidity or transportation may accept quick transactions and lower prices compared to the generally prevailing market prices. Farmers usually sell their cowpeas to rural assemblers, who in turn sell them to urban wholesalers directly, or through intermediaries. Wholesalers sell large stocks when prices are high enough to pay for transaction costs (procurement, storage, and handling) plus a profit margin. They may also be involved in trade of other commodities, such as cereals and groundnut.

There is no reliable source of year-to-year cowpea production or marketing data in Africa. Information on cowpea marketing and trade is scarce and data on cowpea production economics are scattered, because marketing research has focused on African export crops such as cocoa, coffee, cotton, and groundnut and to a lesser extent cereals (Van der Laan cited by Langyintuo *et al.*, 2003).

In West Africa, protein products traditionally move south from the drier areas to the more equatorial humid areas, while carbohydrate products move north. Thus, it is that cowpea is also traded from West to Central Africa because of the comparative advantage that the drier areas of West Africa have in protein production—cowpea being a major source of protein (Langyintuo *et al.*, 2003). At least 285 000 tons of cowpea are shipped among countries in the region each year. This is probably an underestimate because the official sources for this estimate did not collect data on all formal and informal flows of grain. In 1998, Burkina Faso imported about 8000 tons from Niger and exported a total of 5500 tons to Togo,

Côte d’Ivoire, Ghana, and Benin. It is estimated that Nigeria’s average annual imports of 260 000 tons per year from Niger accounts for about 73% of Niger’s surplus production. Cowpea trade between Nigeria and Benin is bilateral. Togo and Ghana, and Ghana and Benin trade bilaterally as well. Gabon depends on Cameroon, Togo, Benin, and Nigeria for cowpea. Mauritania, Gambia, and Guinea Bissau rely on Senegal (Langyintuo *et al.*, 2003; INPhO (FAO), 2007).

1.4 Cowpea Losses Due to Pests and Diseases

Insects are the major causes of crop losses in cowpea. In some years and areas, grain yields can be reduced to nearly zero, if the crop is not sprayed with insecticide. Aphids, flower thrips, legume pod borers (*Maruca vitrata*), and a complex of pod-sucking bugs each and collectively cause yield losses. In northern Nigeria, for example, untreated cowpea plots yielded 76 kg ha^{-1} , while fields treated with the carbamate insecticide Carbaryl (at 1.12 kg ha^{-1}) yielded 1382 kg ha^{-1} . Similar yield increases were seen after treatment with chlorinated hydrocarbons (Raheja, 1976).

The insect problems of cowpea are not limited to the field. When the harvested grain is subsequently stored, beetles of the family Bruchidae (*Callosobruchus maculatus*) cause major losses—estimated at US\$ 30 million annually in Nigeria alone (Ogbuinya, 1997). The insect problems of cowpea are compounded by the fact that chemical insecticides not officially approved for cowpeas (e.g., cotton insecticides) are often used on them anyway, both in the field as well as in storage. Concerns about the negative ecological and health consequences of such insecticide use—although not yet systematically surveyed for cowpea—are growing in the minds of the farmers themselves, and the problem of insecticide misuse has begun to catch the attention of scientists, extension agents, and policy makers (James *et al.*, 2003). Murdock (2002) pointed out that traditional methods and chemical insecticides have largely failed to stop insect-caused losses. In addition to their high cost and uncertain availability, insecticides require sprayers, proper protection practices, and training for (i) effective use and (ii) to lower the health and pollution

hazards. Murdock (2002) states that “If we decided to solve the problem of insect control in cowpea solely by using insecticides, we would have to spray insecticides at probably a minimum of 250 g of insecticide per hectare on the 8.8 million hectares of cowpea grown in Africa, that is, spreading 2.2 million kilograms of insecticide into the African environment every year, not only onto the plants of course, but also into the soil, the air and the water.” This, of course, would result from a one-time spray. In some areas of West Africa, multiple sprays are used each season.

Cowpea is subject to many diseases and viruses but the losses they cause tend to be sporadic and local and are smaller, in general, than those caused by insect pests. The major foliar diseases are bacterial blight, bacterial pustule, *Ascochyta* blight, *Cercophora* leaf spot, brown and false rusts, and web blight. Stem diseases include *Pythium* stem rot, ashy stem blight and *Phytophthora* stem rot (Emechibe and Shoyinka, 1985). *Spaceloma* scab affects all above-ground parts of the plant. Eight viruses have been recorded on cowpea, most of which are insect borne. These include cowpea yellow mosaic comovirus, mottle virus, aphid-borne mosaic potyvirus, cucumber mosaic, cucumovirus, cowpea golden mosaic virus, mild mottle virus, and sunn-hemp mosaic tobamovirus (Thottappilly and Rossell, 1992). Root-knot and other nematodes are widespread and potentially injurious to cowpeas. Ongoing studies in West Africa are finding common occurrence in cowpea fields of root-knot *Meloidogyne* species as well as *Scutellonema cavenessi* (Boukar *et al.*, 2007). Two parasitic plants, *Striga gesnerioides* (Willd.) Vatke and *Alectra vogeli* (Benth.) cause nearly total crop losses in areas where the parasites are abundant (Atokple *et al.*, 1995; also see below).

1.5 Traditional Breeding

The goal of all cowpea breeding programs is to develop consumer-preferred varieties with high yield and resistance to biotic and abiotic constraints to production. Traditional plant breeding approaches to cowpea improvement have had many successes over the last 30 years. Much of the progress is due particularly to B.B. Singh, cowpea breeder at IITA Kano Station in Nigeria, and the large number of advanced breeding materials

and methods he and his students and colleagues produced over a quarter century of work. Bean/Cowpea CRSP scientists, particularly Tony Hall of the University of California at Riverside and his African students, have likewise made great contributions, as has Richard Fery of US Department of Agriculture (USDA)/Agriculture Research Service (ARS) working in the United States. Thanks largely to these and the work of other breeders, recent figures (2004) from the Food and Agriculture Organization (FAO) of the United Nations show a tremendous increase in the productivity of cowpea globally.

1.5.1 Breeding methods

1.5.1.1 Pedigree breeding

Three principal methods are used in breeding self-pollinated crops like cowpea: pedigree, mass selection, and single-seed descent. The pedigree method, often with slight modifications, is the one most frequently used. Segregating populations generated from crosses are selected at the F_2 and subsequent generations. Selections are based largely on the main character of interest, for example, resistance to the parasitic weed *Striga*. Detailed data on maturity, time to flower, growth habit, and grain and fodder yield are collected and the most promising single plants selected for advancement. Other traits of interest are selected for, as well, including seed color, seed texture, seed size, and leaf yield. The relative importance of these traits varies with the particular breeding program. For example, leaf yield is more important in eastern and southern Africa while West and Central African breeding projects lay more emphasis on grain and fodder yield.

When the pedigree breeding method is used, between 60 and 200 F_2 single plants are scored using a negative selection approach—those with undesirable traits, for example, black seeds, are discarded. Next, each of the selected F_2 plants is used to plant a progeny row. In the subsequent F_3 generation still more stringent selection criteria are used to reduce the population size further. Those F_3 families that breed true for the principal character are advanced to the F_4 generation. At F_4 evidence of segregation for the principal character is enough to prevent a family from

being advanced to the succeeding filial generation. From F_5 and F_6 onwards, attention is placed mostly on quantitative traits such as seed size, fodder yield, grain yield, and maturity. At this stage, all families must have been fixed for the principal character and within-family variation must be minimal—in other words family members need to be phenotypically uniform. At the F_6 generation, most of the families must have been fixed for not only the principal character of interest but also for most of the secondary characters. Subsequent evaluations of the F_6 through F_8 families are conducted mainly to assess yield and maturity performance. At each stage of selection, the breeder may save a portion of the seed of each family as insurance against crop failure.

The fully characterized families are then grouped on the basis of maturity group into early, medium, and late; or by seed color into brown, white, or other colors. Each group is then subjected to a preliminary yield trial (PYT). The trial is replicated at one or more locations. Locations are carefully selected to represent the typical production ecologies. The more promising entries, usually a maximum of 20, are pulled together from the various groups to form an advanced yield trial (AYT). The number of replications as well as test sites is greater for AYT compared to PYT. AYT can require 2 or 3 years depending on the breeder and the trait under consideration. The main aim of the AYT evaluation is to ensure rigorous evaluation of the genotypes under all possible stressful conditions. This helps to ensure that superior genotypes are selected. Eventually the AYT will have identified three or four most-promising lines; these are recommended for release. In some African programs national policy may require an additional on-farm evaluation by farmers to corroborate the breeder's claim about the superiority of the new lines over local commercial varieties. Some varieties developed using this approach are listed in Table 1.

The pedigree method of breeding requires meticulous records on the characteristics of each F_2 plant and its subsequent progenies through to the F_6 or F_8 generation. This makes the method cumbersome and time consuming. However, it has the advantage of allowing the breeder to compare the various families tracing their performance back to the antecedent F_2 plant. In addition, the genetic dynamics of several genes in the population

Table 1 Some cowpea varieties developed using the pedigree breeding method

Variety	Origin	Country of release
IT845-2246-4	IITA Ibadan ^(a)	Nigeria
IT89KD-245	IITA Ibadan	Nigeria
IAR-48	IAR2 Samaru ^(b)	Nigeria
IT90K-76	IITA Ibadan	Nigeria
IT89KD-374-57	IITA Ibadan	Nigeria
KVX-176B	INERA3, Ouagadougou ^(c)	Burkina Faso
IAR 355	IAR Samaru	Nigeria
IT90K-277-2	IAR Samaru	Nigeria

^(a)IITA, International Institute of Tropical Agriculture

^(b)IAR, Institute for Agricultural Research

^(c)INERA, Institut National Environmental Recherche Agricole

can be studied. Therefore, despite its drawbacks when used with self-pollinating crops like cowpea, the pedigree method is best for most situations.

1.5.1.2 Single-seed descent

To advance each selected F_2 plant derived from a cross or selfing program, a single representative seed from each plant is selected. This method has been adopted less widely in cowpea breeding. The few reported instances involve the development of pure lines from local landraces. Cowpea lines IT88DM-345, IAR-1696, and possibly IT85ID-985 are examples. A single seed is selected from each selfed plant from the various landrace collections. After the plants are evaluated for genetic purity, a single seed is then selected from each of the plants having the desired trait. This process is repeated until substantial phenotypic homogeneity is attained. Finally at the F_{6-8} level, seeds from the most promising strains are multiplied for further field evaluation, if the objective is to develop a pure line cultivar or if the line is to be included in a hybridization program to produce further improvement.

1.5.1.3 Backcross breeding

As with other crops, the backcross breeding method seeks to improve the genetic value of a locally adapted cultivar that has a few genetic defects such as susceptibility to diseases, low oil

or sugar content, etc. In practice, the hybrid between the adapted variety receiving the gene for further improvement—known as the recurrent parent—and the source of the gene of interest—the donor parent—are backcrossed again or several times, preferably as the male parent to the recurrent parent. With each backcrossing, in addition to acquiring the gene of interest, 50% of the genome of the recurrent parent is transferred into the backcross hybrid. For example, BC₁, BC₂, BC₃, and BC₄ are 50%, 75%, 87.5%, and 93.75% recurrent parent genome, respectively. Some of the varieties developed using this method include IT89KD-245, IT89KD-260, and IT90K-277-2. Seed size and general adaptation are the most common target traits for improvement using this approach.

1.5.1.4 Selection techniques

The overall success of a cowpea breeding program depends largely on the effectiveness of the selection tools used to identify desirable genotypes through their phenotypes. If the precision of the tools is low, improvement of the population for the trait will be small. Even when there is an effective tool for selection, the genetic variation in the genome must be sufficient to warrant exploitation—as is often not the case for major insect pests of cowpea. Barriers to genetic recombination between genotypes must also be surmounted to facilitate introgression of desirable genes into different backgrounds.

1.5.2 Major breeding objectives

1.5.2.1 Diseases

Breeders have employed different techniques to select disease-resistant genotypes. The development of disease-resistant cowpea cultivars is limited by the lack of screening techniques. These are not well developed for many diseases and hence, progress made from screening has been minimal. However, there are exceptions. To identify cowpea lines resistant to the *Cercospora* leaf spot disease, for example, Lane *et al.* (1994) inoculated cowpea accessions with spores of *Cercospora cruenta* in the greenhouse. Out of the 313 lines, 15 were

ranked as immune while 7 were judged resistant. Singh (1998) likewise relied on natural inocula to identify resistance to *Ascochyta* blight, *Septoria* leaf spot, and smut. He planted several hundred lines in fields with a history of the diseases—so-called hotspots. Lines failing to show any disease symptoms were tagged as resistant. Singh (1999) reported the development of improved lines with multiple resistances by planting rows of plants highly susceptible to a particular disease next to neighboring segregating lines. The susceptible line (spreader row) served as an inoculum bank, spreading the pathogens. This approach strongly aids selection for resistance. Based on reactions to three virulent strains of bacterial blight, Wydra and Singh (1998) used an artificial infection technique to identify three out of 90 varieties that were resistant to all the three blights. A similar approach was used by other researchers to identify resistant genotypes. The use of the spreader row is also the most efficient way of screening for resistance to *Sphaceloma* scab since artificial inoculation with an isolate of the pathogens fails to produce any symptoms of the disease even in susceptible genotypes (Mungo *et al.*, 1998). The success of developing disease-resistant cowpea varieties, therefore, lies largely with the availability of appropriate inocula together with optimum environmental conditions for symptom development.

As practical as field screening may seem, it is generally not as reliable as artificial inoculation. This is because natural inoculum abundance varies from season to season, making disease phenotyping subjective. However, field screening is the only available method of screening for resistance with diseases like *Ascochyta* blight, ashy stem blight, *Cercospora* leafspot, and smut diseases. Amusa and Okechukwu (1998) reported that planting of spreader rows 2 weeks before the test lines was more effective than simultaneous planting. Only 9 out of the 45 lines previously rated as resistant on the basis of simultaneous planting of spreader and test lines turned out to be actually resistant.

Although screening techniques are critical for the development of improved resistant genotypes, their usefulness is limited if a source of resistance is not present within the cowpea genome. Diseases, for which sources of resistance have been identified and used in breeding resistant cowpea varieties,

include brown blotch, rust, damping off, *Fusarium* wilt, and bacterial canker. Strain variation in disease pathogens makes artificial inoculation more critical for the development of resistant cultivars. This is because race-specific resistance will allow the development of varieties adapted to specific locations. To achieve a broader spectrum of adaptation, the resistance must encompass a series of strains of the pathogen. This is frequently the case for viral diseases. To identify sources of resistance as well as segregants possessing the genes in a breeding program, pure isolates of the viruses are usually sprayed on the test plants. In this way, Singh and Hughes (1999) identified sources of resistance to cowpea yellow mosaic virus (CYMV), black eye cowpea mosaic virus (BCMV), and aphid-borne mosaic virus (ABMV). Similarly, van Boxtel *et al.* (2000) reported multiple-strain resistant cowpea genotypes for BCMV and ABMV. Often, breeders use the disease reaction of cultivars with known resistance as the standard for strain-resistance breeding. van Boxtel *et al.* (2002) suggested that using the previously characterized cultivars IT86D-880, IT86D-1010, IT82D-889, IT90K-277-2, and TVU 201 and by introgressing all these parents in a breeding program, it is possible to develop a genotype with resistance to at least 13 different strains of the cowpea virus. However, the sustainability of the approach assumes that no new virus strains will develop or will develop slowly. To ensure sustained resistance, the breeder should continue to use the pathology tests to confirm resistance.

1.5.2.2 *Striga* and *Alectra*

The parasitic weeds *S. gesnerioides* and *Alectra vogelii* cause major yield loss in cowpea, sometimes reaching 100% in susceptible cultivars (Singh and Emechebe, 1990; Atokple *et al.*, 1995). Sources of resistance to *S. gesnerioides* have been identified in cultivar B301 (Singh and Emechebe, 1990) and IT82D-849 (Atokple *et al.*, 1995). Identification of resistant genotypes has been made easier thanks to a modified unispore culture technique like that used for disease pathogens. The soil in which the plants being evaluated are grown is first inoculated with the seed of the parasite; typically, about 800 seeds of *S. gesnerioides* per pot. Susceptible cowpea genotypes trigger *Striga* seed germination

and attachment to the host roots. Subsequently, the *Striga* plants emerge from the soil. Visual counts of emerged *Striga* plants in the pot alone are not sufficient to classify a cowpea plant as resistant or susceptible. The cowpea plant roots must be carefully washed to reveal any attachments, including immature haustoria. Fully resistant plants are free of *Striga* attachments while susceptible ones have one to several *Striga* attached. Although this method is laborious and time consuming, it is effective. Atokple *et al.* (1995) showed that in a pot containing two plants, one of them can support several *Striga* attachments, indicating susceptibility, while the other may be free of any attachments and resistant. However, in other pots both plants may be either susceptible or resistant. Another method, known as the *in vitro* technique, utilizes exudates from cowpea root cuttings and petri plates infested with *Striga* seeds. Root cuttings from susceptible cultivars elicit germination of the *Striga* seed while no seed germination is observed in resistant cultivars (Berner *et al.*, 1995). This method allows for the rapid screening, but it requires skill to use successfully. Lane *et al.* (1994), who screened and characterized *Striga* collections from West Africa, reported that there are 5 races—race 1 found in Burkina Faso, 2 in Mali, 3 in Nigeria and Niger, 4 in Benin, and 5 in Cameroon. Line B301 is resistant to all the races except 4, while resistance to race 4 is found in the variety IT81D-994. Varieties with multiple resistance to the various races have been developed using a combination of pot and field screening in so-called *Striga* hotspots (Singh, 2002).

The current extensive trade in cowpea grain in different West/Central African subregions means that cowpea cultivars with resistance to only one or two local races will not be effective because seeds of the parasite are also transported from one region to another along with the cowpea grain or fodder. The screening methods described earlier can be applied to the identification of resistance to all races of *Striga*.

Screening methods similar to those used to breed *Striga*-resistant cultivars have helped to identify *Alectra* resistance in B301 and IT81D-994. Genetic studies of the two genotypes revealed: (1) a pair of dominant resistance genes in B301, (2) a single dominant resistance gene in IT81D-994, and (3) that the two genes are nonallelic (Atokple *et al.*, 1995).

Table 2 List of selected cowpea varieties with resistance to *Striga gesnerioides* and *Alectra vogelii*

Genotype	Phenotype	
	Striga	Alectra
B 301 (1235) ^(a)	Resistant	Resistant
IT81D-994 (124) ^(b)	Resistant	Resistant
IT90K-76	Resistant	Resistant
IT90K-59	Resistant	Resistant
IT93K-693-2	Resistant	Resistant
IT97K-499-35	Resistant	Resistant
IAR-00-1009	Resistant	Resistant
IAR-01-1006	Resistant	Susceptible
IT97K-819-154	Resistant	Resistant

^(a) 1235 designates source of resistance to races 1, 2, 3, and 4

^(b) 124 designates source of resistance to races 1, 2, and 4

The pot culture technique has been effective as a breeding tool in the development of *Striga*- and *Alectra*-resistant cowpea varieties. However, employing it to screen for multiple strain resistance is not feasible because no single country has all the *Striga* strains. Furthermore, the different forms of *Striga* cannot be identified with precision using morphological markers. Table 2 shows examples of varieties with resistance to the *Striga* and *Alectra*.

1.5.2.3 Insect pests

Success in breeding cowpeas carrying resistance to insect pests depends primarily on the screening methods developed by entomologists. Progress has been made in rearing aphids, *Maruca* pod borer, cowpea bruchids, and to a lesser extent flower thrips. The insects thus made available are used to artificially infest cultivars and segregating populations to help to identify resistant plants.

Aphids can be maintained on highly susceptible cultivars grown in the screenhouse or glasshouse and then one or a few pairs of male and female adults transferred to test plants held in screening boxes. Susceptible plants support high colonization rates while the resistant lines remain free of any colony. The aphid-resistant genotypes TVU-3000 and TVU-801 were identified this way. This bioassay technique is now being used to identify resistance among segregating populations derived from crosses between aphid-resistant and -susceptible parents. Nkansah and Hodgson (1995) used this method to identify resistant

genotypes and transferred these genes into other backgrounds through backcrossing. Bata *et al.* (1987) screened segregating populations by planting them in a wooden box filled with topsoil. The box was covered with a screen to prevent the aphids from escaping. At intervals of about 10cm alternate rows of susceptible and resistant cultivars were planted. Resistant segregants did not support colony development, whereas the susceptible plants suffered high infestation and often were killed. Unfortunately, geographical variation in aphid strains makes the resistance in TVU-3000 ineffective in some locations, for example, in the United States and the Philippines though it is useful in Africa (Singh, 2002). Examples of aphid-resistant varieties include IT90K-76, IT90K-59-2, and IT84S-2246-4, all deriving their resistance from TVU-3000.

Screening of segregating cowpea populations for resistance to the cowpea bruchid (*C. maculatus*) involves rearing the adults on susceptible cowpea lines. Some 100–200 seeds of the test plant contained in a glass jar are infested with two pairs of adult bruchids. The test lasts 4–10 weeks. Resistance is indicated by the lack of holes on the grain while susceptibility is reflected in the number of emergence holes as well as number of adult weevils hatched. Landrace TVu 2027 has served as a source of resistance to develop bruchid-resistant cowpea cultivars. This resistance lasts only for about 90 days postinfestation, after which bruchid populations become high. Shade *et al.* (1999) reported that bruchid populations from some areas in Nigeria can overcome the resistance of TVu 2027, which establishes that there are strain variations in bruchids. The ultrasonic biomonitor device developed at Purdue University detects the presence of bruchid larvae feeding hidden within seeds by means of the ultrasonic signals the larvae generate as they feed (Shade *et al.*, 1990). This has the potential to be a useful tool for breeders because it could enable rapid and high-throughput screening of cowpea genotypes compared to the traditional glass jar methods. However, it should be noted that thus far no cowpea cultivar has been discovered that is immune to the bruchid beetle; in short, the current known resistance of TVu 2027 is only relative resistance.

Screening techniques for resistance against thrips have not been standardized. Breeders rely on natural infestation in the field to score genotypes.

Thus far, only a low level of resistance has been reported.

A well-documented screening technique for detecting resistance to the legume pod borer (*M. vitrata*) is available (Oghiakhe *et al.*, 1995). However, there is no known cultivar with more than weak resistance to this destructive insect. Interestingly, one relative of the cowpea, *V. vexillata*, is completely resistant to *M. vitrata*. Unfortunately strong genetic incompatibility prevents the cross from being made, and this renders *V. vexillata* of no value because it cannot be introgressed into cowpea by conventional crossing techniques (Barone and Ng, 1990). For other insect pests such as the lygus bug (Ehlers *et al.*, 2002), which belongs to the sucking bug complex (Ishiyaku and Amatobi, 2004), screening is solely based on natural field infestations where the breeder compares the performance of F₃ families or advanced breeding lines with and without pest protection (insecticide treatments). Some insect-resistant cultivars appear to give relatively high grain yields (Bata *et al.*, 1987). Even so, conclusions from such evaluations are difficult to make, especially during those seasons where the levels of natural pest infestation are low or moderate as this can lead to misleading results. Sources of resistance to the important sucking bugs, such as *Acanthomia* and *Aneplomis* species, do not appear to be present in the cowpea genome, making it impossible to develop resistant cultivars using a conventional breeding approach.

1.5.2.4 Physiological characters

Cowpea breeders give particular attention to the physiological characters of drought and heat tolerance, photoperiod sensitivity, maturity, and yield.

Cowpea is more drought tolerant than maize, sorghum, or even millet (Singh *et al.*, 1999a) and it displays significant genetic variation in response to moisture stress. Singh *et al.* (1999b) reported about a 15-weeks survival/development viability difference between the drought-susceptible cowpea line TVU-7778 and a drought-tolerant line TVU-11979. The evaluation was based on the stay-green trait.

The single dominant gene conferring drought tolerance was transferred into drought-susceptible

lines. Drought-resistant segregating lines were identified using the box screening technique. The test plants were grown together with the checks in a wooden box filled with a 1:1 mixture of sand and top soil. The plants were watered until the first trifoliate leaf emerged. Watering was then stopped and the time to permanent wilting as well as percent wilted plants among the test genotypes was determined to classify them as resistant or susceptible. This method is cheap and simple. However, it can be unreliable because of the lack of a record of soil moisture tension. Relying on time of withdrawal of water as an indicator of drought is problematic because the drought condition is related to the physical characteristics of the soil used. Nevertheless, drought-tolerant variety IT96D-604 was developed using this method and has been found to withstand relatively high soil moisture stress in the field.

After genetic variation for heat tolerance was identified in cowpea (e.g., Hall, 1992), efforts were directed to the introgression of heat tolerance into several different genetic backgrounds. Screening of segregating populations is done under high ambient temperatures for example, 43 and 24 °C as maximum and minimum temperatures, respectively. In Nigeria, the hot condition is characterized by temperatures of 24–27 °C minimum and 38–42 °C maximum. Heat-tolerant lines in the United States and Nigeria (Singh, 2002) were identified by planting in the field during the hot season. Tolerant lines will support full flowering and pod set while susceptible lines show high flower bud and flower abortion with very low or no pod set (Ehlers *et al.*, 2002; Singh, 2002).

Improvement of cowpea phenology for the weather of the production area has been essential for the development of varieties for an agroecology. Photoperiod sensitivity is the primary determinant of phenological adaptation of popular landraces. Photoperiod sensitive lines flower toward the end of the rains, thereby allowing for timely production of good seeds. Most past breeding efforts focused on the development of day-neutral cultivars. Breeders used the longest day length part of the season to screen for this trait. The longest day length in the cowpea growing belt of sub-Saharan Africa is only slightly longer than 13 h. As short as it may look relative to 16 h-long days of the temperate zones, the long day in Africa is effective in eliciting delayed flowering among sensitive

genotypes such as the landrace Kanannado, IT89KD-288, IAR-00-1074, IT89KD-245. Day-neutral varieties include IT84s-2246-4, IAR 48, IT97K-499-35, IT88DM-345, and IT97D-941-1. Putative day neutrals are characterized by early flowering, requiring less than 45 days (Ishiyaku, 1997). To screen segregating populations, the natural day length can be extended by the use of incandescent lamps.

Yield is an important trait in cowpea, whether for grain or of hay. Although yield is often measured directly by weighing the total grain (or hay), some breeders use other traits such as pods per plant, seeds per pod, plant height, number of branches per plant as an indirect measure of yield. In common with all crops, there is nothing more important than improving yield.

1.5.2.5 Quality traits

Nutrition-related traits, though are invisible to the consumer, are the most important and yet most difficult to measure in cowpea. Total crude protein in grain has high genetic variation, ranging from 14% to 28%. Screening segregating populations for this trait is based on F₃ seeds, which provide the information on the F₂ plant. Other quality traits include the microelement contents of the grain such as iron, zinc, copper, and molybdenum. Breeding programs seldom pay much attention to these traits. Recently, it has become fashionable to enhance the nutritional value of crops and investments are being made to develop biofortified varieties especially in the cereals. Grain size in cowpea is important as consumers pay a premium (Langyintuo *et al.*, 2003) for the desired seed size. Consumers in Nigeria, for example, prefer large-seeded (>20 g/100 seeds) varieties.

1.5.3 Challenges for conventional cowpea breeding

Conventional approaches to breeding cowpeas have materially increased the productivity of the crop globally. Varieties are available that yield more than 1 ton per hectare, even in the face of severe *Striga* or *Alectra* infestations, leaf spot diseases, or aphid, and nematode infestations. This success owes to resistance genes against these

constraints that exist in the cultivated cowpea genome and which were moved into different genetic backgrounds by breeders. Over the years, these improvements have resulted in more than a doubling of the average yield of the crop, from about 100 kg ha⁻¹ to about 350 or so kg ha⁻¹. However, even this still-modest level of productivity can only be guaranteed if one or two insecticide sprays are applied. This is because those resistance genes mentioned above only affect plant growth while a fully developed resistant cultivar needs a whole series of insect resistance genes to protect the reproductive organs so they can develop into mature pods with well-filled grain.

Unfortunately, there are no utilizable resistance genes for postflowering insect pests in the cowpea genome. It is true that resistance to some insects such as the legume pod borer, *M. vitrata*, exists in a distant relative of cowpea, *V. vexillata*, but interspecific genetic barriers prevent hybridization. Lack of genes in the cultivated species and high barriers to crossing between related species have limited the success of conventional breeding in solving the major cowpea production constraint—insects. What is true for *M. vitrata* is also true for the cowpea bruchid, and for pod-sucking bugs and thrips. Lack of resistance genes is a major bottleneck that limits the success of conventional cowpea breeding. The breeder must resort to modern biotechnological tools to find these genes outside the cowpea genome and transfer them into cowpea. Given the successes with other crops such as maize, tomatoes, sweet potato, and cotton, genetic transformation to introduce insect resistance is feasible for cowpea.

1.6 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional breeding and screening have led to notable successes in improving cowpea for certain traits but these tools are only useful for genetically improving cowpea when the cowpea gene pool contains the desirable genes. This is frequently not the case. Further, complex polygenic traits are hard to breed for, as are traits for which there are no good bioassays or markers. As mentioned earlier, conventional breeding is limited or even practically

useless for resistance to many of the insect pests of cowpea, insects being the pre-eminent pre and postharvest pests of the crop because the cowpea gene pool is devoid of the necessary genes. Likewise, there is little prospect for genetic improvement of cowpea by wide crossing. Cowpea is extremely well isolated from other *Vigna* species that might provide sources of resistance genes. Many efforts have sought to create viable wide crosses between cowpea and its nearest relatives but the gulf has proven too wide.

Three constraints to cowpea production and utilization are currently targets for improvement through genetic transformation.

The first is the legume pod borer, *M. vitrata*, which by itself can cause severe, even devastating losses. *M. vitrata* belongs to the Pyralidae, the family to which the European corn borer (ECB) belongs. ECB, a major pest of maize in the eastern United States, can be controlled by means of maize hybrids genetically engineered to express the *cry1Ab* gene from *Bacillus thuringiensis* (often referred to as *Bt*). In the USA Corn Belt, about one-quarter of maize now carries the *cry1Ab* gene. The protein product of this gene has been shown to be extremely toxic to *M. vitrata* when fed in the diet ($LC_{50} = 0.03 \mu\text{g g}^{-1}$ diet; L.L. Murdock, W. Moar, and R.E. Shade, unpublished observations). Accordingly, genetic transformation of cowpea to express the *cry1Ab* protein has an excellent prospect of imparting *M. vitrata* resistance, a trait otherwise not available.

Another constraint not adequately addressable through conventional breeding is resistance to cowpea weevil. While it is true that there are cowpea cultivars derived from the landrace TVu2027 with moderate resistance to cowpea weevil, this resistance has already been incorporated into many cowpea varieties and has been widely disseminated, both in Africa and beyond. It now appears that there are populations of cowpea weevil that can overcome the existing resistance. If these resistant weevil populations expand, the TVu2027 trait will decline in usefulness to the point of being useless for protection against weevils. Numerous genes have the potential to confer resistance to the cowpea weevil if transferred into cowpea and expressed in the seed. The most advanced of these involves transferring into cowpea an α -amylase inhibitor (αAI) gene from common bean. In common bean that gene

through its protein product protects the seeds against cowpea weevil and certain other bruchids though not against the common bean weevil. When that gene was linked to a seed-specific promoter and transferred into garden pea using gene technology, the garden pea seeds, which are normally susceptible to cowpea weevil proved to be highly resistant (Shade *et al.*, 1994). By transferring the common bean α -amylase inhibitor into cowpea and expressing it in the seeds, it should be possible to introduce a new source of weevil resistance into cowpea. Some uncertainty hangs over this undertaking, however, because of the possibility that the αAI gene will not be expressed in the recipient plant exactly as it is in the donor parent. This has been observed with αAI expressed in garden peas. The αAI protein from garden peas had small mass difference from that of the protein from common bean, a difference probably due to a variation in the degree of post-translational modification in the recipient species. The possibility that this variant protein—which still inhibits insect α -amylase and blocks weevil growth and development—might cause toxicity or allergenicity in consumers of the transformed seed has to be addressed (Prescott *et al.*, 2005). Several approaches such as alteration of the secondary or tertiary structure of the αAI protein or modification of the primary amino acid sequence to remove or change glycosylation sites are available to address this issue (Lehrer and Bannon, 2005).

Cowpea grain contains modest but significant amounts of oligosaccharides, such as stachyose and raffinose. Some individuals, and especially small children, suffer discomfort, sometimes severe, when they ingest cowpeas. As a result, some adults do not eat cowpeas at all. Furthermore, many mothers will not use cowpeas—despite its high protein content—to prepare important weaning foods because of the ensuing discomfort when babies consume cowpea-based foods. Galactinol synthases are key factors in the biosynthesis of oligosaccharides. Efforts are now underway to characterize the galactinol synthase genes in cowpea with a view to eliminating one or more of them using RNA interference (RNAi) and thereby reducing the levels of oligosaccharides in the seeds.

RNAi refers to the interference resulting from the introduction of double-stranded (ds) RNA into

cells. It represents a gene regulation strategy based on sequence-specific targeting and degradation of RNA. This phenomenon is also known as RNA silencing, or quelling in fungi. In plants, it functions both as an antiviral defense mechanism employing viral encoded suppressors of silencing as well as a mechanism in plant gene regulation. RNAi participates in a whole complex network of cellular defense mechanisms and numerous silencing signaling pathways involved in gene regulation at the transcriptional, RNA stability, and translational levels (Waterhouse *et al.*, 1998; Dunoyer and Voinnet, 2005). RNAi is highly specific, requires dsRNA to be effective and is extremely potent requiring only a few molecules of the homologous dsRNA to effectively shut down the targeted homologous messenger RNA (mRNA).

The RNAi approach has been used in gene manipulation and biotechnological applications. To date the technique has been used to make healthier cooking oils, virus-resistant plants and improved pharmaceuticals, and holds immense promise in the manufacture of new drugs for the control of both animal and human diseases. It has proven to be highly effective in plant disease-resistance applications (Lindbo and Dougherty, 2005). The RNAi technique could, therefore, be applied as a novel strategy for viral resistance in cowpea to complement the already successful conventional breeding and other possibilities, such as coat protein-mediated and RNA-mediated resistance strategies.

RNAi techniques are currently being explored for use in altering the oligosaccharide levels. For example, work at the University of Zimbabwe has focused on elucidating the presence of galactinol synthase (*golS*) genes in cowpea, a key regulatory enzyme in the biosynthesis of raffinose family oligosaccharides (RFOs) (Saravitz *et al.*, 1987). This work is aimed at down-regulating the presence of RFOs that have been implicated as flatulence-causing factors in cowpea. The RFOs are α -galactosyl derivatives of sucrose and comprise the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. While a total of seven homologs have been identified in *Arabidopsis*, work is underway to establish the number of homologs present in cowpea, their expression levels, and distribution patterns in order to determine the appropriate homolog for silencing (Taji *et al.*, 2002).

2. DEVELOPMENT OF TRANSGENIC COWPEAS

As mentioned earlier, cowpeas are a good source of protein and highly digestible energy but its yields remain critically low, largely because of insect pests. Cowpea germplasm contains little or no resistance to these major insect pests and a gene technology approach for adding insect-protection traits has been a high priority for many years (Ehlers and Hall, 1997).

The first reported transformation experiments with cowpeas were conducted by Garcia *et al.* (1986, 1987) and although kanamycin-resistant callus was obtained, no plants were regenerated. Later, Penza *et al.* (1991) and Muthukumar *et al.* (1996) used longitudinal mature embryo slices and mature de-embryonated cotyledons, respectively, as target tissues. Although Penza and colleagues could not demonstrate stable integration of the transgenes, Muthukumar *et al.* (1996) obtained transgenic plants after selection on hygromycin. However, transmission of the transgenes to the next generation could not be demonstrated. Similarly, Ikea *et al.* (2003), using the particle gun method for cowpea transformation, found that the transgenes were transmitted to only a small proportion of the progeny and that there was no evidence for stable integration of the transgenes. A very promising regeneration and transformation system was described by Kononowicz *et al.* (1997) and although not pursued at the time, it did form the basis of a system that is reproducible and that obeys Mendelian rules of inheritance (Popelka *et al.*, 2006). This is the first cowpea transformation system capable of producing transformants on a field scale. Critical features of this system include suitable explants from cotyledonary nodes or embryonic axes and a tissue culture regime without auxins in the early stages, but which includes the cytokinin, 6-benzylaminopurine (BAP), at low levels during shoot initiation.

2.1 Transformation Methodologies

Mature dry seeds are the preferred starting material although seeds harvested from immature pods at maximum fresh weight of seeds can also be used. Dry seeds are threshed by hand to avoid cracking of seed coats and thus reduce

contamination with microorganisms. The protocol published by Popelka *et al.* (2006) has undergone significant modification. An updated method follows.

Dry seeds or immature pods are submerged in 70% ethanol for 2 min, rinsed with sterile deionized water and then treated for 30 min in 20% commercial bleach (8.4 g l^{-1} sodium hypochlorite final concentration). Immature seeds are removed aseptically from pods while mature seeds are imbibed overnight. Two different explants are routinely used for multiple shoot production, i.e., the embryonic axis and the cotyledon itself (Figure 1a, b). From an initial comparison of 19 cultivars and lines, it is now clear that most lines of cowpea can be transformed, the only caveat being that different tissue culture conditions need to be optimized for each line.

The selectable marker genes, *bar* (Molvig *et al.*, 1997) or *nptII* (Mazodier *et al.*, 1985; Boevink *et al.*, 1995) can be used for transformation. The *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991) is the preferred strain for cowpea transformation. *Agrobacterium* containing the T-DNA is cultured overnight at 28°C on a shaker at 180 rpm and the suspension is centrifuged at $8000 \times g$ for 10 min and resuspended in Medium 1 (MS-basic medium; Murashige and Skoog, 1962) diluted one in ten and containing 30 g l^{-1} sucrose, 20 mM 2-(N-morpholino)-ethanesulfonic acid (MES), adjusted to pH 5.6 prior to autoclaving, supplemented with filter-sterilized MS-vitamins, 100 mg l^{-1} myo-inositol, 1.7 mg l^{-1} BAP, 0.25 mg l^{-1} gibberellic acid 3 (GA3), 0.2 mM acetosyringone, 250 mg l^{-1} Na-thiosulphate, 150 mg l^{-1} dithiothreitol, and 0.4 g l^{-1} L-cysteine). The explants are submerged without shaking in the bacterial suspension for 1 h following wounding in the meristematic regions with a scalpel. The treated explants are then blotted on sterile filter paper and transferred to solidified Medium 2 (Medium 1 containing 0.8% agar). After 4 days of co-cultivation, explants (Figure 1c, d) are transferred to Medium 3 (full strength MS medium, supplemented with 100 mg l^{-1} myo-inositol, 150 mg l^{-1} timentin, 30 g l^{-1} sucrose, 3 mM MES, 1.7 mg l^{-1} BAP, 5 mg l^{-1} phosphinothricin (PPT) or $25\text{--}50 \text{ mg l}^{-1}$ geneticin or 150 mg l^{-1} kanamycin, 0.8 g l^{-1} agar and adjusted to pH 5.6) for shoot initiation. After 2 weeks the first shoots (Figure 1e, f) have been initiated and they are then

separated from the explant tissue. The cotyledon is removed from the “shooting” cotyledonary node region and the hypocotyl and root section of the embryonic axis are removed leaving the initiated shoot attached to 5 mm of hypocotyl tissue. Cultures are transferred to fresh Medium 3 every 2 weeks following removal of dead and dying tissue (see Figure 1g, h for shoot development after 6 weeks). After four to six such subcultures, the surviving green shoots are transferred to Medium 4 (Medium 3 without BAP but supplemented with 0.5 mg l^{-1} GA3, 50 mg l^{-1} asparagine, 0.1 mg l^{-1} 3-indoleacetic acid, 150 mg l^{-1} timentin, and either PPT (10 mg l^{-1}), geneticin (50 mg l^{-1}) or kanamycin (150 mg l^{-1}), for shoot elongation. The shoots are subcultured every 2 weeks until single shoots are more than 1 cm long. These larger shoots are transferred from petri dishes to culture jars (80 mm height) for further shoot growth under selection and eventual rooting.

The majority of the regenerated shoots can be rooted *in vitro* on Medium 4, but shoots that are slow to root can be directly grafted onto 10-day-old seedlings by removing the seedling's cotyledons and primary leaves and replacing them by the *in vitro* regenerated shoot with the aid of a silicon ring. Difficulties in rooting of cowpea shoots on hormone free medium are well known (Brar *et al.*, 1999; Machuka, 2000).

Grafted or directly rooted plants are transferred to soil and allowed to establish in a high humidity chamber for 14–21 days before transferring to the greenhouse where they are gradually acclimatized to ambient humidity.

Legumes are generally regarded as recalcitrant to genetic transformation (Popelka *et al.*, 2004). Several methods and meristematic tissues have been tried including somatic embryogenesis and organogenesis (Kononowicz *et al.*, 1997; Machuka, 2000). Higgins and co-workers have found that undifferentiated, apparently embryogenic callus has failed to regenerate viable embryos and plantlets under their conditions. They therefore opted for regeneration via organogenesis, which has been a successful platform for transformation protocols in other plants, including several legumes (Schroeder *et al.*, 1993; Kononowicz *et al.*, 1997; Molvig *et al.*, 1997; Olhoft and Somers, 2001; Saini *et al.*, 2003; Sarmah *et al.*, 2004). The cotyledonary node or embryonic axis appears to constitute the most

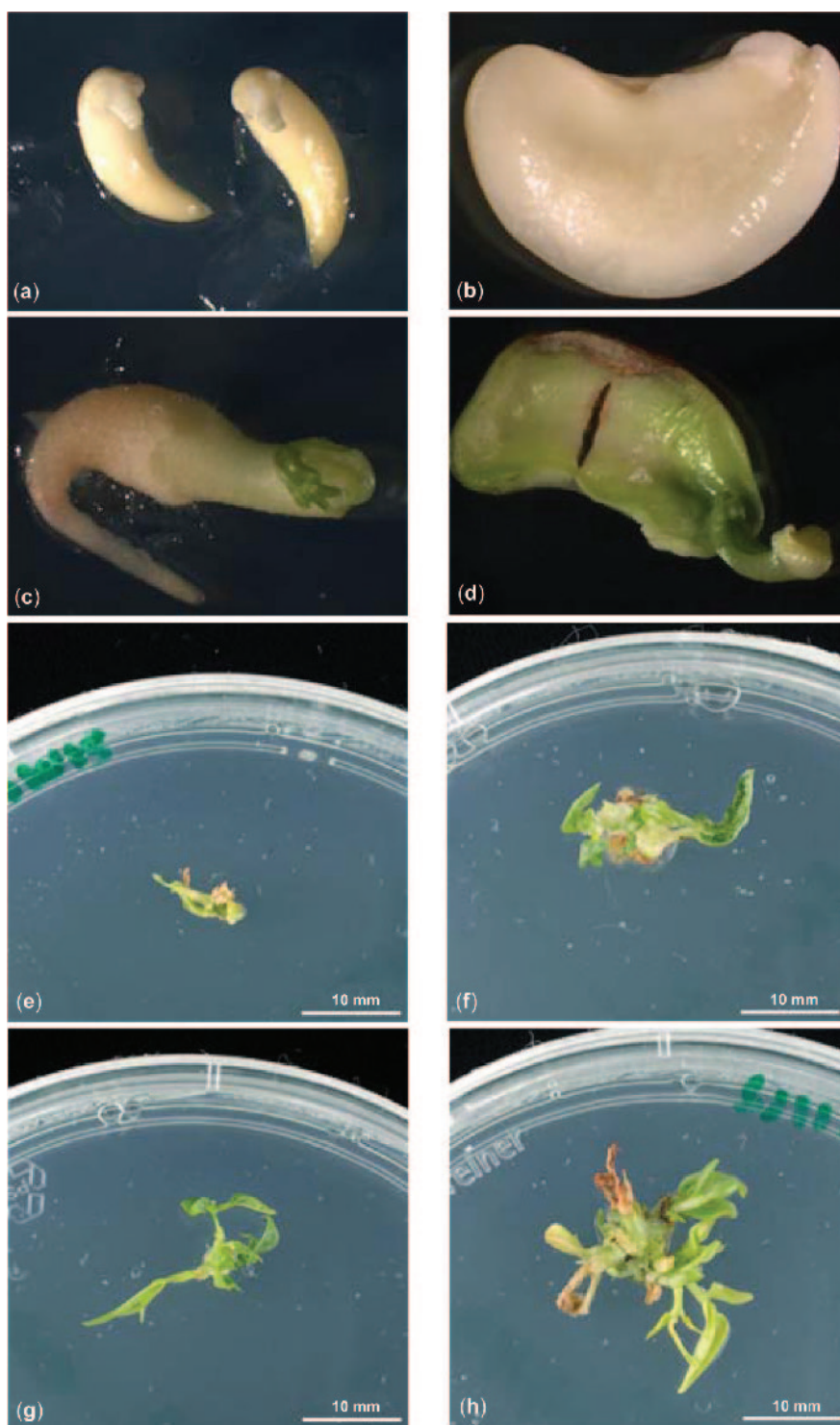


Figure 1 The embryonic axis and cotyledonary node used for cowpea transformation. Dry seeds are imbibed overnight and next day the embryonic axis (a) is removed from the cotyledon (b) with its nodal region exposed. Embryonic axis (c) and cotyledonary node (d) after 4 days of co-cultivation in tissue culture. Embryonic axis (e) and cotyledonary node (f) after 2 weeks under selection in tissue culture. Shoots developing from embryonic axis (g) and cotyledonary node (h) after 6 weeks under selection in tissue culture

Table 3 Protocol for cowpea transformation

Time	Activity	Medium	Details
Day 1	Mature seed sterilization and overnight imbibition	Water	20% commercial bleach for 45 min followed by thorough rinsing with sterile distilled water
Day 2	Explant preparation	-	(a) Longitudinally bisected embryonic axes with shoot and root apices removed, attached to cotyledons or (b) isolated embryonic axes
Day 2	Infection	Liquid	<i>Agrobacterium</i> strain AGL1 with <i>bar</i> or <i>nptII</i> genes, incubated with wounded cotyledonary nodes or embryonic axes
Days 2–6	Co-culture of <i>Agrobacterium</i> and cowpea explants	M1 Solid	4 days on thiol-containing solid medium
Day 6	Shoot initiation	M2 M3 (with selection)	Multiple shoot induction on BAP containing medium; begin suppression of <i>Agrobacterium</i> with timentin and start selection
Days 14–18 and 2 weekly	Remove initiated shoots from explants	M3 (with selection)	(a) Remove cotyledons at day 14 and embryonic axes 5mm below shoot apex at day 18 (b) Subculture on selective medium every 2 weeks from day 6 (c) Remove brown tissue parts at each subsequent subculture
Weeks 8–14 Weeks 14–17	Shoot elongation (a) Root initiation or grafting (b) Transfer to soil in greenhouse (c) Molecular analyses	M4 (with selection) M4 (with selection)	Selection of single healthy shoots (a) Root stocks of nontransgenic cowpea seedlings germinated for 10 days prior to grafting of putative transgenic shoots (b) Growth in high humidity chamber for up to 3 weeks prior to transfer to ambient conditions (c) Analyze expression of selectable marker and reporter genes and conduct PCR analysis
Week 20–32	Flowering and seed set Analysis of progeny		Self-pollination Expression of selectable marker and reporter genes, integration analysis by Southern blot and PCR

suitable explants for successful transformation experiments in legumes (Schroeder *et al.*, 1993; Pigeaire *et al.*, 1997; Olhoft and Somers, 2001; Popelka *et al.*, 2004).

To enhance gene transfer to cowpea, co-culture media is supplemented with thiol compounds, as described earlier for soybean (Olhoft and Somers, 2001; Olhoft *et al.*, 2003). The addition of L-cysteine, dithiothreitol, and sodium thiosulfate reduces browning of wounded tissue. Large numbers of cowpea explants can be processed in a simplified protocol (Table 3). In brief, the protocol consists of the following steps: (1) imbibition of sterilized mature seeds overnight in water, (2) explants are derived either by longitudinally bisecting embryonic axes with shoot and root apices removed but still attached to the cotyledon, or by isolating intact embryonic axes without

cotyledons, (3) infection with *Agrobacterium* strain AGL1 aided by local wounding in the meristematic regions, (4) co-culture on medium containing thiol compounds over 4 days at 25 °C in light, (5) shoot initiation and elongation on medium containing selective agents, (6) shoots are usually rooted *in vitro* or occasionally grafted onto nontransgenic seedlings and transferred to greenhouse conditions for flowering and seed setting, (7) polymerase chain reaction (PCR) or enzyme analysis of putative transgenic plants, and, (8) screening of next generation progeny by PCR.

The progeny of transgenic T₀ plants are normal in phenotype (Figure 2). The transgenes are transmitted to the progeny and homozygous T₂ plants, can be identified by screening their T₃ progeny for enzyme activity or by PCR (Popelka *et al.*, 2006).



Figure 2 Transgenic cowpeas in the greenhouse

Using this transformation system, it is now possible to produce 1–3 transgenic plants per 1000 explants, similar to other legume systems (Popelka *et al.*, 2004). Depending on the cultivar or line to be transformed, this protocol requires 5–8 months from explant preparation to harvested T₁ seeds. Enhancements to the level of transformation frequency may lead to a more efficient system (Chaudhury *et al.*, 2007).

2.2 Regulatory Measures—General Overview for *Bt*-Cowpea

Genetic transformation of cowpea has focused primarily on the development of *Bt*-cowpea, with other applications (traits) expected later. Accordingly, the following discussion will concentrate on *Bt*-cowpea only. Expectations are that it will be grown and consumed almost exclusively in Africa. Its developers have pledged to test and produce *Bt*-cowpea only in those African countries with functional regulatory systems. Most African countries are just now (2006–2007) in the process of developing the laws, regulations, guidelines, and institutional processes for safety assessment and testing of genetically modified (GM) crops. These are critical to the success of *Bt*-cowpea since lack of a transparent, science-based regulatory system is one of the major impediments to transfer of GM technology to developing countries (Traxler, 1999). For *Bt*-cowpea, as with other biotech crops, the regulatory dossier will need to encompass food, feed, and environmental safety. Successful commercialization will initially require regulated and contained production of *Bt*-cowpea seed in Australia, where the trans-

formation system was perfected (Popelka *et al.*, 2006). This will be followed by export from Australia and import into one or more West African countries where it will be assessed in confined field trials. Alternatively, contingencies for contained and/or confined evaluation in the United States are also being considered. Since Africa is the center of origin of cowpea the issue of potential gene flow to wild cowpea species will have to be assessed in the context of the overall risk assessment and approval process. Once regulatory approvals are attained, successful commercialization will result in release of *Bt*-cowpea into the farming environment. Because cowpea is widely traded across West African countries, the transboundary movement of *Bt*-cowpea seeds, vegetable matter, and processed products will also need to be addressed, since these African countries are signatories to the Cartagena Protocol on Biodiversity.

2.2.1 The Cartagena protocol on biodiversity and the biosafety protocol

The Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity (CBD) is an international instrument, ratified by more than 140 countries who are Parties to the CBD (CBD, 2005). The CPB also provides guidance regarding regulations, management, and risk control for the international trade of living modified organisms (LMOs). Key elements of the instrument are: (i) conservation and sustainable use of biodiversity, (ii) sharing of information (e.g., the basis for regulatory approvals), and (iii) based on an “adequate” level of protection. Many cowpea-producing African countries are signatories to the CPB, which went into effect in September 2003 (<http://www.biodiv.org/biosafety/signinglist.asp?sts=rtf&ord=dt>). The CPB requires every signatory to have a functioning regulatory framework in place to implement the CPB. Of particular importance is Annex III of the CPB that addresses environmental risk assessment of LMOs such as GM crops. A key benchmark for regulatory progress of *Bt*-cowpea will be the ability to test material in the field under confined field trials and then, once approved, under commercial field production. The CPB outlines procedures for the intentional release of

GM crops into the environment by the country of import. To date, the process involves import of the test material following the regulatory regulations and guidelines of the specific importing country.

For *Bt*-cowpea, the issue of transboundary movement of LMOs used for Food, Feed, or Processing (LMO-FFP) will have to be addressed because cowpea is routinely traded across West Africa. The CPB contains simplified procedures for the safe handling and transport of LMO-FFPs. In particular, Article 11.4 states that a country can use its domestic regulatory framework to address LMO-FFP as long as it is consistent with the objectives of the CPB. Article 11.6 addresses developing countries with economies in transition and states that for LMO-FFPs countries need to provide a risk assessment consistent with Annex III, a decision within 270 days and communication through the biosafety clearing house. These provisions will be useful to African countries that have not finalized their regulatory frameworks and guidelines for GM crop production in country, but which still need to import cowpea food and feed from a neighboring African country. This is particularly important in Africa where many countries import commodity grain each year as part of humanitarian relief efforts.

2.2.2 Annex III of the biosafety protocol—environmental risk assessment

Under Annex III of the CPB the objective of risk assessment is to identify and evaluate the potential adverse effects of LMOs on the conservation and sustainable use of biological diversity taking into account risks to human health. Annex III provides a set of principles, general methodologies, and points to consider in conducting environmental risk assessments. These principles are consistent with virtually every legitimate functional regulatory system described in this chapter. Most of the major GM-producing countries are not signatories to the CPB, however, whatever the source, the guidelines follow many of the same principles, the most important of which is that they be science based (Hill, 2005). An important difference is the use by the CPB of the poorly defined precautionary approach, or principle as it is sometimes called, which has been used by some as a mechanism to deny

import approvals (Hathcock, 2000; Conko, 2003). International treaties and other obligations are also part of the decision-making process. Recent activity at the World Trade Organization between the EU and several GM crop-producing countries illustrates this dynamics.

Some of the general principles of the Annex include:

1. The risk assessment should be science based, transparent, and make use of expert opinion and guidelines developed by relevant international organizations.
2. Lack of scientific knowledge or scientific consensus should not necessarily be interpreted as indicating a particular level of risk, an absence of risk, or an acceptable risk.
3. Risks associated with LMOs or LMO-FFPs should be considered in the context of the risks posed by the nonmodified recipients or parental organisms.
4. Risk assessment should be carried out on a case-by-case basis, i.e., *it should not be prescriptive*. The intended use and likely receiving environment are key determinants.

The methodological framework for the risk assessment involves the following steps:

1. An identification of any novel genotypic and phenotypic characteristics of the LMO.
2. A determination of the likelihood of adverse effects taking into account levels of exposure.
3. An evaluation of the consequences should adverse effects occur.
4. An overall risk estimate based on the likelihood and consequences of the adverse effects occurring.
5. A recommendation as to whether the risks are acceptable or manageable, including strategies for risk management or monitoring.
6. The risk assessment takes into account all of the relevant technical and scientific details as outlined earlier for the Food Safety Assessment. In addition, it also contains information regarding:
 - a. The biological characteristics of the recipient or parental organism, including taxonomy, centers of origin, and genetic diversity and a description of the habitat where the organisms may persist or proliferate.

- b. The methods of detection and identification and their specificity, sensitivity, and reliability.
- c. Information relating to the intended use of the LMOs, and
- d. Information on the location, geographical, climatic and ecological characteristics, including information on biological diversity and centers of origin of the receiving environment.

2.2.3 The regulatory process with the US regulatory system as a model

The US regulatory approval process for a GM crop illustrates the key features needed to attain a regulatory approval of a GM crop in the United States (Chassy, 2002). The US model is not necessarily the best model, but it has been used successfully to evaluate and approve nearly every commercialized GM-crop product released to date. Accordingly, institutions in Africa will have to develop or acquire the capacity to similarly scientifically evaluate the food, feed and environmental safety of *Bt*-cowpea.

The US regulatory process has nine stages or steps, as follows:

1. Biosafety Committee—National Institutes of Health Biosafety Guidelines.
2. USDA greenhouse standards and inspections.
3. USDA field trial authorization.
4. USDA authorization of transport for field trials.
5. USDA determination of nonregulated status.
6. Environmental Protection Agency (EPA) experimental use permit (EUP) approval.
7. EPA determination of food tolerance or tolerance exemption.
8. EPA environmental assessment and product registration.
9. FDA food and feed review process.

The process begins with the initial laboratory experiments performed under an institution's biosafety committee following US National Institutes of Health (US NIH) guidelines. Transgenic plants then enter an interagency coordinated framework for the regulation of biotechnology products. The three lead US Regulatory agencies are: the US Department of Agriculture (USDA),

the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA) (CAST, 2001; USDA, 2006).

The FDA is the US federal authority that determines food and feed safety, importation, and proper labeling of foods (CAST, 2001; USDA, 2006). In the consultative process the FDA reviews safety data supplied by the GM crop's developer to determine if there are, for example, any food safety issues with allergenicity or altered nutrient levels. While the US FDA uses a consultative process other countries, for example, the EU, Canada, and Japan, require formal food approvals. After commercialization, the FDA has the authority to suspend or restrict sales if a safety issue is found.

The USDA Animal and Plant Health Inspection Service (APHIS) is charged with protecting the US agricultural environment from pests, diseases, and weeds (USDA, 2006). The role of APHIS centers largely on assessing whether a new plant variety can harm native plants or other organisms or whether a GM plant variety has the potential to become a pest (i.e., a weed). The potential for gene flow from a new GM crop variety and effects on nontarget organisms (NTO) are special concerns.

The US EPA regulates the sale, distribution, use, and testing of GM plant varieties modified to express pesticides such as *Bt* toxins and the herbicides applied to GM herbicide-tolerant crops (USDA, 2006). It also sets tolerance limits in food and feed. Key activities include the issuance of EUPs for field testing and conduct of the environmental risk assessment and the endangered species risk assessment. The US EPA is also responsible for the insect resistance management plan (IRM) requirements, which are postcommercial conditions of registration.

Three crucial safety assessments will have to be conducted for *Bt*-cowpea to receive the regulatory approvals needed for registration and commercialization. These deal with food, feed, and environmental safety.

2.3 Safety Assessments for *Bt*-Cowpea

2.3.1 Food safety assessment

The Codex Alimentarius Guideline CAC/GL 45-2003 produced by the FAO/WHO Food

Standards Program is used by regulatory bodies worldwide as a guideline for food safety assessment (Codex Alimentarius, 2003; Chassy *et al.*, 2004). The guideline is based on the concept of substantial equivalence, which says that safety is best assessed by comparing defined differences between a transformed plant and its most similar untransformed line (EuropaBio, 2003). The approach relies heavily on compositional analysis (Chassy, 2002; Chassy *et al.*, 2004; ILSI, 2006). Below is a summary of the GL 45-2003 guideline and a description of some of the key general information that will need to be addressed for *Bt*-cowpea. Needed are:

- A. A description of the recombinant-DNA-cowpea plant. This will cover the crop, the transformation event, and the type and purpose of the genetic modification.
- B. A genotypic, phenotypic, and taxonomic description of cowpea and its use as food and feed. Needed also is a breeding history and identification of any traits, for example, allergens that may have an adverse effect on humans and animals. The history of cowpea as a food will also be covered, addressing the fact that virtually all parts of a cowpea plant are consumed including the seeds, pods, and leaves. In addition, these are consumed at both the immature and mature stages.
- C. A taxonomic and biochemical description of the donor organism, addressing especially any naturally occurring toxins, allergens, or pathogenicity.
- D. A description of the genetic modification(s) including the transformation method, the DNA and genetic elements, markers or other regulatory elements of the gene of interest, their size, orientation, and function.
- E. A complete molecular and biochemical characterization of the genetic modification(s) including the number of insertion sites, copy number, and sequence information flanking the insert as well as any open reading frames resulting from the insertion. Information on tissue-specific expression levels will be included. It should be noted that, to date, no direct correlation has ever been made between the molecular (genotypic) analysis and safety. Accordingly, the emphasis of the safety assessment is on the phenotypic analysis.

- F. A safety assessment to address toxicity and allergenicity (Goodman *et al.*, 2005). A compositional analysis of key biochemical components and metabolites will be provided.

2.3.2 Feed safety assessment

Because cowpea leaves are fed to animals, a feed safety assessment will be needed. The approach to assessing feeds varies widely by country. In the United States, feed is treated legislatively in the same way as food while in other countries such as Canada there is feed-specific legislation (OECD, 2003). In general, many of the issues associated with the safety assessment for food also apply to feed since both humans and animals frequently eat the same materials. For example, the *Bt*-cowpea safety assessment of food or feed will both provide a full characterization of the introduced genetic elements, information on any substances that may result from the modification, and whether any unintended effects occurred as a result of the transformation (OECD, 2003).

As is the case for the food safety assessment, the basis for the feed safety assessment is substantial equivalence (Kleter *et al.*, 2001; Chassy *et al.*, 2004; Berton and Marsan, 2005). However, animal feed, including cowpea used as feed, frequently differs from human food in terms of the form (e.g., silage), the degree of processing (raw vs. uncooked), quantity consumed, and specific tissues fed to livestock. Most importantly, consideration will be given to the quality and nutritive value of the *Bt*-cowpea as used in feed for livestock and for the safety of consumers of animal products derived from the livestock (OECD, 2003).

2.3.3 Environmental risk assessment

Environmental risk assessment (ERA) is an evaluation process that determines the likelihood of adverse effects—as well as the uncertainty associated with that determination—occurring to the environment after exposure to one or more stressors (e.g., a *Bt* toxin) (US EPA, 1998; Wilkinson *et al.*, 2003; EFSA, 2004). In some cases, the ERA may include risks to both humans and the environment, and may address direct, indirect, immediate, and delayed effects (EC, 2001/18/EC).

A risk assessment comprises problem formulation, hazard identification, hazard characterization, exposure assessment, and risk characterization (US EPA, 1998; EFSA, 2004). Because no technology is without risk, risk management and mitigation must also be considered.

A GM crop destined for commercial release requires an extensive ERA be conducted in the country(ies) of production (EC, 2002; EFSA, 2004; Mendelsohn *et al.*, 2004). All currently commercialized products have been assessed for environmental risks based largely on the US EPA's ecological risk assessment guidelines (US EPA, 1998; Mendelsohn *et al.*, 2004; Pew, 2004). Some countries, for example, Australia, Canada, and the European Union, have their own guidelines, but historically much of the data were built off the USA dossier (OECD, 1993; CFIA, 1994; US 7 CFR §340, 1998; EU, 2001; EC, 2002; EuropaBio, 2003; EFSA, 2004; OGTR, 2006; CPB, 2007). Detailed summaries of the evaluation process are provided in the US EPA Biopesticides Registration Action Documents (US EPA, 2008).

Because of the intended pesticidal nature of *Bt*-cowpea, there will need to be an extensive assessment of its potential effects on NTO. The best guidance will be to use the science-based and -tested surrogate species, tiered approach, used for assessing all current commercially approved biotech products (EuropaBio, 2004; Garcia-Alonso, 2006; US EPA, 2007; Romeis *et al.*, 2008). In the United States, regulatory guidelines for NTO risk assessment of insect-protected crops were developed by the US EPA and refined through Science Advisory Panels (SAP) (US EPA, 1996, 1998, 2001a–c, 2008). This approach will be based on potential exposure scenarios and characterization of hazard (toxicity). The selection of the test organisms and test materials will be based on the characteristics of the trait, i.e., Cry1Ab, and the expression pattern in the plant. Although northeastern Africa appears to be the center of origin of cowpea, an assessment of the potential for gene flow (often used synonymously with the term “outcrossing” or “cross-pollination”) will have to be assessed for cowpea grown in West Africa. While cowpea is a closed-pollinated crop and it may be able to outcross naturally, albeit at very low rates and influenced in part by flowering times, the proximity of alternative pollen sources as well as

temperature and humidity, available pollinators, and field architecture. Given all of these factors, a thorough assessment of the potential for gene flow will be necessary.

2.4 Safety Evaluation of *Bt*-Cowpea in Africa

2.4.1 Production of *Bt*-cowpea in Australia—regulatory issues

Bt-cowpea was created with a *cry1Ab* gene construct in the laboratory of T.J.V. Higgins, CSIRO, Canberra, Australia. The *Bt*-cowpea product concept is control of the legume pod borer. Since the borer occurs in Australia, CSIRO will also be able to conduct the initial line selections in Australia. Accordingly, the first set of regulations to address *Bt*-cowpea will be those of the Commonwealth of Australia. Activities (“dealings”) associated with GM Crops are regulated in Australia under the Gene Technology Act of, 2000 (Linacre *et al.*, 2006; OGTR, 2007). Under the terms of the Act, several government agencies are responsible for regulating genetically modified organisms (GMOs). These include the Office of the Gene Technology Regulator (OGTR), which regulates the use of GMOs and the Food Standards Australia New Zealand (FSANZ), which regulates food produced through the use of biotechnology. The point person in the OGTR is the Gene Technology Regulator (GTR), whose responsibilities and decision-making authority address GMOs in research, manufacturing, production, experimental trials, commercial release, and importation. Every dealing with a GMO must be licensed by the Regulator, unless the dealing is (i) exempt, (ii) a notifiable low risk dealing (NLRD), or (iii) on the Register of GMOs. The *Bt*-cowpea being developed in Australia is classified as a NLRD because the dealing is considered low risk since it will proceed only under containment in the laboratory and glasshouse with no intentional release into the Australian environment. This level of authorization requires Institutional Biosafety Committee (IBC) oversight and notification of the Regulator. Because *Bt*-cowpea is being developed for use in Africa and will not be released into the Australian environment or sold within Australia,

a food and feed safety assessment for submission to FSANZ will not be necessary.

2.4.2 Confined field trials in Africa

Once produced in Australia, cowpea seed will be shipped to one or more West African countries for use in confined field trials (CFT). CFT serve two purposes (Linacre and Cohen, 2006). First, they allow plant breeders to collect and assess important agronomic data. Second, data are generated in the CFT to assess human, animal, and environmental safety and provide a key element in the risk assessment. Countries regulate CFT through an application process that requires biological information outlined in the discussion of the food, feed, and ERAs, information on the trial site(s) itself, as well as confinement measures and contingency plans (OECD, 1992; CropLife, 2005; Linacre and Cohen, 2006). The ability of the plant to escape confinement and persist in the environment is a key issue to be addressed. To this end, guidelines will address spatial separation from other crops and plants, and control of reproductive structures and harvesting (OECD, 1992). As has been discussed for CFT in East Africa (Linacre and Cohen, 2006), regional harmonization of applications and guidelines are likely possible for West African countries.

2.4.3 The Burkina Faso regulatory model

As mentioned earlier, many African countries are in the process of formulating their biosafety laws, regulations, and guidelines. This situation with regard to regulatory status is changing rapidly. However, as of early 2007, only one major cowpea-producing African country, Burkina Faso, has developed a fully functional regulatory system and was in fact testing a GM crop under CFT. Given its development, Burkina Faso may illustrate what the regulatory climate might look like in other cowpea-producing African countries. Burkina Faso Law No. 005-2006/AN establishes the regulatory conditions for using GMOs and their derivative products (Burkina Faso, 2006). This comprehensive law addresses risk assessment, risk management, and information and control mechanisms involved in the use, dissemination and transboundary movement of GMOs and products

derived from them. It addresses GMOs that could have harmful effects on the environment, human, and animal health, or which are capable of affecting the sustainable conservation and use of biodiversity. This Law also addresses the development, experimentation (including confined and field tests), production, diffusion, storage, and destruction of GMO products and their derivatives.

The regulatory structure created in Burkina Faso comprises a supervisory agency and three consultative bodies. The National Biosafety Agency (ANB) is the overall national authority on the issue of biosecurity. The National Biosafety Observatory (ONB) is a consultative body responsible for biosafety, surveillance, and education. The National Scientific Committee on Biosafety (CSNB) is a consultative body responsible for biosecurity. The Internal Scientific Committee on Biosafety (CSIB) is the expert body responsible for biosafety scientific evaluation. The process used in Burkina Faso comprises several steps including:

- The import of GM products or products derived from them requires written authorization from the ANB.
- The ANB may ask for additional information before delivering a decision.
- To obtain authorization for the import, confined use, dissemination, and marketing of a GMO or derivative product the applicant must conduct a risk assessment to determine risks according to a timeline set by the ANB.
- The ANB issues an authorization after establishing that the import, confined use, dissemination, and marketing of a GMO or a derivative product can benefit the country without causing any risk to human and animal health, biodiversity, or the environment, and contribute to sustainable development and cause no harm to the socioeconomics of Burkina Faso and comply with ethical rules.
- The applicant must provide the ANB with evidence that it is capable of fulfilling its obligations, as specified in No. 005-2006/AN.
- Authorization can be revoked or suspended if conditions of the registration are not met or if the ANB obtains information that the GMO or derivative product presents risk to human and animal health, biodiversity, or the environment.

2.4.4 Regulatory status of cowpea-producing African countries

The following is a summary of the current state of regulation for GM crops in cowpea-producing African countries as of April 2007 (cf. http://www.absf africa.org/php/about_absf.htm).

Benin has signed, ratified, and put the CPB in force. Benin was involved in the UNEP-GEF Biosafety Framework project but not in the implementation project. At present, Benin has no biotechnology policy, biosafety guidelines, or regulation (ABSF, 2008).

Burkina Faso has signed, ratified, and entered into force the CPB. Burkina Faso was involved in the UNEP-GEF Biosafety Framework project but was not an implementation project. However, Burkina has passed and signed into law its national biosafety legislation. Implementation by laws have been drafted and scheduled for approval by the Council of Ministers by the first quarter of 2007.

Cameroon has signed, ratified, and entered into force the CPB. Cameroon participated in the UNEP-GEF Biosafety pilot program and the Biosafety Implementation Project. Cameroon has drafted biosafety guidelines. National Biosafety legislation is in draft form (ABSF, 2008).

Chad has signed the CPB but has not ratified it. Chad was also involved in the Biosafety Development Project. Chad has not enacted any Biosafety legislation (ABSF, 2008).

Côte d'Ivoire has not signed the CPB. Côte d'Ivoire was involved in the Biosafety Development Project. Côte d'Ivoire has not enacted any Biosafety legislation.

Ghana has ratified and entered into force the CPB. Ghana participated in the Biosafety Development Project. Currently Ghana is developing its biosafety policy and guidelines (ABSF, 2008).

Mali has signed, ratified, and entered into force the CPB. Mali participated in the Biosafety Development Project. Mali does not currently have any biosafety policy in place (ABSF, 2008). A National Biosafety Framework has been prepared but has not been reviewed by the Government and the National Assembly (CPB Interim Report 9 September 2005).

Mauritania ratified and entered into force the CPB. Mauritania participated in the Biosafety Pilot Phase. Mauritania does not currently have

any biosafety policy, guidelines, or legislation in place (ABSF, 2007).

Niger has signed, ratified, and entered into force the CPB. Niger participated in the Biosafety Development Project. Niger does not currently have any biosafety policy, guidelines, or legislation in place (ABSF, 2007).

Nigeria has signed, ratified, and entered into force the CPB. Nigeria participated in the Biosafety Development Project. Nigeria currently has national biosafety guidelines and a national biosafety committee in place (ABSF, 2007).

Senegal has signed, ratified, and entered into force the CPB. Senegal participated in the Biosafety Development Project. Senegal is finalizing its biosafety regulations and biotechnology policy (ABSF, 2007).

Togo has signed, ratified, and entered into force the CPB. Togo participated in the Biosafety Development Project.

3. FUTURE ROAD MAP

Biotechnology-based improvements of cowpea are likely to have major impacts on cowpea productivity in sub-Saharan Africa, and on incomes and welfare of cowpea producers, consumers, and other end users involved in the value chain. Cowpea yield potential is as high as 2500 kg ha⁻¹, yet current yields are far less—in the 350–700 kg ha⁻¹ range. The present low yields are due to pests, diseases, poor access to improved seeds and field, and storage pest control equipment and pesticides, as well as limited market outlets. Insect pests remain the most important limitation to cowpea production and post harvest storage (see below).

Plant breeding research at IITA, by the Bean/Cowpea CRSP (Collaborative Research Support Program) and by national research systems has developed high-yielding dual-purpose (fodder and grain) cowpea types that (i) have some tolerance to biotic and drought stresses, and (ii) high nitrogen and phosphorus use efficiency. Use of these materials (e.g., IT90K-277-2, IT97K-499-35, IT98K-131-2) in combination with improved and integrated crop management practices has the potential to increase cowpea productivity and incomes by 300% in West Africa (Singh *et al.*, 1997). These technologies are now available for

wider dissemination (James *et al.*, 2003), but additional research is needed to develop and adapt other options to specific agroecological and socioeconomic conditions.

Higher productivity of cowpea and more effective pest control with minimal concomitant health and environmental costs requires more than conventional breeding and frequent sprays of highly toxic pesticides. Breeding and development of cowpea varieties with natural resistance to the major insect pests has limited prospects. Extensive screening of cowpea germplasm over many years to find natural resistance to legume pod borer (*M. vitrata*), thrips, pod-sucking bugs, and cowpea weevil has revealed that the cowpea gene pool is poor or even devoid of useful genes for resistance to some of these pests. This means that certain of these constraints to cowpea productivity simply cannot be relieved via conventional breeding and screening alone. A needed adjunct is to use the tools of biotechnology to bring new genes into the cowpea gene pool (genetic transformation) and to use the tools of marker-assisted selection to more efficiently assemble multiple useful genes in single cowpea cultivars. Genetic modification to introduce vital genes can be used to address specific crop productivity problems that cannot otherwise be easily solved. One step in the process is the identification and cloning of genes that eventually could be used to transform cowpea (Fatokun, 2002; Murdock, 2002).

Using the tools of biotechnology the development of genetically improved cowpea lines with resistance to major pests would contribute significantly to (1) increased production and incomes, (2) improved nutrition, (3) lower prices and a greater supply of cowpea for consumers in the growing cities of Africa, and (4) improved soils and soil stability. Sithole-Niang *et al.* (2001) reported that small-holder farmers in South Africa benefit proportionately more from *Bt* cotton than larger-scale commercial farmers.

Biotechnology applied to cowpea offers a cost-effective solution and a substantial opportunity for crop improvement. The legume pod borer (*M. vitrata*), which belongs to the family Pyralidae, is highly sensitive to certain *Bt* crystal toxins. Work by T.J. Higgins and colleagues at CSIRO (see above) has led to the development of a reliable transformation system for cowpea. Specific *Bt* proteins (*cry1Ab* and others) active against

M. vitrata have been identified at Purdue University. This work has set the stage for the development of a *Bt*-cowpea for deployment in West Africa for use in controlling *M. vitrata*.

In sub-Saharan Africa, the controversy surrounding biotechnology appears to be related to the uncertainty about the risks or safety associated with GMOs. Many applications of biotechnology are not known to urban or rural consumers, yet could have tremendous impact on agricultural productivity. *Bt*-cowpea or other pest-resistant genetic improvements create the hope for a significant reduction in crop losses, lower pesticide residues, and improved food security. Like any other innovation, the products of biotechnologically improved cowpeas will be adopted if they provide concrete benefits for farmers and are not rejected by consumers. Langyintuo and Lowenberg-DeBoer (2003) predicted substantial benefits from *Bt*-cowpea for producers in Sahelian regions and for consumers everywhere in West Africa based on a regional trade model. Similar results have been described by Gbegbelegbe *et al.* (2006). However, Gbegbelegbe *et al.* (2006) have also pointed out that there are consumer concerns about *Bt*-cowpea in Nigeria.

Seed systems play a key role in the introduction and wide diffusion of GM crops worldwide and in resistance management plans necessary for the long-term sustainability of the genetic improvement. Unfortunately, seed production systems in West Africa are weak. Lambert *et al.* (2004) reported that seed producers in Senegal, Ghana, and Niger are interested in the idea of *Bt*-cowpea, but are also leery of a consumer backlash and of the responsibilities of resistance management.

3.1 Expected Products

There are several value-added genetic improvements of cowpea currently under development. The first GM cowpea will likely carry only a single *Bt*-gene, but this will likely be replaced quickly with a two-*Bt*-gene cowpea. Pyramided *Bt*-resistance genes whose protein products target different sites in the insect have both increased killing power as well as increased durability, i.e., are less likely to evoke the emergence of insect biotypes that overcome the resistance and render it useless. An

example of a pyramided GM cultivar is the newly released Bollgard II™ cotton that expresses two *Bt* genes with different sites of action in the insect (Greenplate *et al.*, 2003).

A new source of resistance to cowpea weevil is badly needed, and it will be possible to develop a bruchid-resistant cowpea that expresses the α -amylase inhibitor-1 gene from common bean. However, as has been observed when this gene is expressed in field pea seeds (*Pisum sativum*) (Prescott *et al.*, 2005), the protein product is slightly different from that produced in the common bean—due to a difference in post-translational modification. This difference raises questions about potential immunogenicity and even allergenicity as well as toxicity of the aberrant protein. As mentioned earlier, there are options available to obviate this concern but these issues will have to be resolved before serious consideration can be given to deploying an α -amylase inhibitor cowpea.

Other GM cowpeas are at earlier stages of development. These include those with reduced levels of oligosaccharides and thus improved consumer acceptance. Lowered oligosaccharides might open the way for cowpea to be used as the base for infant weaning foods.

Other genetic improvements not easily attainable by conventional breeding lie farther in the future. For example, several insect pests of cowpea, particularly thrips and the complex of pod-sucking bugs, cannot be controlled through plant resistance introduced through conventional breeding. Unfortunately, little or no effort has been expended to discover genes for resistance to these pests. As is the case with the *cryIAb* gene used for the development of *Bt*-cowpea, the genes are likely to come from an industrial laboratory. Until the needed bioassays are developed and applied to discover effective genes, resistance to these additional pests will remain out of reach.

3.2 Risks and Concerns

African Agricultural Technology Foundation (AATF) recently commissioned an assessment of the likely impact of deploying a high quality insect-resistant *Bt*-cowpea cultivar in West Africa; the study was carried out in 2006 by IITA and Purdue University (IITA and Purdue University, 2006)

scientists in major cowpea growing countries of Benin, Niger, Nigeria, Burkina Faso, and Mali. Its aims were to assess producer and consumer preferences, acceptability, willingness-to-pay, and adaptability of a GM cowpea to local conditions in West Africa. The preliminary results indicate that: (1) Information exchange and awareness are important for the adoption and region-wide diffusion of *Bt*-cowpea, (2) cowpea growers prefer *Bt* to conventional cowpea seeds in the sense that they would be willing to pay a higher price for *Bt*-cowpea seeds, (3) given the potential of reducing health hazards by lowering the use of toxic synthetic pesticides, both farmers and consumers would be willing to pay a premium price for *Bt*-cowpea as an alternative to harmful cotton pesticides. The opportunity costs of using cotton insecticides include the economic losses encountered by the farm household when a family member is sick due to the misuse of chemical insecticides, (4) urban consumers in regional markets believe that *Bt*-cowpea may be safer than conventional cowpea treated with chemicals, (5) *Bt*-cowpea will raise incomes substantially at the farm, household, community, and regional levels.

3.2.1 Ex ante impact assessment of *Bt*-cowpea

Economic analyses have been carried out in a few countries to estimate the impact of *Bt*-cowpea. They suggest that *Bt*-cowpea would provide an aggregate expected gross benefit (for rural and urban consumers collectively) estimated at US\$ 1.2 billion per year in Benin, 3.1 billion in Niger, and 8.4 billion in Nigeria. These estimates are at the high end as they are based on the probably false assumption that the seed costs for *Bt*- and conventional cowpea are equal. The unit cost of supplying *Bt*-cowpea seed may be substantially higher than for conventional cowpea. Even so, the benefits for resource-poor farmers and low-income rural and urban consumers of *Bt*-cowpea could be worth billions.

Surveys indicate that the average Nigerian consumer buying cowpea mostly for resale does not have a preference for any of the types of cowpea and would derive the same level of satisfaction from *Bt*- or conventional cowpea. The aggregate results across rural producers and consumers in

Table 4 Estimated gross benefit from *Bt*-cowpea in Niger

		Gross benefit from <i>Bt</i> -cowpea		
Rural consumers and producers	1 652 306 648 908	FCFA/year	3 055 752 790	US\$/year
Urban consumers	52 872 561 407	FCFA/year	97 781 775	US\$/year
Total benefit from <i>Bt</i> -cowpea—Niger	1 705 179 210 316	FCFA/year	3 153 534 565	US\$/year

Niger implies a willingness to pay a premium for *Bt*-cowpea, and this translates into a total benefit of \$US 3 billion with the adoption of *Bt*-cowpea (Table 4). Health reasons (which includes the *Bt*-cowpea is safer than conventional cowpea because it contains no insecticide) could explain the predicted behavior of the average urban consumer in Niger. The total gains for both producers and consumers with the introduction of GM cowpea in Niger would be US\$ 3 billions/year.

The estimated economic impact of *Bt*-cowpea in Nigeria—the largest cowpea-producing nation in the world, is presented in Table 5.

We conclude from the modeling that rural producers and consumers in Nigeria would experience a welfare gain of about \$US 5.1 billion once *Bt*-cowpea became available in Nigerian rural markets. Two reasons could explain why the average rural household in Nigeria might prefer *Bt*- to conventional cowpea:

1. *Bt*-cowpea is safer than conventional insecticide-sprayed cowpea in terms of consumption.
2. *Bt*-cowpea would increase the income of the rural farm household and therefore, increase its welfare.

The model predicts a gross welfare gain of about \$US 3.3 billion for all urban consumers in Nigeria once *Bt*-cowpea is made available in Nigerian urban markets. This assumption is based on the survey results that were conducted in Kano, Sokoto, and Maiduguri which showed that Maiduguri consumers tend to discount

Bt-cowpea, contrary to consumers in Kano and Sokoto who on average tend to prefer *Bt*- to conventional cowpea.

3.2.2 Efforts toward sustainable biotechnology opportunities in West Africa

In the United States and other countries where GM crops have been deployed, viable markets have developed and sustainable business models have emerged (James, 2006). These benefits have been especially evident for low-resource farmers in developing countries (Huesing and English, 2004). In many such environments, GM crops have proven profitable both for seed producers and farmers and have provided benefits to consumers such as reduced mycotoxin levels in foods (Hammond *et al.*, 2004). To raise awareness of needs and opportunities associated with GM crops in Africa, a workshop on “Appropriating Biotechnology for Investment Opportunities in Nigeria” was organized by IITA, the Nigerian Agricultural Biotechnology Project (NABP), the National Biotechnology Development Agency (NABDA), Ahmadu Bello University (ABU), and the Sokoto State Ministry of Agriculture and Natural Resources, and sponsored by USAID. The workshop laid the foundation for a sustainable biotechnology program in Nigeria by building institutional and scientific capacity to conduct research, implement priority regulatory guidelines and policies to create an environment for biotechnology development, and create awareness

Table 5 Estimated gross benefit from *Bt*-cowpea in Nigeria

		Gross benefits from <i>Bt</i> -cowpea		
Rural consumers and producers	658 038 817 706	NAIRA/year	5 131 907 332	US\$/year
Urban consumers	422 491 532 978	NAIRA/year	3 294 923 244	US\$/year
Total benefit from <i>Bt</i> -cowpea	1 080 530 350 684	NAIRA/year	8 426 830 576	US\$/year

of the benefits of biotechnology (C. Fatokun, personal communication 2006).

3.2.3 Adoption, perceptions, and profitability studies of conventional cowpea in West Africa

The introduction of *Bt*-cowpea into West Africa will necessarily occur in the context of a long-established process of cowpea improvement through traditional breeding (Eicher *et al.*, 2006). To assess what may await *Bt*-cowpea, a survey explored farmers' perceptions of cowpea pests in three different agroecological zones in Benin, particularly the legume pod borer, *M. vitrata* (Adetonah, 2005). Most of the farmers report that they start spraying to control insect pests 3 or 4 weeks after sowing. A little over half reported using synthetic insecticides recommended for cotton, while less than a quarter use synthetic insecticides recommended for cowpea. Others use botanical insecticides. A large majority of farmers were able to recognize *M. vitrata* larvae. Lower percentages of farmers were able to recognize adults or knew of the link between adults and larvae.

A study carried out in two agroecological zones in southern Benin demonstrated that the use of some chemical pesticides without adequate protection would expose farmers to headaches and respiratory, skin, eyes, and stomach ailments. Misuse of pesticides is a widespread problem in the developing world, including Africa, and farmers need more information and awareness about the health hazards linked to pesticide misuse as well as alternative pest control techniques like botanicals (IITA/Purdue University, 2005). This highlights the need for alternative technologies for the control of cowpea insect pests.

A third producer study indicated that the profitability of cowpea cropping systems depends mainly on the varieties used (local or improved), the cropping and management practices (use of chemicals including fertilizers and pesticides), and the access to input and output markets (Aïtchédjé, 2001). The results showed that only cropping systems using improved cowpea technologies—mainly improved varieties—were profitable even under tight credit constraints compared to local technologies (local cultivars and current storage techniques). A profitable

cowpea technology is composed of an improved variety and a chemical insecticide. The added value (gross margins) on cowpea production systems is mainly due to higher yields from the improved varieties. The pesticide used for field treatment and the storage technologies used after harvest can make a significant difference (Aïtchédjé, 2001). Financial benefits are probably higher because environmental and health-related costs, including lost workdays, associated with pesticide use were not taken into consideration in this financial profitability analysis. The misuse of pesticides, in general, has been causing deaths every year in the cotton- and cowpea-producing areas of Africa. These factors should be used by farmers in assessing the benefits of using pesticides.

3.2.4 Requirements for cowpea export and quality assurance

An exploratory market study of cowpea products in Ghana using data from case studies shows that cowpea flour, the main value-added product, is typically sold in bulk or in unbranded small packages through retail and wholesale outlets and directly to consumers including individuals, institutions, and the catering industry. Cowpea flour is less prone to insect pest attack than are whole cowpeas and consequently is a major source of food during the dry season and the period between harvests. However, the growth in the dietary share of cowpeas has been constrained by long preparation time and labor requirements, undesirable product characteristics including beanie flavor, low digestibility, and abdominal distress as well as postharvest grain losses to insect pests. There are several cowpea foods based upon cowpea flour and these provide a varied nutritious diet and have added desirable attributes, which include availability, easy cooking, and good taste. Although a high proportion of food processors are aware of new cowpea utilization technologies that are being developed, only a low percentage can expand their businesses to accommodate these new technologies. The full impact of new utilization technologies will be realized over several years and only following substantial private capital investment in processing, marketing, and strategic promotional activities. The market study in Ghana also established consumer preference for selected

product attributes measured in terms of responses to “agree/disagree” to declarative statements about selected product attributes including cooking quality, availability, taste, keeping quality, and comparison with soy flour (Nyankori, 2002). In many areas of Africa fresh cowpea leaves are regularly harvested and consumed often as a part of the typical “sauce” (Bean/Cowpea CRSP West Africa Mission).

3.2.5 Women and cowpea in Africa

In Africa, cowpea is a major source of cash income for both rural and urban women who are involved in cowpea-based food sales. (Okike, 2000). In Cameroon and Senegal, for example, most of the cowpea green pod marketing is handled by women. In other African countries, women harvest and sell cowpea to retailers or consumers at markets or on roadsides. In northern Nigeria, women are involved in cowpea production, cooking, and small ruminant feeding with cowpea hay (CGIAR, 2000). Women use CRSP storage technologies, particularly solar heaters, because they often do not have access to storage insecticides (Bean/Cowpea CRSP, 1997). A rapid appraisal survey of adoption of cowpea varieties and storage techniques carried out in 1996 in the northern Peanut Basin of Senegal indicated that both men and women use the improved varieties and the CRSP-developed metal drum technology for grain storage. Recent studies have revealed widespread adoption of another CRSP technology, bagging the grain in multiple plastic bags (M. Bokar, unpublished)

3.2.6 Remaining challenges

Numerous challenges to enhancing the cowpea value chain in sub-Saharan Africa remain. These include the participatory development and diffusion of improved cowpea varieties adapted to changing rainfall patterns, environmentally sound pest control, value addition, and market access. The Bean/Cowpea CRSP, IITA-PRONAF (Projet Niébé Pour l’Afrique), and national agricultural research and extension systems have contributed to these challenges through research and stakeholder capacity building. As has been demonstrated with

other crops, biotechnology as applied to cowpea has the potential to contribute to a significant increase in productivity and improvement in quality and competitiveness of cowpea-based products. Consumer preferences will be important in developing products with high potential to be adopted by farmers and other end users. Integrated pest management including resistance to pests like *Maruca*, which is not effectively controlled by conventional breeding or pesticide, will be a key for cowpea competitiveness for trade (lower pesticide) and lower health costs due to a significant decrease in toxic pesticide misuse.

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Pea

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1. INTRODUCTION

Pea (*Pisum sativum* L.) is an important crop worldwide and ranks second only to dry bean in worldwide production (FAO, 2006). It is consumed as a staple in many developing countries or fed to livestock for its nutritional value. In addition to its agronomic benefits and nutritional value, pea has served as the subject of numerous genetic studies and as a model for plant physiologists for more than one century. Pea has been chosen for these studies due to its wealth of genetic variation, short generation time, and ease of culture. These characteristics led to Mendel's choice of pea for his experiments and, though others worked with pea previously, Mendel is often credited as being the first to study the genetics of pea when he discovered the Laws of Segregation and Independent Assortment (Mendel, 1866).

Genetic study of pea continued during the 20th and 21st centuries and has traversed many landmark achievements. A myriad of simply inherited genes have been characterized and assembled into the seven linkage groups. Application of modern molecular DNA technologies toward genetic mapping in pea has produced a number of dense genetic maps and identification of specific markers linked to genes of agronomic importance that can be used for marker-assisted selection (MAS). Discovery of traits adversely affecting production and quality of pea has been shown to suffer from limited favorable genetic variation in germplasm. As a result, application of gene technology to introduce novel genes from

distant or unrelated species has been used to overcome these production constraints. A detailed description of this technology to pea improvement is the subject of this contribution.

1.1 History, Origin, and Distribution

Pea is a cool season annual legume crop that originated in the Near East and Mediterranean regions and has been grown since early Neolithic times (Zohary and Hopf, 1973). Evidence from carbonized remains indicates that pea has been cultivated with cereal crops, such as wheat and barley, since domestication. Limited historical evidence makes it difficult to determine the exact location of domestication; however, soon after domestication pea and other legumes spread to other parts of the world with the movement and activities of man. Currently, pea is produced on all continents except Antarctica. Pea was established in cool-temperate areas of Europe and the former Soviet Union as well as India and China. Introduction into North America occurred soon after discovery by Columbus (Hedrick, 1928).

1.2 Botanical Description

The genus *Pisum* is a member of the family Papilionaceae tribe Viciae and is composed of two species, *P. sativum* L. and *P. fulvum* Sibth. and Sm. The species *P. sativum* includes five subspecies, *P.s. ssp. sativum*, *P.s. ssp. elatius*, *P.s. ssp. humile*, *P.s.*

ssp. *Arvense*, and *P.s. ssp. hortense*. The literature indicates that ssp. *elatius* and ssp. *humile* are the progenitors of cultivated pea *P.s. ssp. sativum* (Zohary and Hopf, 1973). *P. sativum* ssp. *arvense* includes the field pea and the “Austrian” winter pea, both possessing colored flowers and variously pigmented seeds. *P. sativum* ssp. *abyssinicum* is a unique subspecies derived from Ethiopia and has been reported to have very narrow genetic base (Weeden *et al.*, 2004).

Genetic composition of pea is approximately 4800 Mbp (mega base pair) spread across $2n = 2x = 14$ chromosomes. The first karyotypes were presented as early as 1931 (Lewitsky, 1931, as cited by Blixt, 1958). The standard karyotype of pea is based on the type line, L110, following improvements over earlier procedures of Caroli and Blixt (1953) (Blixt, 1958; Hall *et al.*, 1997). Centromere location in chromosomes 1 and 2 is metacentric, while in the remaining five chromosomes it is submetacentric. Chromosomes 4 and 7 contain secondary constrictions and possess satellites. *Pisum fulvum* contains a third secondary constriction on chromosome 5 (Ben Ze’ev and Zohary, 1973). Chromosomes 2 and 5 have experienced a translocation event, referred to as the Hammarlund translocation (summarized by Kvostova, 1983). Numerous other chromosomal rearrangements have also been reported (Lamm and Miravalle, 1959; Kvostova, 1983; Folkesson, 1990a, 1990b).

1.3 Economic Importance

Dry pea production worldwide has averaged 11 million metric tons on slightly greater than 6 million hectares during the past decade (FAO, 2006). Fresh pea production is largely controlled by private industry and it is difficult to estimate the production and area due to proprietary issues and lack of reporting in other countries. Pea plays an important role in crop rotation in cereal-based cropping systems by providing an opportunity to break cereal disease cycles, control grassy weeds, and improve soil tilth and fertility through association with the soil bacterium, *Rhizobium leguminosarum*, which fixes atmospheric nitrogen into ammonia for use by the plant. Residual fixed nitrogen is available to subsequent crops

reducing the need to apply supplemental sources of inorganic nitrogen.

Rich genetic variation in pea has led to the development of many market classes including yellow or green cotyledon dry pea, wrinkle-seeded fresh peas for canning or freezing, edible-podded peas, and most recently the snap pea. All market classes harvested as fresh product are used for human consumption while the dried pea crop is used predominantly as an animal feed, but is also a component of soups for human consumption. In addition, the harvested dry pea can be processed industrially for starch or protein preparations. A more specialized type of green pea, the marrowfat pea, is also produced and has extremely large and irregularly shaped seeds. The marrowfat pea is used in snack food preparations in the Far East and in a thick soup preparation in the United Kingdom referred to as “mushy peas.”

Legumes in general are nutritionally rich. Pea contains 18–33% protein, 4–7% fiber, and 37–49% starch and is an excellent source of carbohydrate, minerals, and vitamins. Though dry peas are nutritionally rich, they contain antinutritional factors, which detract from their value for some uses. For example, trypsin inhibitors reduce the feed value when fed to livestock and presence of raffinose family oligosaccharides may cause intestinal and social discomfort when dry pea is consumed for food. Presence of phytic acid limits the availability of mineral elements and other dietary components including starch and some protein.

The most widely produced pea in the world is the yellow cotyledon dry edible pea, although the green cotyledon dry pea is quite popular in some regions. Green dry peas are typically split and used almost exclusively for human consumption while yellow peas are primarily used as a supplement in animal rations. A portion of yellow peas are used for human food, but due to limited reporting from many developing countries it is difficult to estimate an exact quantity. The Austrian winter type pea with colored flowers and pigmented seed is another form of dry pea which when harvested at maturity is commonly used as an ingredient in bird feed. Due to its excessive biomass production, the Austrian winter pea is commonly used as a green manure crop to improve soil tilth and fertility. A rather minor dry pea type produced for the snack industry in the Orient is the marrowfat pea. It is also harvested dry and is characterized by

extremely large and irregularly shaped seed with a dimpled surface.

1.4 Traditional Breeding

Traditional breeding approaches to genetic improvement of pea have been remarkably successful. Pea breeding has taken advantage of the rich genetic variation available in the germplasm. Breeders have achieved modest increases in yield and have altered plant morphology significantly over the past three decades. Early varieties were characterized by a long vine, normal leaflet morphology, and ranging growth habit. The canopy collapsed at maturity as the plants lodged making it difficult to harvest and creating an environment favorable for disease development. Discovery of the single gene mutation, *af*, for tendrilled leaf morphology led to development of modern semi-leafless cultivars with improved standing ability. The semi-leafless morphology

(*afafStSttltl*) combined with the recessive *le* allele for shortened vine length resulted in a plant type that was sturdy and remained upright throughout the growing season. Improved stem strength properties and the ability of tendrils from neighboring plants to intertwine and provide mutual support resulted in the erect canopy. The upright canopy allows greater air flow reducing disease incidence and improves harvest ease.

The pea crop is challenged with both biotic and abiotic stresses. Biotic stress agents include insect pests and fungal, viral, and bacterial pathogens (Table 1). Genetic resistance is present in available germplasm for many of the pathogens; however, little genetic resistance to insect pests is currently available. Plant breeders have introduced resistance to many pathogens, especially those controlled by single genes, but many of the more quantitatively inherited resistances have been more difficult and remain a focus for future improvement. The most devastating pathogen worldwide is *Aphanomyces euteiches* (Drechs.) and

Table 1 Biotic stress agents constraining US dry pea production

Biotic stress	Causal organism	Resistance mechanism
Disease		
Fusarium root rot	<i>Fusarium solani</i>	Polygenic
Aphanomyces root rot	<i>Aphanomyces euteiches</i>	Polygenic
Fusarium wilt	<i>Fusarium oxysporum</i> f. sp. <i>pisi</i> Race 1, 2, 5, 6	<i>Fw</i> , <i>Fwn</i> , <i>Fwf</i>
Pea enation mosaic virus		<i>En</i>
Pea streak virus		Not defined
Bean leafroll virus		<i>lr</i>
Pea seed-borne mosaic virus		<i>sbm-1</i> , <i>sbm-2</i> , <i>sbm-3</i>
Powdery mildew	<i>Erysiphe pisi</i>	<i>er-1</i>
Downy mildew	<i>Peronospora pisi</i>	Not defined
Ascochyta blight	<i>Phoma medicaginis</i> , <i>Mycosphaerella pinodes</i> var. <i>pinodella</i> , and <i>Ascochyta pisi</i>	Polygenic
Nematodes		
Pea cyst nematode	<i>Heterodera goettingiana</i>	NA
Root-knot nematode	<i>Meloidogyne</i> spp.	NA
Root-lesion nematode	<i>Pratylenchus penetrans</i>	NA
Insects		
Pea leaf weevil	<i>Sitona lineatus</i>	NA
Pea seed weevil	<i>Bruchus pisorum</i>	Not defined
Pea aphid	<i>Acyrtosiphon pisum</i>	NA
Loopers	<i>Autographa californica</i> (Speyer) <i>Anagrapha falcifera</i> (Kirby)	NA

genetic resistance is controlled by several loci and is inherited in a quantitative manner (Pilet-Nayel *et al.*, 2002). Genetic resistance has recently been identified and is currently being introduced into new cultivars in several breeding programs. Powdery mildew, caused by *Erysiphe pisi* DC. is important in all pea production regions and is controlled by a single recessive gene, *er*; however, it has been hypothesized that a second gene may also be involved in resistance (Tiwari *et al.*, 1997). The *er* gene is durable and has effectively conferred resistance since its initial description (Harland, 1948). Genetic resistance to the important viruses is simply inherited and controlled by single genes; *En* for pea enation mosaic virus, *lr* for bean leaf roll virus and *sbm-1* for pea seed borne mosaic virus, and have been incorporated into many modern cultivars.

Seed quality traits are less well characterized genetically, but breeders have been able to select improved types through visual selection. Dry pea is sold into two primary markets for food or feed and each requires different quality attributes. The food market is driven primarily by visual appearance of the seed's size, shape, and color. However, splitting ability and integrity of whole seed during rehydration and canning are also important quality criteria for the food market. Splitting ability comprises traits including, dehulling efficiency and resistance to cracking and breakage. Retention of bright cotyledon color is a primary quality criterion for all peas, both yellow and green cotyledon types, in food markets. Quality of fresh market peas for freezing and canning is based on bright, attractive color, flavor, seed size, and skin toughness as it impacts seed integrity through processing. Resistance to bleach or "blonding" is important for all green pea markets, dry or fresh market types. Green cotyledon pea seed harvested dry can bleach in the presence of high humidity or rain and intense sunlight. Bleached seed in a harvested sample cannot exceed 1.5%, 3.0%, or 5.0% to be graded no. 1, 2, or 3, respectively. Excessive bleach reduces the quality and value of the crop to the grower.

Quality criteria for the feed markets focus on the internal seed composition rather than appearance. Energy value derived from the starch component, protein content, amino acid profile, and presence of antinutritional factors, such as phytic acid and trypsin inhibitors, which reduce palatability and

digestibility of the pea seed are also important. Significant genetic variation for protein content is available in germplasm, but only modest effort has been made to increase seed protein content.

Modern breeding programs either use bulk population, pedigree selection, or a combination of these procedures to identify superior breeding lines (Muehlbauer *et al.*, 1988). The bulk population method has the advantage of easy population maintenance while allowing natural selection to occur prior to selection of superior nearly homozygous types in the F₄ or F₅. A disadvantage of the bulk procedure is that certain genotypes may be lost reducing the range of variation present for selection. The pedigree method maintains a wider range of genetic variation initially present and allows specific undesirable genotypes to be removed from the population. The pedigree method is time consuming, labor intensive, and requires that individual plants be space planted for data collection. Wide plant spacing has the disadvantage that data collected may not be representative of growth in a solid stand.

Population improvement in pea has been accomplished using biparental mating or backcrossing followed by selection and has been successful in transferring disease resistance from landraces and wild germplasm into adapted legume germplasm. Three-way and double crosses are also used to combine useful traits among diverse germplasm. Male sterility is present in *Pisum* germplasm; however, due to limitations associated with cross-pollination in the male sterile parent, development of hybrid cultivars has not been successful. Recurrent selection, though time consuming and laborious in self-pollinated crops, has been used to pyramid favorable alleles for disease resistance (Lewis and Gritton, 1992). Specific crossing designs, such as the diallel and top cross, are commonly used to combine various traits and to determine combining ability among parental types (Griffing, 1956).

Mutation breeding has been used worldwide for improvement of grain legumes through increased genetic variation and creation of novel alleles. Pea cultivars resulting from mutagenesis have been released with improvements including increased yield, lodging resistance (afila leaf trait), larger seed, increased protein concentration, and modified maturity (Micke, 1988a). Mutation breeding of pea has been centered in many European

countries including Sweden, the Netherlands, Germany, Italy as well as several eastern European countries and Russia (Micke, 1988b).

Conventional breeding methods have been successful in improving pea germplasm toward development of superior cultivars. However, genetic studies have shown that improvement of several important agronomic characters in pea is difficult and would benefit from MAS. Detailed genetic maps have been generated for pea placing many genes of interest on individual linkage groups and associating them with DNA markers derived from several methods (Weeden *et al.*, 1998; see McPhee, 2006 for a review). The diversity of procedures employed to develop the markers currently available limits the application of MAS in breeding programs. Optimum application of MAS requires that markers be (1) tightly linked to the trait of interest, (2) based on one or few technologies, and (3) co-dominant to allow selection in early generations. Loridon *et al.* (2005) reported a consensus map of pea that comprises randomly amplified polymorphic DNA (RAPD) and co-dominant microsatellite markers that will not only serve as a reference map for the research community, but also offers a framework to which additional co-dominant markers can be placed near important disease resistance or other genes of agronomic importance.

Major accomplishments for plant breeding in pea include improved agronomic performance through an erect plant type with an upright canopy, multiple disease resistance, and improved crop quality for current markets. Improved disease resistance is primarily for those traits where single genes are responsible for resistance and additional effort is needed for those traits controlled in a quantitative manner. Though significant improvements have been made through conventional breeding, lack of favorable genetic variation or inability to identify superior selections provides opportunities for genetic modification using genes introduced from beyond natural hybridization barriers.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Traditional plant breeding efforts in pea has made significant advancements; however, many impor-

tant traits suffer from limited genetic variation and knowledge of genetic control mechanisms. Specific avenues for improvement include increased content of sulfur-containing amino acids and reduced level of antinutritional compounds, such as phytic acid, galactose sugars RFOs (raffinose family oligosaccharides), and trypsin inhibitors. Though genetic resistance is present in available germplasm, application of broad-spectrum virus resistance may be possible based on common genetic mechanisms among viruses that affect peas. Pea has a significant advantage to other legumes and crops in general due to its broad use as foodstuff in developing countries and its reputation as a healthy food. Gene modification to improve nutritional value through increased mineral or micronutrient content would be an effective means by which to address malnutrition in many undernourished populations. Starch modification in pea seed could be an effective way to convert pea to a functional food and thereby add value as a health food.

2. DEVELOPMENT OF TRANSGENIC PEAS

The primary objective of genetic transformation in pea, with few exceptions, has been to prove the potential for transformation and establish a functional system for genetic transformation (Table 2). A few genes of agronomic importance have been incorporated into pea with functional outcomes and, unfortunately, those which have been proposed for deregulation have been unable to overcome the regulatory hurdles.

2.1 Donor Genes

2.1.1 Bruchid resistance

Schroeder *et al.* (1995) transformed pea cv. Greenfeast with the α -amylase inhibitor (αai) gene from common bean (*Phaseolus vulgaris* L.) to induce resistance to *Bruchus pisorum*, an important seed pest. The coding region was engineered to include the coding region of αai flanked by the 5' and 3' regions of the PHA gene, *dlec2*, from bean. The selectable marker gene *bar*, flanked by the 35S promoter and ocs 3' terminator region were included in the transfer DNA (T-DNA). Explants were transformed according to procedures described in Schroeder *et al.* (1993).

Table 2 Genetic transformation of pea

References	Gene(s)	Genotypes	Explant
De Kathen and Jacobsen, 1990	<i>nptII</i> and <i>hpt</i>	'Madria'	Epicotyl node
Puonti-Kaerlas <i>et al.</i> , 1992	<i>hpt</i>	'Puget' and 'Stivo'	Epicotyl segments
Schroeder <i>et al.</i> , 1993	<i>bar</i> and <i>nptII</i>	'Greenfeast', 'Rondo'	Sliced embryonic axes
Davies <i>et al.</i> , 1993	<i>nptII</i> and <i>uidA</i>	'Puget'	Cotyledonary nodes
Schroeder <i>et al.</i> , 1995	α - <i>AI</i> and <i>bar</i>	Greenfeast	Sliced embryonic axes
Grant <i>et al.</i> , 1995	<i>nptII</i> and <i>bar</i>	'Bolero', 'Trounce', 'Bohatyr', 'Huka'	Cotyledonary nodes
Bean <i>et al.</i> , 1997	<i>bar</i>	Puget	Cotyledonary nodes
Chowrira <i>et al.</i> , 1998	PEMV-CP	'Sparkle'	<i>in vivo</i> axillary meristems
Grant <i>et al.</i> , 1998a	<i>nptII</i> , AMV-CP, PSbMV-CP	94-A26, Bolero, 'Hadlee', 'Crown', 'Courier', 89T46.UK	Cotyledonary nodes
Polowick <i>et al.</i> , 2000	<i>uidA</i> , <i>nptII</i>	Greenfeast, 'CDC Vienna', S2-90-25E, 93-4-18G, MP1338, MP1382, AWPNZ66, AWP1512	Sliced embryonic axes
Nodalska-Orczyk and Orezyk, 2000	<i>nptII</i> , <i>hpt</i> , <i>dhfr</i> , <i>bar</i>	'Laser', 'Heiga'	Cotyledonary nodes
Morton <i>et al.</i> , 2000	α - <i>AI-1</i> , α - <i>AI-2</i> , <i>bar</i>	'Laura'	Sliced embryonic axes
Svabova <i>et al.</i> , 2005	<i>uidA</i> , <i>nptII</i>	'Adept', 'Komet', 'Lantra', 'Olin', 'Oskar', 'Tyrkys'	Cotyledonary nodes
Collins <i>et al.</i> , 2006	<i>nptII</i> , α - <i>AI-1</i>	'Excell'	Sliced embryonic axes

2.1.2 Alfalfa mosaic virus (AMV)

Grant *et al.* (1998b) transformed six cultivars or breeding lines of pea, 94-A26, 'Bolero', 'Hadlee', 'Crown', 8554, and 89T46.UK. These lines represent the fresh processing, maple, and dry pea market classes. Two binary vectors were designed to transfer the AMV coat protein into pea. Both vectors included the *nos-nptII* gene for resistance to kanamycin as the selectable marker and AMV-cp coding sequence downstream of the 35S promoter. In addition to the 35S promoter, the second plasmid contained the tobacco mosaic virus (TMV) enhancer region immediately upstream of the AMV-cp coding sequence. Cotyledonary explants from field grown seed were used as explants and transformation was accomplished using *Agrobacterium tumefaciens* strain AGL1.

Timmerman-Vaughn *et al.* (2001) also transformed pea cv. Crown and two breeding lines, 94-A26 and 89T46.UK13, representing three different market classes; maple pea, fresh processing pea, and field pea, respectively. These lines were transformed with the AMV strain NZ1 coat protein coding sequence acquired by polymerase chain reaction (PCR) amplification from plasmid DNA. Two binary plasmids were used to transfer T-DNA to the plant. The first contained the AMV-cp downstream of the 35S promoter and the second contained the TMV Ω' enhancer in addition to the

35S promoter. Selection was accomplished using a *nos-nptII-nos* gene for kanamycin resistance. Immature cotyledons were used as explants (Grant *et al.*, 1995) and transformed with *A. tumefaciens*.

2.1.3 Pea enation mosaic virus (PEMV)

Chowrira *et al.* (1998) transformed pea cv. Sparkle with the PEMV coat protein coding sequence from an existing clone, pPCP4-5, flanked by the 35S promoter and a 3' *rcbs* terminator sequence. *In planta* transformation of axillary meristems was accomplished via microinjection and electroporation of decapitated plants. Selection was not conducted on the plants and R₁ progeny derived from seed harvested from shoots that developed from electroporated buds were tested for the presence of the PEMV-cp by PCR and southern hybridization.

2.1.4 Pea seed-borne mosaic virus (PSbMV)

Grant and Cooper (2003) report unpublished results of transforming 10 pea genotypes with three different constructs containing the PSbMV coat protein—two in the sense orientation and one with a nontranslatable sequence. Though independent transformed plants were obtained, none of the

primary transformants were resistant to PSbMV infection and the gene(s) were not inherited based on progeny analyses.

Jones *et al.* (1998) used four different constructs to transform the replicase gene, *Nib*, from PSbMV strain DPD1 (pathotype 1) into pea cultivar Puget to induce resistance to PSbMV. Transformation was accomplished using the procedure of Bean *et al.* (1997). Three constructs contained two tandem 35S promoters followed by the *Nib* coding sequence and the cauliflower mosaic virus (CaMV) PolyA terminator sequence. Construct #2 included a tandem repeat of the *Nib* coding sequence and Construct #3 included an inverted repeat of the *Nib* coding sequence. The fourth construct contained a *nos* promoter and *ocs3'* terminator surrounding the *Nib* coding sequence. Thirty-five independent transformants were generated with

one to three copies of the transgene and transformants with each of the constructs were recovered.

2.2 Methods Employed

Pea has been genetically transformed using two methods, *A. tumefaciens*-mediated and direct delivery through microinjection coupled with electroporation. Direct delivery using the gene gun technology has been attempted but only transient gene expression was reported (Warkentin *et al.*, 1992). The preferred method of genetic transformation in pea is through the use of *A. tumefaciens* (Figure 1). Although, microinjection and electroporation have been reported (Chowrira *et al.*, 1998), this methodology has not been adopted in the scientific community.

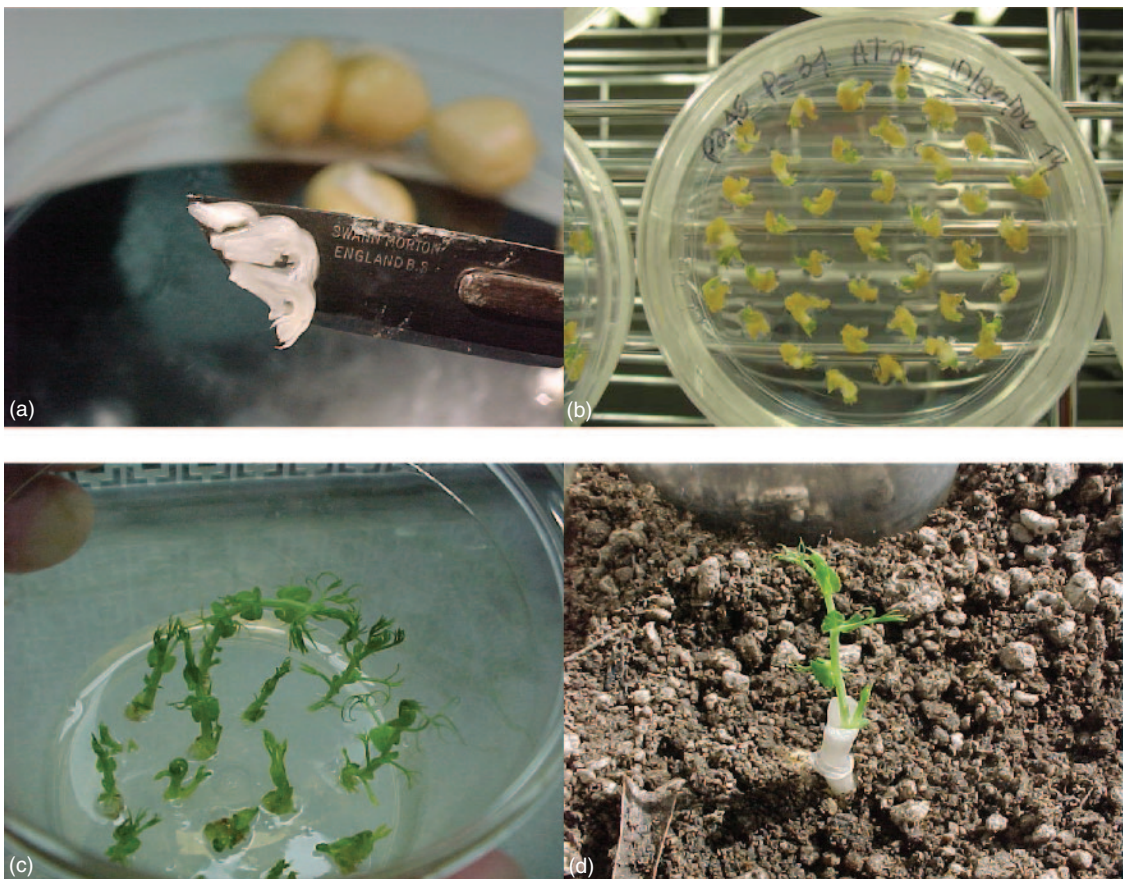


Figure 1 Stages of pea transformation via *Agrobacterium tumefaciens*. (a) Thin slices of immature embryonic axis, (b) explants on selection media, (c) putative transformed shoots on selection media, (d) transformed shoot being grafted to root stock

Early efforts in transformation of pea focused on identification of appropriate explant tissue and tissue culture procedures. Due to difficulties associated with regeneration through organogenesis and embryogenesis from callus generally in legumes intact meristematic tissue has been used as explant for genetic transformation, and *A. tumefaciens* is the selected vehicle for gene transfer. Efficiency of genetic transformation in pea ranges from <1% to 5%.

2.3 Selection of Transformed Tissue

Selection of transformed pea has been accomplished with both antibiotic and herbicide resistance genes. Antibiotic selection has been accomplished using *nptII* for kanamycin resistance and *hptII* for hygromycin resistance. Selection using herbicide resistance genes has been accomplished using *ahas3r* for resistance to chlorsulfuron and *bar* for resistance to Basta. Though kanamycin has been considered inadequate for selection, it has been used successfully at concentrations of 50 mg l⁻¹ (De Kathen and Jacobsen, 1990; Lulsdorf *et al.*, 1991; Polowick *et al.*, 2000), 75 mg l⁻¹ (Grant *et al.*, 1998a), and 100 mg l⁻¹ (Davies *et al.*, 1993; Svabova *et al.*, 2005). Hygromycin has been used at concentrations of 5–10 mg l⁻¹ (De Kathen and Jacobsen, 1990; Grant *et al.*, 1995; Bean *et al.*, 1997; Jones *et al.*, 1998), 15 mg l⁻¹ (Puonti-Kaerlas *et al.*, 1992), and 25 mg l⁻¹ (Lulsdorf *et al.*, 1991). Herbicide resistance to Basta conferred by the *bar* gene has been successfully used at concentrations of 10 mg l⁻¹ (Polowick *et al.*, 2000) and 15 mg l⁻¹ PPT (Schroeder *et al.*, 1993) (Figure 1c). The only report using the *ahas3r* gene for resistance to chlorsulfuron used a concentration of 10 mg l⁻¹ (Polowick *et al.*, 2000).

2.4 Regeneration of Whole Plant

Regeneration of whole plants occurs in two stages using current methodology. Shoots are generated from existing meristems in the explants and successively subcultured during selection. Once shoots are considered transformed they are either induced to root on a high auxin medium or grafted directly onto existing rootstocks in the greenhouse or growth chamber (Figure 1d). Induction of roots is rather time consuming and can take as long as

4–6 weeks, whereas grafting can be accomplished immediately. Both systems will incur some loss, but grafting can shorten the time required to produce T₁ seed by several weeks.

2.5 Testing

Identification of successful transformants among plants surviving selection in tissue culture has been accomplished using a variety of methods. Evaluation in the early attempts at transformation made use of the florescent nature of the β -glucuronidase (*GUS*) gene, *uidA*, and was tested in callus or cell extract (De Kathen and Jacobsen, 1990; Lulsdorf *et al.*, 1991). Phenotypic tests, such as fluorescence and herbicide paint assays, were used in later experiments where young plants were regenerated. As PCR became more routine, PCR-based methods were used to amplify a region of the inserted gene or the T-DNA to test the T₁ or primary transformant. Enzyme assays have been used as a means to test functionality and expression level of the gene. Final proof of transformation involves DNA or RNA blotting using probes derived from the T-DNA. Positive results from southern or northern blot tests in later generations showed both that the gene was heritable and that it was stably incorporated in genomic DNA.

Puonti-Kaerlas *et al.* (1992) were the first to report the inheritance of an inserted gene (*hpt*) in regenerated plants and progeny. They performed a comprehensive evaluation using phenotypic reactions, nucleic acid tests, chromosome analyses, and morphological inspections. R₁ seeds harvested from the primary regenerants were tested for resistance to hygromycin by placing a drop of hygromycin on the third leaf and recording the presence of local lesions. In addition, leaf explants were placed on media containing hygromycin and production of callus was recorded. R₁ plants were allowed to self, and R₂ seeds collected and tested for presence of the transferred DNA by southern blotting. Gene expression was tested based on northern blots using an *hpt* probe. Southern analysis showed that all hygromycin resistant R₁ and R₂ plants possessed *hpt* and those that were sensitive lacked the gene. They analyzed 12 R₁ or R₂ plants derived from two clones and all were shown to be tetraploid. They concluded

that the chromosome doubling occurred during tissue culture since analysis of the parents showed that they were diploid. Detailed morphological analysis of the transformants showed that for some traits the transgenic plants were significantly different than the control genotype, but that overall the deviations were slight. Various leaf measurements showed the greatest difference between the control and transgenic offspring. Variation among offspring clones derived from the same callus ranged significantly from values statistically different from the parents to those that did not differ significantly from the control.

Davies *et al.* (1993) tested for stable integration and expression of the transferred DNA using southern blots and PCR amplification of a portion of the *GUS* gene. They reported the selection and regeneration of 28 T₀ plants, which were derived from four independent transformation events. PCR evaluation indicated that all plants tested contained the transferred gene while the control did not produce a product. *GUS* positive plants were tested multiple times and were shown to confirm the reaction. Though a positive *GUS* reaction was observed in all instances, activity in one of the plants was significantly lower by as much as 50-fold. Pollen fertility among these lines was greater than 90% in three of the four clones, but was 29% in the fourth and seed set was only 17.1 seed per plant while the other three lines produced from 32 to 71.8 seed per plant. Southern blot analysis was performed with two probes, an internal probe and one that spanned the *GUS*/RB junction and showed the presence of multiple *GUS* genes in all four lines. The numbers of *GUS* genes, based on the internal probe, contained in three of the lines were 1, 5, and 20, respectively. A band was not detected for the fourth line using the internal probe, therefore, the positive result was due to the RB/T-DNA junction. Combined analysis of the digests and southern blots indicate that the four lines contained, 1, 3, or 5 independent loci. Those lines with more gene copies than independent loci contained tandem arrays of insertions.

Schroeder *et al.* (1993) used the *bar* gene for resistance to the herbicide, Basta, and tested for phosphinothricin acetyl transferase (PAT) enzyme activity among putative transformants. They performed northern blot analyses with a probe encompassing the entire *nptII* coding region. In addition to enzyme assays and northern blots

they tested the plants phenotypically using a leaf paint assay where individual leaflets were wetted with a solution of the Basta herbicide and localized reactions were recorded. PAT activity varied 20-fold among the lines tested and could be detected in progeny plants of the R₁ generation. They reported that the *bar* and *nptII* genes were transferred to the next generation following a 3:1 Mendelian ratio for presence/absence.

Grant *et al.* (1995) used the *bar* gene and *nptII* to identify transformants. Verification of gene insertion and expression were performed using enzyme and leaf paint assays similar to Schroeder *et al.* (1993). Transformation was confirmed by southern blot analysis using the *bar* and *nptII* genes as probes. Two primary transformants were evaluated cytologically and were shown to have the expected diploid chromosome complement of $2n = 14$. T₁ progeny from transformants for three of the lines showed expected Mendelian segregation ratios, while all plants from the fourth line were positive. It is possible that the 100% positive result was due to this line containing three copies of *bar*; however, few individual plants were available for testing. Regenerated plants were tested for the possibility that they were chimeric. This possibility was dismissed since only one pod (with two seeds) that was negative for the *bar* gene and the two seeds in that pod could have been negative by chance.

Bean *et al.* (1997) used the *bar* gene to identify transformants during culture and transformants were phenotypically verified using the herbicide paint assay and a PAT enzyme assay. Insertion into genomic DNA into primary transformants was accomplished by southern blotting with a 550-bp fragment of the *bar* gene from the plasmid, pJIT84. Segregation in the T₁ indicated that two of eight events segregated according to the expected 3:1 ratio while the remaining lines were chimeric with differing segregation ratios.

Jones *et al.* (1998) used the same procedure as Bean *et al.* (1997) and used leaf paint assays with PCR-based tests to confirm the primary transformants. DNA and RNA blots were also used to verify incorporation into genomic DNA and expression of the *Nib* gene. Progeny lines were tested through the F₃ (equivalent to T₃) and the gene was shown to be stably inherited and expressed. The plants that were tested and shown to be transgenic were shown to achieve equivalent plant height to the nontransgenic control.

Chowrira *et al.* (1998) did not use selection during generation of primary transformants (R_1). PCR analysis of 23 R_1 plants identified three with the expected band. These three plants were then tested by southern hybridization to confirm the PCR results. Plants testing positive by southern blotting were tested for protein level by western blotting. Progeny in the R_4 showed resistance to PEMV indicative that the coat protein gene was heritable and maintained function.

Evaluation of transgenic pea developed by Commonwealth Scientific and Industrial Research Organization (CSIRO) Plant Industry in Australia containing the α -amylase inhibitor gene, (αAI) for bruchid resistance is the most complete and comprehensive evaluation of transgenic pea with regard to expression, inheritance and of human and environmental impact. Schroeder *et al.* (1993) reported the stable integration of the amylase inhibitor gene from *P. vulgaris* in pea cv. Greenfeast and cv. Rondo. Verification of transformation included enzyme assays for PAT and nptII activity and Northern analysis for bar expression. Inheritance of the *bar* and *nptII* was tested in the R_1 and fitted the expected 3:1 (presence: absence) ratio. Inspection for morphological aberrations indicated that the R_0 and R_1 plants did not have adverse effects from insertion of the T-DNA.

Schroeder *et al.* (1995) grew five of 18 transformed plants to the T_1 and used a nondestructive test for presence of the α -ai protein in the cotyledon. Individual seeds expressing the protein were sown to produce T_2 seed for bioassays with *B. pisorum*. Six plants from one of the lines (F10) selected for high expression levels of α -AI grown to the T_4 along with six control plants were used in a second bioassay experiment. Six to eight weevil eggs were placed on the pods of the test plants such that the larvae could hatch and infest the seed. Emergence of adult weevil was monitored regularly for 140 days and emergence of adults was recorded. Southern, northern, and western analyses were conducted to quantify gene expression in transgenic seeds. Homozygous T_4 plants were indistinguishable from nontransgenic control plants and seed yield was equal.

A subsequent study by Morton *et al.* (2000) investigated the effectiveness of a variant of the α -AI gene, $\alpha AI-2$, on pea weevil development and the potential impact on strategies for crop protection in the field. The $\alpha AI-2$ gene is less

effective than $\alpha AI-1$ and serves to delay emergence of the adults by as much as 3–40 days, but does not kill the larvae. Deployment of the $\alpha ai-1$ gene would provide severe selection pressure on the bruchid risking development of a resistant population. Morton *et al.* (2000) suggest that even though $\alpha AI-2$ does not provide complete resistance, combining it with $\alpha AI-1$ would not effectively reduce the selection pressure since it functions in a similar manner. Feeding trials using peas expressing $\alpha AI-1$ indicated no adverse effects on rats (Pusztai *et al.*, 1999) and field trials conducted in three diverse locations and over several years demonstrated that agronomic performance of the transgenic plants was equal to the nontransgenic controls and that the resistance to the seed weevil was effective.

The most recent evaluation of peas expressing the $\alpha AI-1$ gene involved feeding trials with broiler chickens (Li *et al.*, 2006). This study indicated that the transgenic peas did not adversely impact health or protein utilization of the chickens; however, starch digestion was reduced indicating a limitation to the use of transgenic peas expressing $\alpha AI-1$ in diets of monogastric mammals. A similar result of normal protein digestion and reduced starch utilization was reported in a feeding trial with pigs using the same transgenic seed line (Collins *et al.*, 2006).

In summary, transgenic peas expressing the $\alpha AI-1$ gene were created using the *A. tumefaciens*-mediated protocol and were tested extensively for gene expression, inheritance of the introduced gene through at least five generations, evaluated in field trials for efficacy of the $\alpha AI-1$ protein against seed weevil, and evaluated in feeding trials to determine the ultimate utility and impact of transgenic pea on the environment and in feed rations. Evaluation of pea seed expressing the $\alpha AI-1$ gene is the most extensive and complete example of its kind in the literature.

2.6 Regulatory Measures

Advent of the worldwide web has opened doors to easy access of information on a wide variety of topics. The regulatory policies in place within the United States and internationally to monitor and regulate genetically modified crops are no exception. The three primary regulatory agencies in the United States are APHIS (Animal and Plant

Health Inspection Service), EPA (Environmental Protection Agency), and FDA (Food and Drug Administration). Each of these agencies has websites containing a wealth of information on the regulatory processes they use to ensure that no adverse effects on the environment or human health will occur as a result of the release and production of a new, genetically modified organism.

The three US agencies act in a coordinated way to ensure that human health and safety of the environment is maintained. APHIS is charged with regulation of plant pests and plants. EPA is charged with regulation of microbial/plant pesticides and new uses of existing pesticides and the FDA is charged with regulation of food, feed, and food additives. A document entitled, Coordinated Framework for Regulation of Biotechnology, published in the Federal Register issue 51 Fed. Reg. 23,302, 23,302 (1986) prescribes how products of US biotechnology will be reviewed and what federal agencies will review them. In general, separate but coordinated oversight is carried out by the United States Department of Agriculture (USDA), EPA, and FDA, depending upon the gene and host crop and their respective roles are to ensure that the new genetically modified crop is safe to grow (APHIS), safe to the environment (EPA), and safe to eat (FDA). Plant incorporated pesticides (PIP) include the gene and the enzyme produced as the result of expression of that gene and are regulated by EPA. FDA policy regards the PIP as a food additive and as a result has authority to regulate the new crop.

Criteria that must be met prior to deregulation include documentation that no risk to human health, nontarget organisms, and the environment exists. In addition, the potential for gene flow must be negligible and a management plan that minimizes development of pest resistance must be developed. EPA assesses potential effects associated with a plant-incorporated protectant, including toxicity, allergenicity, and skin and eye irritation, as well as long-term effects including cancer, birth defects, and reproductive and neurological system disorders. These potential effects are also assessed in relation to the public's potential exposures to these pesticides and take into account all potential combined sources of the exposure (food, drinking water, etc.) to determine the likelihood that a person would be exposed at levels that would pose a health risk.

It is critical that these factors that are used to evaluate a novel genetically modified crop be taken into consideration at the outset of a given research project. As genetic transformation can be a long and expensive process, especially for legumes, it is in the best interest of the research project to design experiments that minimize adverse effects and remove potential road blocks to deregulation. In addition to the health and environmental concerns listed above, proprietary issues for all processes and components must be reconciled prior to complete implementation and deregulation.

3. FUTURE ROAD MAP

3.1 Expected Products

Dry pea production is challenged with a number of biotic and abiotic stresses. As new information is generated regarding resistance mechanisms and novel traits are discovered to introduce resistance, it is expected that new genetically altered pea varieties addressing plant stress could be developed. Drought is a major limiting factor for production in many regions worldwide. In addition, salinity and/or mineral toxicity are production constraints that may be addressed through novel gene introduction.

Seed quality issues such as seed size, digestibility, and appearance are important for marketability and consumer acceptance. Altered expression of invertase has been studied in chickpea and could be applied to pea to increase seed size for niche market classes. Starch modification toward altered digestibility and functional starch is a novel concept that has significant dietary application. Increased protein content and improved amino acid composition through increased content of S-containing amino acids, cysteine, and methionine, would benefit both food and feed uses.

Broad-based virus resistance and resistance to fungal pathogens through incorporation of anti-fungal genes offer opportunities to significantly improve crop production and possibly return pea production to areas where disease pressure has made production impossible.

Functional genomics and gene discovery in pea will continue to expand and be a focus of research. Virus-induced gene silencing (Constantin *et al.*, 2004) and RNAi (RNA interference) applications

hold great promise in all crop species, but transformation efficiency will need to be improved before this technology can be fully implemented. Knockout mutants resulting in the reduction or elimination of antinutritional factors such as trypsin and chymotrypsin inhibitors, raffinose oligosaccharides, and phytic acid would improve digestibility and consumer appeal.

3.2 Addressing Risks and Concerns

Risks and concerns related to development of transgenic pea varieties to human health and the environment depend largely on the introduced gene. Careful selection of the gene and thorough evaluation of the new plant will significantly reduce the chance of adverse effects when the new variety is produced. Traditional cropping systems and production practices may change with the possibility that herbicide resistance is introduced into pea. Impacts of this technology have been debated and are well documented.

Pea is a self-pollinating crop with <1% outcrossing that reduces the risk of gene transfer via pollen. Escape of a transgenic crop into roadsides or neighboring fields is of concern with regard to creation of a weed problem that cannot be controlled. Escape of pea from cultivated areas has not been observed in North America and combined with the low levels of outcrossing make pea a relatively low risk crop to the environment and for deployment of transgenic varieties.

A primary concern over transgenic crops is that unintended effects on plant phenotype and seed content may occur as a result of introducing a foreign gene. Charlton *et al.* (2004) reported a novel application of proton nuclear magnetic resonance (NMR) to quantify metabolite differences in leaf extracts and using that information to test the hypothesis that introduction of a gene results in marked differences in a transgenic plant. Comparison of transgenic lines, null transformants (plants lacking the gene yet passing through the tissue culture process), and wild-type plants showed that significant differences could be detected between the three plant types, but that these differences could not be attributed solely to introduction of the gene. Due to the wide variation present among wild-type samples and reduced significance of differences

between the wild type and later generations of transgenic lines they concluded that differences detected by NMR were likely due to passage through tissue culture and progressive loss of epigenetic effects. They also suggest that NMR may be a viable means by which to show that transgenic pea lines lie within the normal range of variation seen in wild-type plants.

3.3 Expected Technologies

Transformation efficiency in the legumes is very low. Variability in efficiency is contributed by genotype, binary plasmid, introduced gene, and variability among personnel. Transformation efficiency in pea ranges from <1% to as high as 5% and methods to improve efficiency are needed to expand application of the technology.

Selectable marker genes that are currently available are adequate and are quite effective. It will be important in future applications of transformation technology that twin binary plasmids are used such that the marker gene and the gene of interest are inserted on independent T-DNA. The independent T-DNA has a likelihood of being incorporated at different positions in genomic DNA. Genetic segregation of these genes in progeny allow for selection against the selectable marker removing concerns over these genes in the transgenic variety.

3.4 Public Perceptions and Political and Economic Consequences

Worldwide importance of pea as food crop offers an opportunity for pea to serve as a vehicle to deliver important added dietary components to malnourished populations. Public acceptance of genetically altered crops for food use will provide resistance to adoption, but if these issues can be overcome this technology could serve as an invaluable tool for human health.

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Faba Bean

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Vicia faba (faba bean) was not among the very first domesticated crops. It was probably introduced into agriculture only in the late Neolithic period (Körber-Grohne, 1987). Cubero (1974) concluded that the center of origin was in the Near East, Iraq, and Iran, and secondary centers evolved later on in Afghanistan and Ethiopia. Before 1000 BC, the culture of faba beans was already very established in Europe, including Britain. Large-seeded types are of recent origin and they were probably developed only 1000–1200 years ago in East Iraq, and from there spread to Asia, across North Africa to Europe, and eventually to America. In China, the crop seems to have arrived only after 1200 AD. The faba bean reached Mexico and South America by the Spaniards. From then on, it experienced there an independent evolution (Körber-Grohne, 1987; Bond, 1995). Several gene bank accessions with promising agronomic features originated from Ecuador. These are mostly large-seeded types. The more recent history has to mention the comprehensive reports of Muratova (1931), Sirks (1931) and Hanelt *et al.* (1972) on genetics, systematics, taxonomy, history, and geographical topics (Bond, 1995).

1.2 Botanical Description

The intraspecific diversity is mainly described by use of seed size. Persoon (1807) described the small-seeded group as *V. faba minor* (roundish seeds of up to 0.6 or 0.7 g weight per seed), the medium-large-seeded group as *V. faba equina* (single seed weight less than about 1 g) and *V. faba major* with its impressive, large, and flat seeds weighing more than 1 g per seed. The name “field beans” indicates to the small and medium sized types, whereas “broad beans” denotes *V. faba major*. Today, often “faba bean” is used to address the species in its total diversity. A wild ancestor of faba bean is not known, and the related vetch *Vicia narbonensis* is considered as the nearest relative to *V. faba* (Zohary and Hopf, 1973). Based on restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) data, more recently Van den Ven *et al.* (1993) placed *Vicia peregrina* and *Vicia michauxii* into the direct taxonomic neighborhood of *V. faba*. These related vetches have seven chromosomes. *V. faba* belongs with *Vicia villosa* and *Vicia sativa* to the genus *Vicia*. With *Lens culinaris* and *Pisum sativum*, it belongs to the tribe *Vicieae* and only at the family level of *Fabaceae* it is united with the *Phaseolus* bean, the soybean and with the lupines (Sitte *et al.*, 2002).

The faba bean crop is annual, sown either in autumn or in spring. It bears a strong, hollow, tetragonal erect stem, with zero or up to five (or even more) basal branches arising from basal leaf axis. There are only noneffective rudiments of tendrils at the leaf tip. Different from pea, stipules are very small, with a distinct black stipule spot on the stipule's down side. The bean has got a robust tap root with profusely branched secondary roots. Nevertheless, the rooting system is not as voluminous as that of cereals like oats or wheat. The roots bear the typical nodules formed by *Rhizobium leguminosarum*, as expected for it being a leguminous species. The first, juvenile leaves bear two leaflets. After four or more nodes the maximum number of leaflets (typically six) is reached. The first, lowermost inflorescence is located in the axis of a leaf with at least four leaflets, inserted on the stem as low as at the fourth node or several nodes later (higher). Two to more than eight flowers per inflorescence occur. The wild type flower color is white with a soupcon of pink traces and a very distinct, satin black spot on both wing petals (Figure 1). Brown, violet, red, and further grades of flower colors exist. Totally white flowers are a pleiotropic effect of an allele for zero tannin in the seed testa, accompanied by grayish testa color instead of buff testa and by absence of the wild type stipule spot. One to two pods with three or four seeds are to be expected per inflorescence; one plant may yield far more than 12 pods, distributed across more than six nodes. In germplasm used for human consumption, often very few, very large pods per plant are realized, with more than 20 cm pod length, more than six

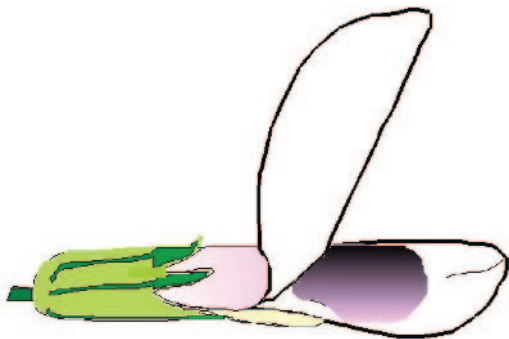


Figure 1 *Vicia faba* flower with white flag petal and black spot on wing petal

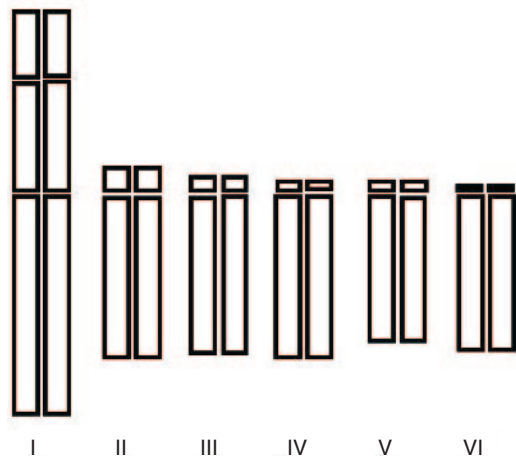


Figure 2 Karyogram of *Vicia faba* [Reproduced from Fuchs *et al.* (1998). © Springer]

seeds per pod and more than 1 g weight per seed. The contrary is realized in some *V. faba minor* ssp. *paucijuga* types from the Hindukush region, showing small, 2 seeded pods with less than 0.3 g weight per seed, growing on small, gracile, tillering plants with very slender leaflets. These types are suspected to represent the most primitive version of *V. faba* (Cubero, 1974).

V. faba is one of the cytogenetically best characterized plants. Its six chromosome pairs contain as much as 1C approximately 13 pg (picogram) of DNA, which corresponds approximately to 13000 Mbp (mega base pair). The first, very large chromosome (about 18 μ m length) is metacentric, with one satellite. There are five similar (approx. 7–9 μ m length) acrocentric chromosomes (Figure 2). The metacentric chromosome I of *V. faba* probably originated from a remote fusion of two telocentric chromosomes. Many cytogenetic phenomena were observed for the first time by studying *V. faba*, for instance, nucleolus formation at the secondary constrictions during telophase, or the existence of an upper tolerance limit for chromosome arm length (Schubert and Oud, 1997; Fuchs *et al.*, 1998).

1.3 Economic Importance

At the worldwide scale, faba bean occupies about 2.6 millions ha, which in 2005 represented 4%

of the total area dedicated to pulses. From the worldwide area occupied by faba bean, 41% was concentrated in Asia, 33% in Africa, only 12% in Europe, and 7% in Oceania as well as in America. China is the largest grower of faba bean in the world with 39% of the worldwide area. In Africa, faba bean is mostly concentrated in Ethiopia (15% of the world wide faba bean area). The worldwide production in 2005 was about 5.8 million tons of which China produced 43% and is, therefore, the largest producer in the world (<http://faostat.fao.org/site/336/DesktopDefault.aspx?PageID=336>).

Faba bean is a very minor crop in Germany (16 000 ha in 2005), in Poland (12 000 ha), and Austria (4000 ha). France did grow a total of 105 000 ha, amounting to one third of the French pea acreage. In the United Kingdom, faba beans were grown in 188 000 ha, this is double the area grown to pea plus lupins. Spain had in 2005 about 53 000 ha of faba bean. Mediterranean types are sown in late autumn. In parts of the United Kingdom and France, where winter is relatively mild, autumn sowing of faba beans is to some extent practiced as well using “true” winter beans that can survive winters north of Pyrenees Mountains and Alps. In the United Kingdom, more or less, half of the faba beans are such winter types. All types of faba bean can survive very mild frosts as young plants (until about -6°C). Beans from the Hindukush do even show some frost tolerance in later stages. Autumn sowing is as well realized in North Africa, in southeast China (along the Yangtze Valley), and parts of Japan.

Cultivated faba bean is used as human food and as animal feed, mainly for pigs, horses, poultry, and pigeons in developing countries and almost strictly as animal feed in developed countries. For the human consumption, it can be used as a vegetable, either green or dried. Feeding value of faba bean is high; with about 30% of protein it is considered in some areas to be superior to field peas or other legumes. It is one of the most important winter crops for human consumption in the Middle East. Faba bean has been considered as a meat extender or substitute and as a skim milk substitute. Roasted seeds are eaten like peanuts in India. The proportion of the dried faba bean used as human food in the developing countries is not defined but data are available for green faba bean and allow assessing the contribution of faba bean

in the human nutrition. Its use as green vegetable amounts to 20% of the total production of faba bean. It is a common food in the Middle East, Mediterranean region, Latin America, China, and Ethiopia. The production of green faba bean in the Mediterranean region accounts for 40% of the worldwide green bean production. The Middle East and Latin America contribute equally with about 19% to the worldwide green bean production. Other uses of faba bean have been identified. Indeed, haulm from faba bean harvest fetches a premium in Egypt and Sudan and is considered as a cash crop. The haulm can also be used for brick making and as a fuel in parts of Sudan and Ethiopia (<http://faostat.fao.org>).

1.4 Traditional Breeding

The breeding objectives for this crop totally depend on the economic and agro-ecological conditions and on the geographical region and use. For combine harvest of dry, mature seeds, all pods and even stem and leaves of the crop have to become mature simultaneously, whereas for manual harvest of vegetable green bean pods, a long-lasting harvesting period is sought. For combine harvesting, nonshattering and nonlodging habits are essential, whereas they are of lesser importance for the production of green pods. For the production of feed, mostly small grains are bred, whereas for human consumption, equina and major types are preferred in most areas (except Ethiopia, where small types are used for food). Moreover, color, taste, and cooking features are important for vegetable type germplasm. If grown for animal feed, primarily mature grain yield and yield stability are sought, and to sustain high performance, resistance against drought (and winter frost in case of winter bean breeding) and against fungi, pathogens, and pests is needed. An additional objective is mature grain quality, depending on the actual animal species to be fed.

Normal faba beans show indeterminate growth; flowers and very young pods grow in competition with the vegetative apex of the stems. Several alternative growth types have been studied as a strategy to strengthen the pods as sinks for assimilates. The so-called *ti*-type (terminal inflorescence, or “topless”) and the so-called stable type (*st*, somewhat stunted habit, and very stiff stem) were

introduced. Dwarfism is known as well. These phenotypes are all caused by a single recessive allele (ICARDA, 1986). Several corresponding cultivars have been bred in Germany, like “Tina” (*ti*), “Tinova” (*ti*) or like “Boss” (*st*) and “Mythos” (*st*). Still, these are not widely used, and no such type is present on the 2005 German list of varieties. On the other hand, very recently, Nadal *et al.* (2005) in Spain proposed the use of the *ti*-type as a solution in broomrape-infested (see below) conditions (production of young pods for fresh consumption). There has been a remarkable input into a type named “independent vascular supply” (*ivs*). Gates *et al.* (1983) proposed to circumvent the physiological interaction and competition between pods and flowers within the same inflorescence by independent vascular traces to each flower, so that direct interaction between flowers and young pods cannot occur, and distinguished this type from the “usual” branched vascular pattern. The conclusion was that selection for *ivs* would be most reliable to improve pod set. By microscopic studies, Ruckenbauer and Mollenkopf (1983) found that a classification into independent and branched vascular bundles is not adequate. In material that was claimed to express the *ivs* features, these authors found no hints on any structural deviation of the vascular traces from “normal” beans.

Faba bean, in spite of its high importance in several semiarid regions like the Mediterranean Basin, is rather susceptible to drought. Its main mechanism to deal with this stress is a very early and sensitive stomata closure. Adapted material escapes terminal drought by earliness, whereas no powerful strategy is known to deal with unpredictable, intermittent drought. Several physiological traits may be used to assist in breeding for drought tolerance. Germplasm from gene banks like ICARDA was used to specifically enlarge diversity. Frost resistance is mostly analyzed as a component of winter survival. Very few genotypes with outstanding frost resistance are known (Stoddard *et al.*, 2006). Similar to drought, physiological traits might help in breeding, such as fatty acid composition of leaves (Arbaoui and Link, 2006). No molecular tools are as yet available in faba bean breeding for these two traits.

The most important fungal foliar diseases are *Botrytis fabae*, *Ascochyta fabae*, and *Uromyces viciae-fabae*; besides, root rot caused by *Rhizoctonia solani*, *Fusarium* species, and other fungi can

occur. *Botrytis* is seen in a wide range of growing conditions, it is often a very serious threat, and still no convincing source of resistance is known. Severe outbreaks are most common in the Nile delta, near rivers in China, rainy coastal areas of the Mediterranean Basin, and the more oceanic climate of western France and western United Kingdom (Tivoli *et al.*, 2006). Differences in susceptibility follow a quantitative genetic pattern. Several less susceptible bean genotypes are known (e.g., some ICARDA lines originating from South America). For *Ascochyta* and *Uromyces*, specific resistances are known and even molecular markers were developed. The line 29H, amongst others, was repeatedly used to improve *Ascochyta* resistance. Bean rust (*Uromyces*) resistance is available in many cultivars. Qualitative resistance is common and widely used by breeders (Sillero *et al.*, 2006). *Phoma* and mildew cause further less well-studied foliar diseases in *V. faba*. Few, if any, convincing sources of resistance against root rot are known. Material with a zero content of tannin (see below) in the seed testa (monogenic recessive feature) seems to be more susceptible at germinating and emergence than tannin-containing germplasm.

Viruses are not a frequent problem for the faba bean producer; still, viral diseases may occur as serious epidemic. The bean yellow mosaic virus, bean leaf roll virus, broad bean true mosaic virus, and the broad bean stain virus must be named. Since no direct pesticide protection exists, the genetic strategy must hold. The two latter viruses are to some extent seed transmitted and not aphid transmitted. Yet, the beetle *Sitona lineatus* (see below) spread these two viruses early in the season (Rohloff, 1980). There has been promising breeding research prior to 1989 in GDR, but these programs were then abandoned (Lötsch, 1989). In 1992 and again in 1999, a new, aphid-transmitted virus (faba bean necrotic yellow virus) occurred at a devastating level in the Nile Valley. Meanwhile resistant genotypes have been identified (e.g., “ILB132” Khaled *et al.*, 2000).

The most important pest is *Aphis fabae*, the black aphid. It occurs very often at significant levels, therefore, insecticides are used. In addition to the direct damage, it is spreading viruses. No useful resistance is known. Even earlier in the season than this aphid, the weevil *S. lineatus* feeds on the first, very young leaves. More important is the damage of its larvae, which feed on the

root nodules and thus cause direct damage and probably increase root rot (Salt, 1983). *Bruchus rufimanus*, a seed-infesting weevil, is present in most faba bean fields and stocks. The female deposits the eggs in the field onto the very young pods. Infested seeds are not accepted for human consumption, thus the beetle is a serious threat for this aspect of production. Additional *Bruchus* and *Callosobruchus* species live in bean seeds (mainly in the Middle East); several of them complete their cycle in the store, the female laying the eggs onto the testa of mature dry seeds. The dry seed coat is a barrier, and not all the larvae can enter and overcome it; there is no connection to the seed coat's tannin content. The present breeding of new cultivars with reduced vicine and convicine content (see below) of the seed can favor the colonization of faba bean by additional weevils such as *Callosobruchus maculatus* that does not infest normal-vicine faba beans (Desroches *et al.*, 1995).

In addition to aphids and beetles, nematodes have to be mentioned. Faba bean may be infested by the stem nematode (*Ditylenchus dipsaci*) and the cyst nematode (*Heterodera goettingiana*). The stem nematode is widespread, and its "giant" race, common in North Africa, is a serious pest, especially in cases where nematode-infested seeds are sown. Small-seeded beans are generally a poorer host, several resistant genotypes were identified, amongst them is the *Ascochyta* resistant line 29H. The cyst nematode is important in many temperate regions. Obviously no resistances have been found (Sharma *et al.*, 1994). Broomrape, *Orobanche crenata*, is a parasitic plant, devastating pulses and other crops in the Mediterranean Basin and Nile Valley. Hand weeding, use of glyphosate, late sowing and breeding is used to fight it. Breeders have up to now not produced a bean with reliable resistance. Screening is mostly done in fields where this parasitic weed occurs naturally, which is a difficult test situation. Evaluations in controlled environments are possible but expensive. These shortcomings make broomrape resistance a problematic trait. Partially resistant genotypes are available; the resistance trait shows a quantitative genetic variation. The rather resistant genotype F402 identified by Egyptians was repeatedly used. Several improved genotypes have been bred from this common source in Egypt ("Giza 402", "Giza 429", "Giza 674")

and in Spain ("Vf1071", "Vf136", "Baraca"). Meanwhile, three quantitative trait loci (QTLs) for resistance were identified (Torres *et al.*, 2006). *Orobanche* resistance is a trait that shows all features to make it a candidate for marker-assisted selection. It is a very serious problem, resistance shows quantitative genetic variation, difficult to phenotype the trait, and there is only one unique, common source of resistance ("F402"). Up to now, the level of cooperation among breeders and scientists in the Mediterranean Basin and Nile Valley is scanty to realize the importance of this pest and to employ modern breeding techniques.

Breeding for improved quality aims mainly at increase of the seed protein content and protein quality. Protein content could easily be increased to over 30%. Still, as long as there is no economic incentive to do so, breeders will not invest significant effort in achieving this goal. Protein quality is mainly limited due to a low sulfur-containing amino acids such as methionine and cysteine. Classical methods are not promising, since the variation is low and there is a negative genetic correlation between seed protein content and sulfur-containing amino acids' content of this protein (Link *et al.*, 2005). Quality, moreover, depends on the content of antinutritional factors like condensed tannins and vicine. Zero-tannin cultivars, for example those for feeding pigs, do exist (e.g., "Gloria"). The recessive monogenetic segregation of this gene and the pure white flower, as its pleiotropic effect, make this trait easy to handle. Low vicine cultivars do as well exist (e.g., "Mélodie"). The trait is again monogenetic recessive, a morphological marker (white hilum) and even molecular markers are available. Vicine may have negative effects on human health. A rare human enzyme deficiency, favism, leads to anemia upon faba bean consumption in affected individuals. Also, vicine negatively effects monogastric animal nutrition like pigs and chicken. Further antinutritional compounds are not of importance in faba beans (Duc *et al.*, 1999).

Classical breeding in faba bean looks back on very marked improvements, e.g., for non-shattering, improved yield and yield stability, highly improved lodging resistance. Still, breeding progress is hampered by the partial allogamy of the bean. The pollinators are bumble bees and honey bees (Link *et al.*, 1994a; Suso and Moreno, 1999). The degree of cross-fertilization is about 50%, with

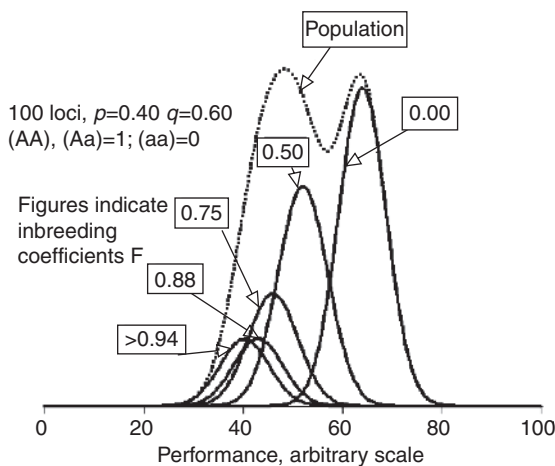


Figure 3 Theoretical distribution of individual plant's performances in case of partially allogamy like in faba bean, here with 50% outcrossing of inbred plants and 30% outcrossing of noninbred plants. The population is shown as composition of cohorts of plants with different levels of inbreeding [Reproduced from Link *et al.* (1994b)]

a large genotypic and environmental component of variation and with marked heterosis; heterozygous plants show on average less outcrossing than homozygous plants. Inbreeding depression for grain yield is marked; F_1 -hybrids outyield their inbred parents mostly by more than 40%. The partial allogamy and the marked heterosis for vigor and productivity cause the genetic variation of a faba bean population to be very much inflated (Figure 3), compared to a situation of pure selfing or pure outcrossing. This reduces markedly the gain from mass selection for these traits. The reason is that the superiority of the selected plants is mainly caused by their high heterozygosity, not by a high breeding value; heterozygosity, however, is not inherited. A solution is to strictly select among entries of identical level of inbreeding, preferentially among pure lines.

The first approaches to realize the production of hybrid cultivars (based on cytoplasmic-genetic male sterility) in faba bean trace back to Bond in Cambridge in 1957 and to Berthelem in Rennes in 1967. Bond worked with the system CMS447, discovered by him in winter beans in Newcastle upon Tyne. Berthelem discovered the system CMS350 in an English bean population. The system CMS350 was found to be sensitive to environmental conditions. The system CMS447

is very peculiar, since a genetic restoration of pollen fertility becomes permanent; offspring that segregates from a heterozygously restored plant does not segregate for the pollen sterility as expected from textbook schemes on cytoplasmic male sterility (CMS) systems. Molecular genetic findings proposed that CMS447 may be the result of an infection with a defect virus, and that restoration corresponds to nontransmittance by seeds (Pfeiffer *et al.*, 1993). In Germany, Link *et al.* (1997) detected two other CMS systems (CMS199, CMS297), yet due to instability of the sterility, especially due to spontaneous reversions to pollen fertility, hybrid breeding still could not be realized in faba bean.

As classical breeding category, line breeding is applied. An important bottleneck in line breeding is the production of purely selfed seed. In the open field situation of a breeding nursery, with small plots and large numbers of genotypes, seed multiplication suffers from uncontrolled contamination with cross-pollen, unless spatial isolation and pure lines are used. In cages, pollinators can be excluded and pure self-fertilization can be enforced. Still, without pollinator visit, most genotypes admit a need of tripping (a mechanical stimulation of the stigma, caused by the pollinator, which induces successful pollination and fertilization). As a consequence of absence of pollinators, yield of purely selfed seed in cages is variable and mostly very low. Germplasm from Southern Europe and Northern Africa often shows a lower or no need of tripping. Tripping can be done manually, to substitute for the missing pollinators in the cages, thereby allowing true selfing and high seed set, but this is a very labor-intensive procedure. A very successful alternative to pure inbred line cultivars is the breeding of synthetic cultivars. Still, due to the limited degree of natural outcrossing, only about half of the potential hybrid vigor is used in a synthetic cultivar.

1.5 Rationale for Transgenic Breeding

The development of gene transfer techniques for faba bean is of commercial interest as they might facilitate the production of cultivars with improved characteristics, such as resistance to biotic and abiotic stresses and enhancing the nutritional value. Chocolate spot, caused by *B.*

fabae and *Ascochyta* blight, caused by *A. fabae* are the most widespread and devastating fungal diseases in all production areas. Viral diseases and the parasitic weed broomrape (*O. crenata*) are the most important factors contributing to the losses in faba bean production in the West Asia North Africa (WANA) region (Hanounik *et al.*, 1993; Robertson and Saxena, 1993). Losses in faba bean caused by broomrape are estimated at the range from 50% to 80% in fields with medium and high levels of infestations, respectively (Gressel *et al.*, 2004). Pests like black bean aphid (*A. fabae*), sitona weevil (*S. lineatus*), stem nematode (*Ditylenchus dipsaci*), and bruchid beetle (*B. rufimanus*) also limit the yields, if no pest control is employed. Abiotic stresses like cold and drought are also major constraints to yield as strong tolerance genes are not available in the primary gene pool of *V. faba*. These constraints are strong arguments for genetic transformation for the improvement of this crop. Recombinant DNA technology if applied to faba bean, however, requires the development of efficient and reproducible protocols for both regeneration and genetic transformation. The combination of conventional breeding approaches, plant tissue culture techniques, and recombinant DNA technology will open the necessary avenues needed for improvement of faba bean in overseeable time.

2. DEVELOPMENT OF TRANSGENIC FABA BEANS

Faba bean exhibits a rather low competence for *in vitro* culture. This is mainly due to the difficulties in the regeneration from callus tissues and the high content of phenolic compounds, which causes cell death if not overcome (Bieri *et al.*, 1984; Selva *et al.*, 1989). Unfortunately, efficient *in vitro* techniques are limited for faba bean as compared to other major economically important crops. Faba bean genetic transformation continues to be problematic even with the intense efforts of the researchers in this field. As a consequence, among the major grain legume crops *V. faba* was the last species where the production of transgenic plants had been reported (Böttinger *et al.*, 2001; Hanafy *et al.*, 2005). In contrast, the closely related species *V. narbonensis* could be relatively easy to be manipulated *in vitro* (Pickardt *et al.*, 1991).

Several investigators worked extensively on faba bean transformation and regeneration of transgenic plants in the past (Schiemann and Eisenreich, 1989; Ramsay and Kumar, 1990; Saalbach *et al.*, 1994; Siefkes-Boer *et al.*, 1995; Jelenic *et al.*, 2000). The first attempts to transfer foreign genes into faba bean were published by Schiemann and Eisenreich (1989). They used *Agrobacterium rhizogenes* containing the binary vector pGSGluc1 carrying *nptII* and *uidA* genes under the control of the bidirectional TR1/2 promoter. β -glucuronidase (GUS)-positive roots and subsequently transgenic calli lines were obtained. A similar study was performed by Ramsay and Kumar (1990), using an *A. rhizogenes* strain containing pBin19 for inoculation of *V. faba* cotyledons and stem tissue, leading to successful transfer of the *nptII* marker gene. However, no transgenic faba bean plants were regenerated in any of these studies. Since the lack of efficient protocol for regeneration of transgenic plants was the main obstacle in faba bean transformation, Jelenic *et al.* (2000) tried to solve this problem by application of bacteria carrying shooty types Ti-plasmids, pGV2215, and pGV2235. Neither of the two mutants appeared to be useful for faba bean regeneration.

Finally, Böttinger *et al.* (2001) recovered the first transgenic faba bean plants from transformed tissues. Their system was based on *de novo* regeneration using thidiazuron (TDZ). The second successful protocol developed by Hanafy *et al.* (2005) was based on direct shoot organogenesis from meristematic cells of mature or immature embryo axes.

2.1 *Agrobacterium*-mediated Transformation and Regeneration of Transgenic Faba Bean Plants

Böttinger *et al.* (2001) reported, for the first time, a method for the production of transgenic faba bean plants. This *Agrobacterium*-mediated transformation protocol, adopted from a plant regeneration protocol for protoplast derived calli (Tegeder *et al.*, 1995), was based on the *de novo* regeneration of shoot initials from callus. The transgenic plants were recovered by inoculation of stem segments with *Agrobacterium* strains EHA 101 or EHA 105, harboring different binary vectors (Table 1). Calli were induced on MS medium (Murashige

Table 1 Summary of successful transgenic faba bean production strategies

Cultivar	Explant	<i>A. tumefaciens</i>	Transgene	Selection	References
Mythos	Etiolated epicotyls	EHA 101, EHA 105	<i>uidA</i> , <i>nptII</i> , <i>bar</i> , <i>sfa8</i> , <i>lysC</i>	Kanamycin, phosphinothricin	Böttinger <i>et al.</i> , 2001
Mythos	Embryo axes	EHA 105	<i>bar</i> , <i>sfa8</i>	Phosphinothricin	Hanafy <i>et al.</i> , 2005
Albatross	Embryo axes	EHA 105	<i>bar</i> , <i>sfa8</i>	Phosphinothricin	Hanafy <i>et al.</i> , 2005
Giza 2	Etiolated epicotyls	EHA 105	<i>bar</i> , <i>sfa8</i>	Phosphinothricin	Hanafy <i>et al.</i> , 2005

and Skoog, 1962) containing 0.5 mg l^{-1} of each TDZ, 2,4-dichlorophenoxyacetic (2,4-D), and naphthylacetic acid (NAA) and 100 mg l^{-1} kanamycin or 2 mg l^{-1} DL-Phosphinothricin (PPT) as a selective agent. Afterwards, transgenic shoots were regenerated *via* organogenesis using a high concentration of TDZ (7.5 mg l^{-1}) and 0.75 mg l^{-1} NAA. Finally, plants were recovered by micrografting. The process required 16–24 months to get seed-producing primary transformants. It was laborious and time consuming because the quantum of regenerable tissue was very limited and it had relatively low regeneration efficiency, prone to somaclonal variation.

Consequently, in an effort to improve the transformation efficiency of *V. faba* and to overcome the difficulties reported by Böttinger *et al.* (2001), an *Agrobacterium*-mediated transformation system based upon direct shoot organogenesis has been developed (Hanafy *et al.*, 2005). After transformation of meristematic cells derived from zygotic embryo axes stable and fertile transgenic plants were regenerated. The use of

embryonic axes, which were cultivated on media containing high concentrations of cytokinins in combination with low auxin, enhanced direct shooting without an intermediate callus phase. In this manner the possibility of somaclonal variation has been reduced to the minimum. This transformation system was an adaptation of the protocol previously reported by Schroeder *et al.* (1993) in *P. sativum*. It allowed obtaining seed-bearing primary transformants within approximately 9–10 months (Hanafy *et al.*, 2005).

2.1.1 Faba bean cultivars and *A. tumefaciens* strains

Successful transformation and regeneration of transgenic faba bean plants has been achieved with three cultivars so far: *Mythos*, *Albatross*, and *Giza 2*. Growth media used for tissue culture, transformation, and regeneration are listed in Table 2. Mature faba bean seeds were surface sterilized (ethanol and sodium hypochlorite solution),

Table 2 Growth media used for the production of transgenic faba bean plants

Media	Composition ^(b)
TNZ	MS + 3% sucrose, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} TDZ
TNZ1	MS + 3% sucrose, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} TDZ, 500 mg l^{-1} Betabactyl
TNZ2K	MS + 3% sucrose, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} TDZ, 300 mg l^{-1} Betabactyl, 100 mg l^{-1} kanamycin
TNZ2P	MS + 3% sucrose, 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} TDZ, 300 mg l^{-1} Betabactyl, 2 mg l^{-1} PPT
MTN	MS + 3% sucrose, 7.5 mg l^{-1} TDZ, 0.75 mg l^{-1} NAA
MB1	MS + 3% sucrose, 1 mg l^{-1} BAP, 1 mg l^{-1} GA3, 100 mg l^{-1} coconut milk, 100 mg l^{-1} Ticarcillin, and 50 mg l^{-1} Combactam
CCM	Gamborg B ₅ medium + 3% sucrose, 0.5 mg l^{-1} kinetin, 1 mg l^{-1} 2, 4-D
SRM	MS basal salt medium supplemented with B ₅ vitamins ^(a) + 3% sucrose, 2 mg l^{-1} NAA, 2 mg l^{-1} BAP, 150 mg l^{-1} Ticarcillin, and 100 mg l^{-1} Combactam
SIM1	MS basal salt medium supplemented with B ₅ vitamins + 3% sucrose, 4.5 mg l^{-1} BAP, and 2 mg l^{-1} PPT
SIM	MS basal salt medium supplemented with B ₅ vitamins + 3% sucrose, 4.5 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, 100 mg l^{-1} Ticarcillin, 50 mg l^{-1} Combactam, and 2 mg l^{-1} PPT

^(a)Reproduced from Gamborg *et al.* (1968)

^(b)Antibiotics and phosphinothricin are added to the medium after autoclaving. Cultures are kept at 21°C under cool white fluorescent light ($80 \mu\text{mol m}^{-2} \text{ s}^{-1}$)

soaked overnight in sterile tap water with shaking and germinated in the dark at 20 °C on $\frac{1}{2}$ MS-basal medium. After about 10 days, internodal segments of the arising main shoots were used for co-cultivation with *Agrobacterium tumefaciens*. The remaining seedlings were kept in dark, and secondary shoots, arising from the cotyledonary buds during the following weeks, were used as explants source for further transformation experiment.

The following *A. tumefaciens* strains have been successfully used for production of transgenic faba bean plants (Böttinger *et al.*, 2001; Hanafy *et al.*, 2005): (1) EHA101 carrying pGSGluc1, harboring *uidA* and *nptII* genes, both under the control of the bidirectional TR1/2 promoter; (2) EHA101 carrying pAN109, harboring the *nptII* marker gene under the nopaline synthase promoter (*Pnos*) and the mutated *Escherichia coli lysC* gene, coding for a feed-back desensitized aspartate kinase III (Shaul and Galili, 1992), driven by the seed specific bean phaseolin promoter; (3) EHA105 carrying pBIOU, harboring the *Pnos-nptII* marker cassette and an intron-containing genomic sequence for the sunflower 2S-albumin 8 (*sfa8*; Kortt *et al.*, 1991), driven by the seed specific *usp* promoter from *V. faba* (Bieri *et al.*, 1984); (4) EHA105 carrying pGlsfa (pGPTV-bar derivative, Becker *et al.*, 1992), harboring the *sfa8* gene under the seed specific legumin B promoter (LeB4) and the *bar* gene under the *nos* promoter.

Bacteria carrying the desirable vector were harvested by centrifugation and resuspended in an equal volume of liquid TNZ medium (*de novo* regeneration protocol) or the bacterial culture was diluted in a rate of 1:5 with liquid CCM (embryo axes transformation system). Etiolated epicotyls were cut into segments of 0.2–0.4 cm in length in the bacterial suspension and incubated for 30 min, then transferred into 250 ml glass containers with solidified TNZ medium and co-cultivated for 48 h at 20 °C in the dark (*de novo* regeneration protocol).

Regarding the embryo axes transformation system, embryo axes of both mature and immature seeds were wounded by the removal of the root tips and slicing of the embryo axes to three or four segments longitudinally and inoculated in the bacterial suspension for 15–20 min (immature embryos) and 30 min (mature embryos) with occasional agitation. After infection, the explants (30–40 per plate) were co-cultivated on solid

CCM medium at 25 °C in dark for 3–4 days. After the co-cultivation period, explants were cultured in a glass container on TNZ1 medium, in the dark at 20 °C for 3–4 days, for recovering without selection pressure. The explants were subsequently transferred to TNZ2K- or TNZ2P-medium (depending on the selectable marker). The explants were subcultured every 2 weeks on fresh medium for a period of 3–4 months. Resistant calli reaching a diameter of approximately 5 mm were transferred to MTN-medium. Calli were subcultured every 3–4 weeks on this medium for a period of more than 12 months. The appearance of shoot primordia varied between 4 and 12 months. Shoot primordia were transferred to elongation medium (MB1) and subcultured every 3–4 weeks.

Healthy resistant shoots, more than 1 cm in length were excised and grafted onto etiolated seedlings of untransformed faba bean of about 7–10-day-old (see Pickardt *et al.*, 1991 for details). After 2–3 weeks, when the shoot developed new leaves, the transgenic plants were transferred to the greenhouse and grown to maturity.

2.1.2 Transgenic faba bean plants

Two successful protocols for regeneration of transgenic faba bean plants have been developed so far. The first protocol was based on *de novo* regeneration using TDZ (Böttinger *et al.*, 2001), and the second protocol was based on direct shoot organogenesis from meristematic cells of mature or immature embryo axes (Hanafy *et al.*, 2005). In both these transformation systems, the control experiments showed that PPT at 2 mg l⁻¹ totally suppressed callus development from wild type faba bean epicotyl segments, cultured on TNZ, or embryo axes cultured on shoot inducing medium (SIM1).

With regard to the *de novo* regeneration protocol, in a series of transformation experiments, resistant callus started proliferating after about 1–2 months on the surface of a number of explants on TNZ2P medium. Within 3–4 months after culturing the explants on TNZ2P medium, 4.3–31.6% of the explants produced resistant calli (Figure 4). PPT resistant calli with a diameter of about 5–10 mm were transferred to the MTN medium to increase the callus viability

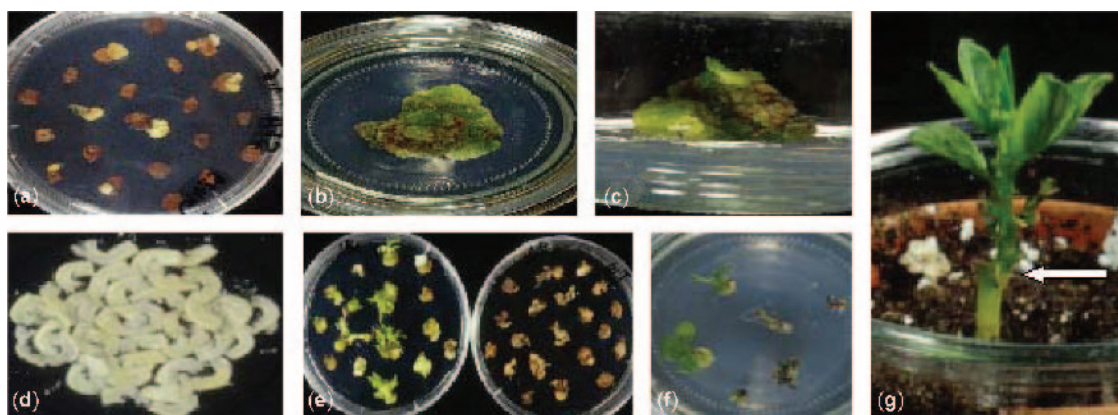


Figure 4 Successful transformation and regeneration of transgenic faba bean. (a) Initiation of resistant callus from stem explants under selection pressure of 2 mg l^{-1} PPT on TNZ2P- medium. (b) Callus proliferation on MTN medium. (c) Shoot regeneration on MTN medium. (d) Explant segments derived from embryonic axes. (e) Multiple shoot regeneration under selection pressure (left), all the control explants (WT) were dead (right). (f) Further selection between PPT resistant and susceptible regenerated shoots on medium containing 2 mg l^{-1} PPT. (g) Recovering the transgenic plant by micrografting

to regenerate (Figure 4). Shoot regeneration occurred after 6–12 months on MTN medium (Figure 4c). Shoot primordia were transferred to shoot elongation medium, MB1. Because of the very low rooting percentage of the regenerants, shoots reaching a suitable size (within 2–4 months) were micrografted onto nontransgenic root stocks of faba bean to recover whole plants. Afterwards, the plants were transferred to soil for acclimatization. Regenerated plants were transferred to the greenhouse for further plant development and production of T_1 seeds. Some regenerated clones showed morphological abnormalities, such as dwarfness and formation of abnormal flowers and subsequently abnormal (or no) pods. Some plants showed narrow leaves and very weak stems with weak apical dominance. Only four clones of the cultivars Mytos and Giza 2 produced normal flowers and pods with seeds. The time needed to obtain T_1 seeds by this process was about 16–24 months.

On the other hand, the feasibility of the transformation strategy based on embryo axis transformation was initially evaluated by monitoring the number of regenerated shoots from the embryo axis explants, cultured on medium with high 6-benzylaminopurine (BAP) concentration (4.5 mg l^{-1}), where routinely 4–5 shoots were regenerated from each explant. In a series of transformation experiments, the explants (immature or mature embryonic axes) were inoculated with

Agrobacterium strain EHA105/pGlsfa (harboring *SFA8* and *bar* genes) alone, or co-transformed with EHA101/pAN109, which carried a mutated *lysC* gene from *E. coli* and *nptII* (Böttinger *et al.*, 2001) (Figure 4d). After 3–4 weeks of culturing on selective medium, all control explants had died. On the other hand, the transformed explants started regenerating (via organogenesis), and about 3–4 shoots appeared from each explant on SIM (Figure 4e). The shoots selected for 4–6 months (Figure 4f) were grafted *in vitro* and finally transferred to the greenhouse to set seeds (T_1) (Figure 4g). A total of seven stable independent transformants (containing *SFA8* linked to *bar* from cv. Mytos and Albatross) have been recovered. Transformation frequencies ranged from 0.15% to 2.0%. Regarding the co-transformation experiments, transformed plants (T_1) were screened for the presence of both transfer DNAs (T-DNAs) by PCR analysis. It was found that only the T-DNA encoding the PPT selectable marker has been successfully integrated. The time needed to obtain T_1 seeds by this protocol was about 9–10 months.

The transgenic plants were analyzed by testing the expression of *bar* gene in the greenhouse by using the leaf painting assay or spraying the young plants with the same dilution of the herbicide. Within two days necrotic spots appeared on the untransformed leaves. Ten days after BASTA[®] (phosphinotrycin glufosinate



Figure 5 Herbicide leaf painting test showing the resistance of transgenic leaf to BASTA[®] (300–400 mg l⁻¹ ammonium glufosinate) application (left)

ammonium) application, the treated transgenic plants and leaflets showed complete tolerance, in contrast to completely necrotic leaflets on nontransformed plants (Figure 5).

3. FUTURE ROAD MAP

Each of the two successful protocols for regeneration of faba bean transgenic plants (Böttinger *et al.*, 2001; Hanafy *et al.*, 2005) has its advantages and drawbacks.

The major advantage in using *de novo* regeneration protocol is regeneration of nonchimeric plants. This process, however, is more time consuming and the main constraints in this protocol are poor regeneration ability, following a callus phase, reduced fertility and high percentage of phenotypic abnormalities in regenerated plants. This can be ascribed to the fact that explants and callus cells of *V. faba* tend to produce high amounts of phenolic compounds, resulting in subsequent intoxication of the tissue (Bieri *et al.*, 1984; Selva *et al.*, 1989). A possible cause of these abnormalities is also due to the long culture time *in vitro* (around 7–16 months). The recovery of seed-producing putative transformed plants under these circumstances takes about 16–24 months, which is a considerably long time period. Transformation strategies, which minimize the *in vitro* culture

period and avoid the callus phase would, therefore, be advantageous.

Transformation of pre-excited meristematic cells on the embryo axes is much simpler. The major success in legume transformation was achieved by methods based on transformation of the pre-existing meristems on the embryo axes, cotyledonary nodes, shoot tips, or nodal explants. An *Agrobacterium*-mediated genetic transformation system using pre-excited meristematic cells on the embryo axes is an effective method for the production of transgenic faba bean, which provides a useful strategy for an efficient insertion of the economically useful genes into its cultivars. It was demonstrated that the embryo axes of faba bean are competent for production and multiplication of shoots and inherited transformation.

The production of commercial transgenic faba bean plants requires an efficient regeneration system. Although transgenic faba bean plants can now be regenerated successfully, the process is still labor-intensive and therefore, requires improvement.

3.1 Expected Products

Some of the problems of classical faba bean breeding might be addressed by applying strategies of genetic modification. *Orobanche* sp. was shown to be sensitive to glyphosate (Lolas,

1994; Goldwasser *et al.*, 2002). In this respect, it would be feasible to introduce genes conferring resistance to glyphosate to faba bean, like 5-enolpyruvylshikimate-3-phosphate synthase from the CP4 strain of *A. tumefaciens* or glyphosate oxidase from *Ochrobactrum anthropi*. These genes are already being efficiently used in commercial transgenic crops like cotton and soybeans (Padgett *et al.*, 1995; Saroha *et al.*, 1998). BASTA (phosphinotrycin-glufosinate ammonium) or bromoxilin herbicide resistance genes could be introduced as well (Cubero and Nadal, 2005). They are not effective against *Orobanche* sp., but could be of help against other unwanted weeds in the field. Similarly, insect pests of faba bean from the orders *Lepidoptera*, *Coleoptera*, and *Diptera* could be controlled by heterologous expression of the Cry proteins from *Bacillus thuringiensis* (Schnepf *et al.*, 1998). Moreover, to reduce losses caused by aphids the *Galanthus nivalis* agglutinin (GNA), a lectin, might be tested in addition to Cry proteins (Wang *et al.*, 2005).

Nematode resistance could be conferred to faba bean by the introduction of the *Mi-1.2* gene from the wild relative of tomato *Lycopersicon peruvianum*. It was shown that this gene contributes resistance against nematodes, but also against aphids, when introduced to cultivated tomato (Rossi *et al.*, 1998). Heterologous expression of the same gene in eggplant (*Solanum melongena*) displayed resistance to nematodes (Goggin *et al.*, 2006). A comprehensive list of other cloned or mapped nematode resistance genes from different species has been published recently (Williamson and Kumar, 2006).

The problem of faba bean fungal diseases could potentially be solved by transformation with genes encoding for plant defensins, a group of small peptides broadly present throughout the plant kingdom. These peptides exhibit a wide-range antifungal activity, when expressed heterologously (Thomma *et al.*, 2002). For example, the *Alium cepa* antimicrobial protein 1 (*Ace-AMP1*; Cammue *et al.*, 1995), a defensin, was shown to be very efficient in conferring *Batrachyomyces* resistance to *Pelargonium* sp. (Bi *et al.*, 1999), powdery mildew resistance to *Rosa hybrida* (Li *et al.*, 2003), and most recently, conferring resistance to several fungal pathogens in wheat (Roy-Barman *et al.*, 2006).

The only two reports so far, describing successful regeneration of transgenic faba bean, raise hope

of the feasibility of nutritional value improvement by transgenic approaches. Böttlinger *et al.* (2001) demonstrated the expression and specific activity of the mutated *lysC* gene in transgenic faba bean plants. Still, the desired change in amino acid composition in the transgenic seeds remains to be demonstrated. In addition, the same group demonstrated the expression of the methionine-rich sunflower albumin gene *sfa8* in faba bean transgenic lines using RT-PCR. However, due to the lack of the specific antibody, it was not possible to demonstrate the accumulation of the corresponding protein in the generated lines. The work of Hanafy *et al.* (2005) made another important step forward. By RT-PCR the authors detected the presence of the *sfa8* transcripts in the T₂ and T₃ generation of their transgenic lines. More importantly, by Western blot analysis the accumulation of the SFA8 protein was demonstrated in transgenic faba bean lines. In the two tested generations (T₂ and T₃), the *SFA8* expression level remained uniform. The exact levels of the foreign protein could not be determined, due to the lack of purified *SFA8*.

Apart from demonstrating that the transgenic approach could be used to improve protein quality in faba bean, these two reports also showed that the *bar* gene (PPT acetyltransferase) could be effectively used as a selective agent of transgenic individuals, instead the unpopular antibiotic resistance. Phosphinotrycin resistance conferred by the *bar* gene, can be used in the early phase of positive clone selection. More importantly, *bar*-based selection is easily conducted in the glasshouse or in the field on fully grown plants.

3.2 Expected Technologies

The introduction of molecular biology techniques revolutionized plant research. In particular, the sequencing of the *Arabidopsis thaliana* genome has produced a wealth of information on virtually every aspect of plant biology. Also, it has set new standards that transformed the way plant research is done: high throughput mutagenesis, genome saturated collections of mutants, construction of dense genetic maps used in positional (map-based) cloning, excellent efficiency of *in planta* transformation, and availability of the whole-genome microarrays. However, *A. thaliana* simply

cannot encompass all the diversity of physiological processes present throughout the plant kingdom, e.g., the nodule development in legumes. Therefore, the development of legume-specific plant models is necessary. Today, two cool-season legume models are established: *Lotus japonicus* and *Medicago truncatula*. Similarly, *Glycine max*, as the economically the most important legume, is the model for the warm-season legumes. The complete genome sequences of the two cold-season model plants should be complete within the next 1–2 years (Young *et al.*, 2005). The soybean genome, due to its size of 1200 Mb, is relatively far from being completely sequenced.

Translating the knowledge gained through these model species, to crop legumes, such as *V. faba*, might improve both classical and transgenic breeding programs. Its 13 059 Mb genome (Zhu *et al.*, 2005) and its rather secondary economic importance, are serious obstacles for sequencing the faba bean genome. So, it is certain that faba bean breeders will have to rely on information extractable from sequences of the model legumes, at least for time being.

Genomics of model legumes represents the basis for understanding the molecular, as well as metabolic basis of synthesis and accumulation of desired compounds (e.g., storage proteins), and to decipher mechanisms by which legumes resist pests and unfavorable environmental conditions. Not less important, genomics is the base for generating large numbers of molecular markers that can be used to assist classical plant breeding.

The legume community has the intention to develop several translational genomic tools that will facilitate the exchange of genetic sequence information among different legume species. These “translational tools” include collections of expressed sequence tags (ESTs), complementary DNA (cDNA) libraries from different plant tissues (seed, leaf, nodule, flower, pod), as well as from various environmental conditions, genomic libraries, and generation of dense genetic maps. Due to limited resources, a priority list of legume species was defined. Faba bean is positioned reasonably high on that list, but only after common bean, chickpea, peanut, and peas (Gepts *et al.*, 2005). Eventually, sequencing of gene-rich regions for this group of crop legumes is planned. In addition, it is aimed to produce a set of at least 500 genetic markers per legume crop species, which

will be linked to whole-genome sequences of model legumes. In order to localize QTLs, such as for yield or seed protein composition, segregating populations for mapping agronomically important traits will be made available. The Legume Crops Genome Initiative (LCGI) predicted that within the next decade the complete *Phaseolus* sp. and *Arachis* sp. sequences will be ready, as well as partial sequences of *Pisum* sp. and *Cicer* sp. (Gepts *et al.*, 2005). At the moment, it is hard to predict whether *V. faba* will reach the same level of sequence information by that time.

For the model species *L. japonicus* and *M. truncatula*, more effort will be put on elucidating their transcriptome, proteome, and metabolome (Colebatch *et al.*, 2004). These datasets are inevitable to fully comprehend the regulation of synthesis of desired products: proteins, lipids, and carbohydrates, as well as secondary metabolites interacting with pathogens, symbionts, as well as beneficial or harmful insects.

As the amount of genomic sequences from both, the model and crop legumes, will grow, the correct annotation of gene function is going to be crucial. To perform functional annotation, reverse genetics approaches similar to those in *A. thaliana* are also being employed in model legumes, e.g., a TILLING facility (targeted induced local lesions in genomes; Till *et al.*, 2003) has been set up with about 5000 *L. japonicus* mutagenized lines (Gilchrist and Haughn, 2005).

The relatively close phylogenetic relationship of faba bean and the model legumes is a strong argument in favor of translational genetic strategies. This was further substantiated by one recent phylogenetic analysis of legumes, based on chloroplast maturase K (matK) sequence (Cronk *et al.*, 2006). In particular, the *Papilionoideae* subfamily, to which crop and model legumes belong, was subdivided into seven informally named clades. Faba bean was classified to the *inverted repeat loss clade* (IRLC), together with legume model *M. truncatula* and crop legumes *L. culinaris*, *P. sativum*, *Cicer arietinum*. The next most related clade *Robinioids* includes the other model legume *L. japonicus*.

The close phylogenetic relationship between faba bean and model legumes implicates the existence of common genomic microstructure and macrostructure. Macrostructural similarity, also referred as synteny implies the presence of the same genes on the same chromosome of two species. On

the other hand, microstructural similarity, referred as microsynteny or colinearity, indicates conserved gene order on a particular chromosome of two species. For example, it was demonstrated that *L. japonicus* and *M. truncatula* are structurally more conserved than, e.g., *Glycine max* and *M. truncatula* (Choi *et al.*, 2004). This is of practical importance for designing strategies for small scale sequencing projects and comparative mapping on faba bean. Another study compared conserved microsynteny between *G. max* and *M. truncatula*, using a hybridization-based approach (Yan *et al.*, 2003). The high degree of microsynteny detected in this study, implied that it should be possible to use the genome sequence of *M. truncatula* as a reference for isolating genes in soybean and other closely related legumes, e.g., faba bean.

A further example for using sequence information gained on model legumes in crop legumes is the cross-species application of EST- and genomic-derived SSRs (microsatellites) for *M. truncatula* in most important European legume pulses: pea, faba bean, and chick pea (Gutierrez *et al.*, 2005). Of 242 *M. truncatula* simple sequence repeats (SSRs) analyzed, approximately 40% gave cross-amplification in faba bean. Detailed sequence analysis of these SSR sequences showed a rather high variability in the microsatellite motif itself, and much more conserved flanking regions. However, amplified microsatellites did not show a size polymorphism in different parental genotypes of faba bean used in the study. This outcome sets limits to their use in mapping projects. Still, the effort to produce a large number of cross-legume markers will continue.

The germplasm of elite faba bean cultivars was reported to have a rather broad genetic base, as demonstrated by a recent amplified fragment length polymorphism (AFLP) analysis (Zeid *et al.*, 2003). So, cultivar improvement by using this intraspecific variability presents an important alternative to the currently unfeasible interspecific hybridization. This approach is currently used to genetically improve faba bean against several important sources of biotic stress, such as fungal diseases caused by rust (*U. viciae-fabae*), mildew (*Peronospora viciae*), chocolate spot (*B. fabae*), *Ascochyta* blight (*A. fabae*), or the parasitic species broomrape (*O. crenata*). In addition to these rather classical breeding approaches, the application of DNA markers and other molecular tools led to

precise mapping and isolation of several faba bean genes. For example, three amino acids permeases, VfAAP1, VfAAP3, and VfAAP4, from faba bean, have been cloned from a seed-specific cDNA library (Miranda *et al.*, 2001). The same group reported the cloning of two putative peptide transporter homologues VfPTR1 and VfPTR2. In the future these candidate genes could be used to manipulate the amounts and composition of storage proteins in faba bean seeds.

The assignment of various linkage groups and even single genes to particular chromosomes was facilitated by the work on faba bean primary trisomics (Torres *et al.*, 1995; Vaz Patto *et al.*, 1999). So far five out of six possible trisomics have been generated (Pozarkova *et al.*, 2002). For the large chromosome 1, for which trisomics could not be obtained so far, a linkage map has been generated with an average 8 cM map interval (Cubero and Nadal, 2005). The integration of genetic and physical map of faba bean is further improved by techniques of flow sorting (Dolezel and Lucretti, 1995; Macas *et al.*, 1996) and *in situ* hybridization (Fuchs and Schubert, 1995). Even more, flow sorting of *V. faba* chromosomes, due to their large size, has become a model for plant chromosome sorting in general. Also, by using flow fractionation of chromosomes (Dolezel *et al.*, 2001), it should be possible to prepare chromosome-specific bacterial artificial chromosome (BAC) libraries. These BACs could be screened with marker-derived probes flanking QTL regions. Sequencing the positive BAC candidates could then lead to the identification of genetic basis of a particular QTL. In a recent flow sorting approach, microsatellite-enriched chromosome-specific DNA libraries were generated, and used for developing of novel DNA markers (Pozarkova *et al.*, 2002). Also, first attempts were undertaken to fine-map genes or QTLs responsible for resistance to fungal diseases (Avila *et al.*, 2003; Roman *et al.*, 2003) or parasitic plants (Roman *et al.*, 2002). Still, the saturation of the faba bean linkage map is rather difficult to achieve due to its huge genome size. A list of linkage maps currently available for the *V. faba* genome has been published recently (Torres *et al.*, 2006).

As a conclusion, it can be stated that the major hindrances in generating transgenic faba bean plants have been overcome. Since the wild progenitor of *V. faba* has not been determined

yet (Cubero, 1974), faba bean is considered as a genetically isolated species, unable to naturally cross with other *Vicia* species (Bond *et al.*, 1985). As a consequence, its gene pool seems rather restricted. Therefore, genetic improvement of faba bean via transgenic approaches is a possible step forward. Since, only recently, reproductive and efficient techniques for regeneration of transgenic *V. faba* plants have been established, the number of transgenes that have been introduced into the faba bean genome is so far limited to five: *bar*, *uidA*, *nptII*, *sfa8*, and *lysC* (Table 1). Nevertheless, these efforts demonstrate a proof of principle that transgenic technology can be used as one of the approaches to improve faba bean breeding. There are numerous traits that would be suitable for improving economic faba bean production. However, to complete the whole procedure of generating a commercial faba bean line, starting with the newly developed transgenic plant, then outcrossing the new trait into economically valuable genotypes, and eventually getting the approval for registration of the transgenic line, is not feasible yet, especially not in the European Union (EU) (Cubero and Nadal, 2005).

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Lentil

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1. INTRODUCTION

Lentil is an annual, herbaceous pulse (grain legume) crop and is probably as old as the agriculture itself. Since its domestication in the Near East, lentil (*Lens culinaris* Medicus) remained as an important source of dietary protein in the area. Despite its importance as a source of protein through ages, the crop has received little attention to improve its yield and quality. Organized collection of germplasm and crop improvement programs have only started at early 1980s with the establishment of international agriculture centers including ICARDA (International Center for Agricultural Research in Dry Areas).

1.1 History, Origin, and Distribution

Lentil was one of the first domesticated plants as were wheat and barley. It is suggested that lentil was first cultivated in the Near East including Southern Turkey where Neolithic agriculture developed about 9000 years ago (Zohary and Hopf, 1988). By the Bronze Age, the plant had been spread throughout the Mediterranean region, Asia, and Europe, which was followed by the New World including Latin America. *L. culinaris* ssp. *orientalis*, which closely resembles the cultivated species *L. culinaris*, is widely accepted as the progenitor species. The conclusion that the cultivated lentil originated in the Near East

are from *L. culinaris* ssp. *orientalis* is based on discoveries of carbonized remains of apparent cultivated lentil in the same region over which *L. culinaris* ssp. *orientalis* is distributed (Muehlbauer *et al.*, 1995).

1.2 Botanical Description

Lentil is under the order *Rosales*, suborder *Rosineae*, family *Leguminosae*, subfamily *Papilionaceae*, and tribe *Vicieae*. After a complex taxonomic history, lentil is eventually placed in the genus *Lens* Miller. The name *Lens* describes the shape of the seed of the cultivated form of lentil. Analyzing previous findings based on origin and spread, morphological, cytological, cytogenetical observations, and more recently on the basis of isozyme and molecular studies (Ferguson and Robertson, 1996), *Lens* was reclassified by Ferguson *et al.* (2000) into seven taxa split into four species:

Lens culinaris Medikus subsp. *culinaris*
subsp. *orientalis* (Boiss.) Ponert
subsp. *tomentosus* (Ladiz.) Ferguson *et al.* (2000)
subsp. *odemensis* (Ladiz.) Ferguson *et al.* (2000)
Lens ervoides (Brign.) Grande
Lens nigricans (M. Bieb.) Godr.
Lens lamottei Czeffr.

Most lentil researchers now accept this latest classification (Sarker and Erskine, 2006). *Lens*

orientalis is the presumed progenitor of cultivated *L. culinaris* Medik. The last name is for Medikus, a German botanist-physician who has given the name to the plant in 1787.

Lentil plants are typically short, slender, semierect annuals varying between 15 cm to 75 cm height depending on the genotype and environmental conditions (Figure 1). Individual plants may bear single stems or may be multibranched. Branches may arise directly from the main stem or from the cotyledonary node below ground or they may rise from other branches depending on the available space in the field and environmental conditions (Saxena and Hawtin, 1981).

The plants have slender tap roots and a mass of fibrous lateral roots. Various types of root systems, ranging from shallow branched roots to deep tap roots, are recognized depending on the texture of soil and climate in the area (Nezamuddin, 1970). The tap root and the lateral roots in the upper layers of the soil carry numerous small, round, or elongated nodules that start to decline before the onset of flowering (Saxena and Hawtin, 1981).

Lens species are diploid plants with $2n = 14$ chromosomes. They all have similar karyotypes consisting of three pairs of metacentric or submetacentric chromosomes, three pairs of acrocentric chromosomes, and one satellited pair of chromosomes (Slinkard, 1985).

Lentil plants complete their life cycle in 3–4 months under optimum environmental conditions. These conditions are usually reached in spring-sown crop, but in winter-sown crop, growth duration delays up to 30–60 days, especially in the earlier stages of growth, because of the suboptimal temperatures. Optimum temperatures for lentil seed germination is in the range of 15–25°C, but above 0°C seeds can germinate. Optimum temperatures for growth and yield are around 24°C.

Due to the hypogeal germination, in which cotyledons remain below the ground, lentil plants are less likely to be killed by freezing, wind, insect damage, and grazing. If the young shoots are damaged, new buds can be initiated from the nodes below ground. The crop is also said to tolerate drought better than waterlogged soils (Hawtin *et al.*, 1980). Throughout the world, a large proportion of the lentil crop is grown in semiarid regions without the benefit of irrigation. In most of these regions, agriculture depends on



Figure 1 Morphological characteristics of lentil [Reproduced from Kamci (2004)]

water conserved in the soil after fall and winter rains. Lentils show adaptability to a wide range of soil types. They are grown in sandy loam soils, alluvial soils, black cotton soils, or in heavier clay soils (Nezamuddin, 1970). On soils with very high

natural fertility and excessive soil moisture, the crop might make excessive vegetative growth but this reduces seed yield (Saxena, 1980).

Lentils grow well on slightly acidic soils (pH 5.5–6.5) to moderately alkaline soils (pH 7.5–9.0). Delayed nodulation and decreased yields have been obtained when the pH of the soil increased beyond 9 (Bharadawaj, 1975).

Most genotypes of lentil are very sensitive to soil salinity. Especially in the irrigated lentil growing areas, this becomes a major constraint in obtaining good yields. Salt tolerance of lentils was reported to be much less than that of most cereals, chickpeas, lupins, and faba beans although it is higher than that of *Phaseolus* bean, cowpeas, and soybeans (Ivanov, 1973).

1.3 Economic Importance

Lentil is one of the principal food crops cultivated in the semiarid regions of the world, particularly in the Indian subcontinent and in the dry areas of the Middle East (Muehlbauer *et al.*, 1995). Of the countries that produce lentil, India, Turkey, Canada, the United States, Australia, and Syria can be considered as the major producers. All the major lentil-producing countries are also major consumers, except the United States, Canada, and Australia. Other countries of the Middle East such as Egypt, Jordan, Algeria, Iraq, and Lebanon are major consumers of lentil but not major producers. Other major lentil importing countries include Spain, Colombia, Sri Lanka, and India.

Lentil has been produced on a commercial basis in the Palouse region of the United States since 1937 (Youngman, 1968). The area is characterized by loess-rolling hills with elevations of up to 900 m. In that region, lentil is most often grown in rotation with cereals where it offers a needed alternative to break cereal disease cycles, provides a crop where grassy weeds can be adequately controlled, and through nitrogen fixation, reduces the demand for nitrogen fertilizers (Muehlbauer *et al.*, 1995). In Canada, lentils are grown primarily in Saskatchewan. This province is now the second largest producer after India, with an area of about 700 000 ha (Sarker and Erskine, 2006). In Turkey, a steady increase in the sown area and production of lentil has occurred since the late 1970s. However,

a major change in Turkey's lentil production took place after 1982 with the implementation of the utilization of fallow areas project but area sown to lentil and the production has been decreasing since 1989 (Bayaner and Holloway, 1998). World lentil production has tripled in the last three decades from 1.05 million metric tons in 1971 to 3.8 million metric tons in 2004, through a 124% increase in sown area and a 58% increase in average national yield from 611–966 kg ha⁻¹ (FAO, 2004).

Lentil is a nutritious food legume. The seeds are rich in protein with concentrations averaging 26%. This value is comparable with that of faba bean, higher than that of chickpea and more than double that of wheat. However, like other grain legumes, there is a shortage of tryptophan and the sulfur-containing amino acids, methionine and cystine, which are relatively rich in cereals (Adsule *et al.*, 1989). The protein in lentils contains significant concentrations of lysine, a limiting amino acid in cereals. Therefore, when cereals and lentils are consumed together, they provide adequate amounts of essential amino acids in the human diet. Seed composition varies with genotype, seed maturity, and soil nutrient availability (Summerfield, 1981; Muehlbauer *et al.*, 1995). Lentils, like chickpeas and faba beans, are good sources of vitamin B, but are poor in carotene and vitamin C.

Even though lentils are considered to be highly nutritious, they contain antinutritional factors such as trypsin inhibitors, hemagglutinins, and oligosaccharides that cause flatulence. The toxic factors are heat labile and are almost completely destroyed after 20 min of autoclaving, but cooking have no influence on flatulence. A major use of lentil as food is in decorticated and split form, which is a principal ingredient in soups and various dishes. Residues from threshing of the lentil crop such as dried leaves, stems, and fruit walls are also essential for livestock feeding. One cultivar of lentil (Indian head), which has the capability of producing an abundance of foliar material, is used as a green manure in Canada to improve soil nutritional status. Also to grow lentils in rotation with other crops has the advantage of fixing nitrogen when effectively nodulated, thus reducing the demand for nitrogen fertilizers and depletion of inorganic nitrogen from soil (Muehlbauer *et al.*, 1995).

1.4 Traditional Breeding

Lentil breeding programs throughout the world have similar objectives with larger and more stable seed yield being the most important (Muehlbauer *et al.*, 1995). The yield limiting factors are lack of seedling vigor, slow leaf area development, high rate of flower drop, low proportion of pod setting, poor dry matter accumulation, low harvest index, lack of lodging resistance, low or no response to inputs, and various biotic and abiotic stresses (Sarker and Erskine, 2006).

Abiotic stresses that affect lentil were listed as cold, drought, heat, salinity, nutrient deficiency, and nutrient toxicity by Singh and Saxena (1993). Of these stresses, drought and heat are considered the most important worldwide (Turner *et al.*, 2001). Cold stress was considered important in the West Asia–North Africa region. Salinity is an important stress factor in the Indian subcontinent and to some extent in West Asia–North Africa. Nutrient deficiency and nutrient toxicity is of lesser importance worldwide but important in localized regions (Muehlbauer *et al.*, 2006).

Foliar diseases are the most serious biotic stresses affecting lentil crops (Muehlbauer *et al.*, 2006). *Ascochyta* blight caused by *Ascochyta lentis* is problematic to various degrees in all lentil growing regions of the world, but especially damaging in Canada (Ahmed and Morrall, 1996; Ahmed *et al.*, 1996), Australia, and Middle Eastern countries (Johansen *et al.*, 1994). Other major biotic stresses of lentil include Anthracnose caused by *Colletotrichum truncatum*, botrytis gray mold caused by *Botrytis fabae* and *Botrytis cinerea*, *Stemphylium* blight caused by *Stemphylium botryosum*, lentil rust caused by *Uromyces fabae* and sclerotinia white mold caused by *Sclerotinia sclerotiorum* (Muehlbauer *et al.*, 2006).

The methods of breeding lentil are similar to those utilized in breeding other self-pollinated crops and include pure line selection or hybridization followed by bulk method, pedigree method, single-seed descent, or some modification of these procedures (Muehlbauer *et al.*, 2006).

Although lentils are of ancient origin, systematic research on lentil improvement has begun very recently. In a recent review, Sarker and Erskine (2006) mentioned about the large number of germplasm that have been collected, evaluated,

and preserved at national and international levels, with ICARDA holding the largest collection of cultivated and wild germplasm accessions. Identification and usage of genotypes with resistance to various biotic and abiotic stresses, particularly resistance to vascular wilt, rust, and *Ascochyta* blight, and the breeding of new genotypes with good standing ability, which are suitable for mechanical harvest in West Asia and North Africa were listed among the major achievements in lentil breeding.

Lentil breeding has also led to high-yielding varieties with greater yield of biomass and breeding for resistance to lodging, pod shattering, and a different plant growth habit has increased the yield potential considerably and facilitated mechanized harvesting (Christou, 1997).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Improvement of lentil by conventional breeding tools is limited by the availability of novel genes in the current breeding germplasm pool. Another constraint in breeding programs is the limited crossability of the cultivated *L. culinaris* with other wild allied species and production of fully fertile progenies. These problems can easily be overcome by the use of transgene technology.

Transgenic plant breeding may also allow elimination of antinutritional factors such as trypsin inhibitors and hemagglutinins by the use of antisense techniques. The technology can also be used for the improvement of nutritional quality of lentil proteins, which are characterized by a shortage of methionine and cysteine. Since lentil is one of the few crops that can be grown in the semiarid regions of the world, this can have a significant impact for the improvement of dietary intakes of people living in areas with poor soils and limited rainfall.

2. DEVELOPMENT OF TRANSGENIC LENTILS

Among all the plants used by humans, legumes are nearly as important as cereals in terms of nutritional value and uses. However, while much effort was spent in the past years on cereals like

corn, wheat and barley, legumes, except the crops including soybean and peanuts, have received little attention. Difficulty in applying the tools of genetic engineering in legumes, their recalcitrance for regeneration to a viable plant and lack of intense attention retarded transgenic plant development in lentil. Limited report on lentil engineering has been published so far and a need for a reproducible transformation system with an efficient selection and regeneration method is not totally fulfilled.

2.1 Donor Genes

Virtually in all of the *Agrobacterium*-mediated transformation optimization studies (Warkentin and McHughen, 1992; Warkentin and McHughen, 1993; Lurquin *et al.*, 1998; Mahmoudian *et al.*, 2002a, b) and also in a study that aims the optimization of gene delivery in lentil cotyledonary nodes using particle bombardment (Oktem *et al.*, 1999), p35SGUSINT vector containing kanamycin resistance gene (*NOS-NPTII-NOS*) and intron containing β -glucuronidase (*GUS*) gene was used. The vector was obtained by cloning *GUS* gene cassette with cauliflower mosaic virus 35S promoter and terminator from pGUSINT plasmid into Stratagene pBS vector (Vancanneyt *et al.*, 1990).

Maccarrone *et al.* (1992) used a different design for liposome-mediated gene transfer. pCaMVCN with chloramphenicol-acetyltransferase (*CAT*) reporter gene, pBI121 and pBI221 both carrying *GUS* reporter gene were used as vectors in the study. Following this study Maccarrone *et al.* (1995) exploited the liposome-mediated transfection of lentil protoplasts for understanding the function of the *LOX* gene. In the study different *LOX* cDNA (complementary DNA) fragments were inserted into pAS6, which is pEMBL with the cauliflower mosaic virus (CaMV) promoter, *GUS* gene and *NOS* terminator in sense and antisense direction.

Chowrira *et al.* (1996) reported electroporation-mediated gene transfer into intact lentil tissues as well as pea, cowpea, and soybean plants. The plasmid pGPT1.0, which is constructed by cloning p35SGUSINT fragment into pUC8 vector, was used in transformation experiments. The fragment contains the *uidA* gene from *Escherichia coli* with cauliflower mosaic virus 35S promoter and *nos*

terminator. The chimeric gene is interrupted by the second intron from the potato *ST-LSI* gene.

Mahmoudian *et al.* (2002a, b) reported a vacuum infiltration-based *Agrobacterium*-mediated gene transfer method for lentil cotyledonary nodes. In the study pGUSINT binary plasmid, which is a derivative of pBI121, was used. It contains coding sequences of neomycinphosphotransferase-II (*nptII*) conferring resistance to kanamycin and an intron containing *uidA* gene.

In another study reporting particle bombardment-mediated transformation of lentil, pBUC19 plasmid with chimeric SuRA/SuRB Hra acetolactate synthase gene (*ALS*) from tobacco, conferring resistance to sulfonylurea herbicides was used in the transformation of cotyledonary nodes (Gulati *et al.*, 2002). The chimeric gene was constructed by inserting a fragment of the mutant *SuRB S4-Hra* gene containing the two mutations into the mutant *SuRA C3* gene (Lee *et al.*, 1988) producing a chimeric gene of 4637 base pairs including the endogenous tobacco *SuRA C3* promoter and terminator.

2.2 Methods Employed

First transformation study in lentil by *Agrobacterium tumefaciens* was performed by Warkentin and McHughen in 1991. In this study oncogenic strains of *A. tumefaciens* are directly used without any modification. Researchers evaluated the susceptibility of lentil to four diverse strains of *A. tumefaciens* at the whole plant level *in vivo* and at explant level *in vitro*. C58, Ach5, GV3111, and A281 strains were capable of inducing tumors at high frequency on inoculated stems of lentil *in vivo*, and on excised shoot apices *in vitro*. GV3111 and Ach5 were the most effective *in vivo* and A280 was the most effective *in vitro*. Transformation was proved by nopaline and octopine assays with negative and positive controls. Also the integration of transfer DNA (T-DNA) into the genome was confirmed by Southern analysis with the probe designed for *NOS* gene plus some 5' flanking sequences. Later in 2002, a similar study on crown gall induction was carried out by Khawar and Özcan successfully to evaluate the virulence of A281 strain on different genotypes of lentil.

Following their study in 1991, Warkentin and McHughen (1992) continued with their

lentil transformation studies by using GV2260 *Agrobacterium* strain and p35GUSINT vector. It was an optimization experiment in which lentil seedlings from the cultivar Laird was used as a source of explants including shoot apex, epicotyl, cotyledonary node and root sections. Variables tested were co-cultivation duration, preculture duration, presence of acetosyringone in co-culture media, and type of wounding. Increase in co-cultivation duration was accompanied by an insignificant increase in the number of transformed explants. On the other hand preculturing had a negative effect. Different wounding strategies along with acetosyringone in co-cultivation media did not enhance transformation efficiency. In general, explant sections proximal to actively dividing centers like root tips, cotyledonary nodes, and apical meristems produced higher GUS scores when compared to other explant zones. However, overall transformation rate was low with a small number of cells transformed.

Warkentin and McHughen (1993) also investigated the susceptibility of the cotyledonary node explants of lentil seedlings (cv. Laird) to GV2260/p35SGUSINT. In the study, cotyledonary node explant was used for its capacity of high frequency shoot regeneration from axil region under 6-benzylaminopurine (BAP) induction. During shoot regeneration on selective media, no GUS positive shoot was recovered. The possible cause was assigned to immense wounding of explants with scalpel followed by bacterial inoculation. Therefore, a procedure that relies on a more gentle wounding technique like microprojectile bombardment was suggested for the transformation of cotyledonary nodes.

Lurquin *et al.* (1998) used longitudinally sliced embryonic axes from pea and lentil mature seeds, co-cultivated with *A. tumefaciens* carrying *GUS* gene to evaluate the efficiency of gene transfer to tissues in different cultivars and co-cultivation conditions, which are two different pH values for bacterial growth media and absence/presence of acetosyringone. The group claims that the technique can be used to evaluate performance of pea and lentil genotypes in co-cultivation with *Agrobacterium*.

In the study by Mahmoudian *et al.* (2002a, b) cotyledonary nodes were infiltrated with *Agrobacterium* strain GV2260. Differing from the preceding works, *Agrobacterium* cells were

concentrated prior to inoculation with explant. A mild infiltration process increased transient GUS activity significantly. The most effective factor for higher rate of transformation frequency was assigned to the use of higher concentration of *Agrobacterium* cells during inoculation.

Chowrira *et al.* (1996) reported the only electroporation-mediated transformation study in lentil. The group obtained transgenic R₂ plants using *in planta* electroporation-mediated gene transfer on lentil nodal axillary buds *in vivo*. The branches that grew out of nodal meristems were reported to be chimeric. Seeds originating on these chimeric branches were shown to be positive by Southern blot hybridization. It was also reported that the same system was applied to pea, cowpea, and soybean plants and transgenic R₁ plants were recovered from seeds originating on the chimeric branches that grew out of the electroporated nodal meristems. The group reported unusual non-Mendelian R₂ segregation ratios that showed bias against transgene presence or expression but did not provide further supporting data.

Oktem *et al.* (1999) reported *GUS* gene delivery and expression in lentil cotyledonary nodes using particle bombardment. In the study an electronic processor controlled GeneboosterTM (Jenes *et al.*, 1997) particle delivery system was used. *GUS* expressing sectors were seen on 4% of the regenerating shoots.

Gulati *et al.* (2002) used particle bombardment-based gene transfer in lentil. PDS 1000/He delivery system (Biorad Laboratories, Hercules, CA) was used with a vacuum pressure of 66 cm of mercury. Different rupture pressures (650–1800 psi) and explant preculture periods (0–6 days) were evaluated. The study provided a reproducible genetic transformation system for lentil.

In another study, five different *Agrobacterium* strains: LBA4404, EHA105, GV2260, C58C1, and KYRT1, and two different binary plasmids: pGUSINT and pTJK136 containing *GUS* and *nptII* genes were tested for the best transformation response in lentil (Celikkol, 2002). In the study, also the factors affecting *A. tumefaciens*-mediated transformation efficiency like wounding method (microcarrier and needle mediated), presence of vacuum infiltration and cultivar effect for Firat-87 and Sultan-1 cultivars were analyzed. The succinamopine type KYRT1 strain harboring the

pTJK136 plasmid exhibited the highest transient *GUS* expression for both cultivars. Microwounding (at different gas pressure and micro-carrier concentrations), either alone or in combination with vacuum infiltration did not significantly enhance gene delivery as scored by transient *GUS* gene expression. By the use of best strain/plasmid couple and needle wounding, transgenic shoots were regenerated from cotyledonary nodes with 0.8% efficiency, micrografted to root stocks, and successfully transferred to soil (Figure 2).

The first successful transfection of lentil protoplasts with liposomes was carried out

by Maccarrone *et al.* (1992). They used cationic liposomes made up of different dipalmitoylphosphatidylcholine (DPPC) and sterylamine (SA). The best results were obtained with a ratio of DPPC to SA of 9, which formed a homogenous population of round vesicles. These liposomes were able to transfect lentil protoplasts with two different reporter genes: *CAT* and *GUS*. The activity of these two enzymes could be found in the cell lysates after 24 h from the incubation of protoplasts with the lipid-DNA complexes.

The same group, in their antisense-based transformation study, used electroporation for



Figure 2 Gus expression and regeneration of transgenic lentil shoots originated from cotyledonary nodes [Reproduced from Celikkol (2002)]

transformation of lentil protoplasts (Maccarrone *et al.*, 1995). For this purpose, protoplasts were transferred into 0.4 cm electroporation cuvettes containing 15 μg of carrier DNA (sheared salmon sperm DNA) and 25 μg of plasmid in a final volume of 800 μL . Samples were electroporated with exponentially decaying pulses at field strength of 400 V cm^{-1} and capacitance of 960 μF . The group successfully transformed the protoplasts of lentil and succeeded in inhibition of *LOX* gene by using full-length sequence of the cDNA. It is obvious that the gene transfer to lentil protoplasts can be a valuable alternative to other transformation systems. It has greater efficiency and easy to handle. The only obstacle on this method is the generation of fertile plants from these protoplasts.

2.3 Selection of Transformed Tissue

Since all of the transformation attempts so far depended on direct regeneration of the plantlets from mature meristematic tissues, selection of transformants was always a problem in lentil genetic modification studies.

In the first transformation study on lentil, by Warkentin and McHughen (1992), selection of transformed tissues was done by the use of kanamycin, which was found to be very inefficient for the type of explant used. When the formation of small transformed sectors on the cotyledonary node explants, which is the result of very low transformation efficiency, combined with the death of adjacent nontransformed tissues under selection pressure, growth of transformed cells were completely inhibited. On the other hand, lower doses of kanamycin caused increase in the number of escape shoots.

Chowrira *et al.* (1996) claimed that upon electroporation on the nodes of 100 plants, 18 GUS positive chimeric R_0 shoots were generated, which were assessed by histochemical GUS staining. The R_1 progeny of 10 of these plants were further analyzed and 22 of 88 individuals were found to be GUS positive. Also it was reported that three R_2 lines were analyzed and 4 out of 34 plants showed GUS expression. These ratios indicate problems about either presence or expression of *uidA* gene in the plants on the basis of Mendelian inheritance patterns.

In the study of Gulati *et al.* (2002), selection of transformed tissues was done by the use of 5 nM herbicide chlorsulfuron. After 4 weeks of selection, proliferated shoots were allowed to elongate by reducing the concentration of chlorsulfuron to 2.5 nM. Green and healthy shoots were excised and micrografted. Out of 600 explants bombarded with 900 psi rupture pressure in sets of 25–30 explants per shot, 36 putative transformants were successfully transferred to soil, resulting in 6% transformation efficiency. In another set of experiments with 190 explants bombarded with 1100 psi rupture pressure, six putative transformants were transferred to soil, providing 3.1% transformation efficiency. Putative T_0 transformants were also tested for herbicide tolerance by using metsulfuron herbicide leaflet painting assay.

2.4 Regeneration of Whole Plant

Legumes are, in general, considered as recalcitrant plants for tissue culture and genetic modification studies, among which lentils are the most difficult to regenerate into whole plants. Since transformation studies ended up with whole plant regeneration and seed formation are scarce, tissue culture studies gain a special importance on the way of developing transgenic lentil. For this reason, in addition to the studies on transgenic plant regeneration, sole plant regeneration attempts are also included in this section.

In vitro propagation techniques are important tools for modern plant improvement programs. Since lentil is recalcitrant to tissue culture, only few considerable plant regeneration systems have been achieved up to date. First report about lentil tissue culture is regeneration from cultured shoot tips (Bajaj, 1979). This study was followed by determination of regeneration capacity of shoot meristems and epicotyls from calli induced by gibberellic acid and kinetin (Williams and McHughen, 1986). The most responsive explant hormone combination was found to be shoot meristem with 10 mg l^{-1} Kin and 1 mg l^{-1} of GA_3 . For this combination 60% of callus bodies gave rise to shoots with mean shoot number of 2.4. Also low regeneration capacity of epicotyls was demonstrated on the same hormone combination.

Calli-derived shoots of cultivar Eston were rooted with 11% efficiency in mist chamber.

Although somatic embryogenesis has been demonstrated in numerous species, plant regeneration of lentil through somatic embryogenesis has only been reported by Saxena and King (1987). In that study, plants were regenerated by the process of somatic embryogenesis from embryo-derived callus cultures of *L. culinaris* Medik. cv. Laird. The callus originated from embryonal axes cultured on modified B₅ medium containing ammonium nitrate supplemented with 1–10 mg l⁻¹ 2,4-D. Formed callus was then transferred to medium containing BAP and indole 3-acetic acid (IAA), then this callus differentiated into several embryoid like structures. Upon further subculture of the embryoid like structures on glutathione-supplemented medium, well-organized embryos were formed and developed into whole plants. Up to this date, no progress has been made toward developing an *in vitro* regeneration system based on somatic embryogenesis.

Polanco *et al.* (1988) reported the influence of different growth regulators and explant types on callus and shoot formation. In their study, three different cultivars (Verdina, Pardina, and Castellana) were used and shoot tip, first node, and first pair of leaves were utilized as explant source. It was reported that 2,4-D induced callus formation in all explants, but no organ regeneration obtained from this calli. Multiple shoot formation from 33% to 92% was obtained from explants supplemented with BAP and NAA (α -naphthaleneacetic acid). Root formation was achieved only in media with NAA or IAA.

Singh and Raghuvanshi (1989) reported direct and indirect organogenesis from lentil shoot tips and nodal segments. Kinetin in combination with 2,4-D was used for callus induction. Callus-derived shoot regeneration or direct shoot regeneration was carried out with high kinetin concentration in tissue culture. For both direct and indirect shoot regeneration studies, shoot tip was found to be the most responsive explant type.

First protoplast study in lentil was carried out by Rozwadowski *et al.* (1990). Isolation was achieved by the use of 1% cellulase, 0.5% driselase, and 0.25% pectolyase. After purification, protoplasts were cultured in medium lacking amino acids and ammonium nitrate but containing 0.35 M glucose and a growth regulator complement of

either 2,4-D, NAA, kinetin, BAP, zeatin, GA₃, or NAA and each of 2,4-D and BAP. The osmotic potential of the liquid medium was gradually reduced by lowering the glucose concentration. About 6% of the dividing protoplasts developed into cell colonies after 3 weeks of culture. In 35–42 days, the microcolonies were about 1 mm in diameter and developed into calli on transfer to agar-solidified B₅ medium supplemented with glutamine. Attempts to regenerate plants from protoplast-derived calli were unsuccessful.

The objective of the study carried out by Malik and Saxena (1992) was to explore the potential of thidiazuran (TDZ) as an inductive signal for shoot initiation potential for seedlings of different legume species (*Pisum sativum*, *Cicer arietinum*, *L. culinaris*, Medik cultivar Eston). Upon optimal TDZ (10 μ M) treatment, secondary shoot buds developed from the axils and bases of multiple shoots of mature lentil seedlings. About 19 shoots per seed were recovered from lentil. Although developing shoots of *P. sativum* were able to form roots, TDZ at 5 μ M or a higher concentration suppressed the development of primary roots and completely blocked secondary root formation in both lentil and chickpea.

In the genetic transformation study of the Warkentin and McHughen (1993) rooting of the regenerated shoots (GUS negative) was carried out in half-strength B₅ with or without NAA and scored within 1–2 months duration. 53% and 34% rooting were scored for B₅ and B₅+NAA containing media, respectively.

In another study that aimed at interspecific hybridization of different *Lens* species, media was optimized for shoot regeneration from nodal segments *in vitro* without callus phase to avoid polygeny (Ahmad *et al.*, 1997). Concentration of growth regulators, MS salts, and presence of sucrose for direct regeneration *in vitro* were analyzed. A protocol was standardized for regeneration of two shoots in average from nodes in MS media with 2.89 μ M GA, 1.11 μ M BAP, and without sucrose. The study claimed that 90% efficiency of root induction was observed upon 5.37 μ M NAA treatment. Since the study reports very low efficiency of shoot regeneration, it can be regarded as unsuitable for *in vitro* micropropagation studies that aims transgenic plant development.

The effect of BAP on root formation from lentil shoots regenerated on BAP containing media was

studied by Polanco and Ruiz (1997). Regeneration of multiple bud shoots and formation of roots were examined on seedling shoot tips, first nodes, bractlets, and immature seeds of the cultivar Verdina. Media with 2.25 mg l^{-1} BAP induced buds and shoots but no roots. Root formation was only induced on media with a low concentration of BAP (0.225 mg l^{-1}) or without BAP. In these media, the highest percentages of explants forming roots were obtained from bractlets and immature seeds on rooting media containing 2 mg l^{-1} IAA. However, these roots were unable to keep the plants alive outside the culture flask. When shoots were maintained for 4 weeks on the shoot initiation media, rooting up to 40% was obtained. The inhibitory effect of BAP on rooting increased with the duration of culture on initiation media, reaching the maximum of inhibition when regenerated shoots were maintained for 8 weeks in culture. In conclusion, given that BAP is routinely added to tissue culture to stimulate shoot regeneration, the concentration of BAP and time in culture must be carefully chosen in the micropropagation process of lentil in order to avoid BAP-induced inhibition of rooting in regenerated shoots.

Efficient and simple method of plantlet regeneration was described from immature seeds of different sizes (1–6 mm) of four different lentil cultivars: Verdina, Pardina, Lupa, and Alpo (Polanco and Ruiz, 2001). Shoot regeneration from the auxiliary meristems of the immature seeds were mediated mainly by the various concentrations of BAP in combination with gibberellins and auxins (NAA, IAA, 2,4-D). In all cultures, the best shoot regeneration response was achieved with seeds that were 5–6 mm in size ranging between 80% and 100%. In this study, it was claimed that the highest rooting efficiency was obtained with the low incubation periods in cytokinin containing regeneration medium (a period of 4 weeks), but it is obviously seen that the highest rooting efficiency in the study, which is 88.9%, is the result of the absence of BAP in regeneration media.

Gulati *et al.* (2001) reported a protocol for micrografting of lentil shoots. BAP at $8.8 \mu\text{M}$ induced the maximum number of shoots (4.3 shoots/explant) within 8 weeks. The regenerated shoots were micrografted on 5–6 days old rootstocks with 96% efficiency. Shoots were micrografted on 5–6 days old rootstocks with 96% efficiency. The successful grafts were transplanted

to pots in Redi-earthTM, hardened off and were grown to maturity with 100% success. The grafted plants were phenotypically normal and produced normal pods and viable seeds. The success of micrografting was independent of the nature and concentration of growth regulator used in shoot initiation medium and the time period for induction of shoots. The protocol was successful with several cultivars of lentil.

In another study, Fratini and Ruiz (2002) reported the effect of kinetin, zeatin, TDZ, and BAP on root regeneration. TDZ and BAP were found to inhibit root development more than kinetin (KIN) and zeatin ribside (ZEA). The study concluded that TDZ and BAP were more inhibitory for root development than KIN and ZEA, which is also in agreement with the study of Polanco and Ruiz (2001).

Khawar and Özcan (2002) tested shoot regeneration capacities of cotyledonary node explants from 21 different lentil genotypes on MS medium containing $0.225 \mu\text{M}$ TDZ. The highest shoot regeneration was 20.6 shoots/explants with Akm 362 genotype. Regenerated shoots were cultured for rooting but root formation was not achieved. To overcome rooting problem, regenerated shoots were micrografted to cultivar Kayı 91 with 100% efficiency.

The major limiting factor in the establishment of a successful plant regeneration system from *in vitro* regenerated shoots for lentil is the low frequency of rooting. There are only a few reports of rooting from regenerated shoots in lentil. It is a slow process taking 4–8 weeks and it is negatively affected from the presence of BAP (which is a very effective and widely used cytokinin for shoot regeneration in lentil) on *in vitro* regeneration medium (Polanco and Ruiz, 1997). Even if rooting is successful *in vitro*, high mortality rates are observed upon transplantation to soil, due to agar medium adapted roots with incompletely developed vascular tissues.

To fulfill the requirement of an improved and reproducible rooting procedure, Fratini and Ruiz (2003) established a rooting method for lentil, which is claimed to be based on explant polarity. Nodal segments of lentil with an axillary bud cultured in an inverted orientation (apical end in medium) showed higher rooting frequencies than explants cultured in a normal orientation (basal end in medium). The highest rooting

percentage (95.35%) and average number of shoots regenerated per explant (2.4) were obtained from explants placed in an inverted orientation on MS with 3% sucrose, supplemented with 5 μM IAA and 1 μM kinetin. The only limitation found was that rooting response was greatly reduced when the nodal segments were obtained from plants after the initiation of flowering.

In another study (Bayrac, 2004), optimization of regeneration system for cotyledonary petioles of lentil was carried out (Figure 3). Since lentil cotyledonary petiole is one of the most responsive tissues for *Agrobacterium*-mediated transformation, an optimized tissue culture system for this tissue will expedite the development of transgenic lentil plants. In the study, eight different medium types were employed to examine the callus induction potency of cotyledonary petiole. Except two, all tested medium yielded more than 80% callus induction. Subsequent to this study

each calli produced in different medium were transferred to nine different medium types to test the potencies of shoot induction. Only the callus induced in medium with 1 mg l^{-1} Zeatin riboside and 1 mg l^{-1} NAA yielded shoots at 8–40% frequency. The most responsive media for shooting were MS basal medium with no growth regulator, medium supplied with 1 mg l^{-1} Zeatin and 1 mg l^{-1} NAA, and medium supplied with 1 mg l^{-1} TDZ, respectively.

Another regeneration study through micrografting (Kamci, 2004), factors such as grafting type, grafting stem height, and the scion health were analyzed. In the study two graft types, namely cleft and whip type, were successfully adapted to lentil. Among the two, the whip type of grafting had better results (Figure 4). Both healthy scions and grafting to a shorter stem height provided enhancements in graft success. Also sealing round the uniting sections of the grafts increased the rate

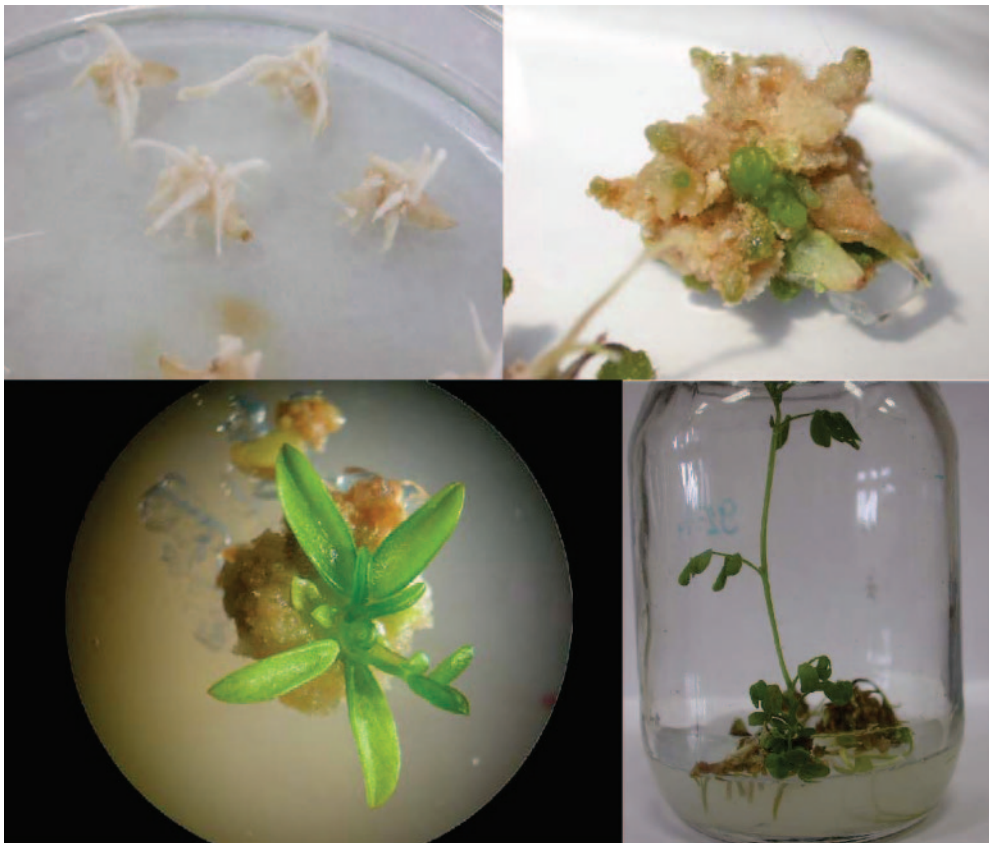


Figure 3 Regeneration of plantlets from cotyledonary petiole tissue of lentil [Reproduced from Bayrac (2004)]

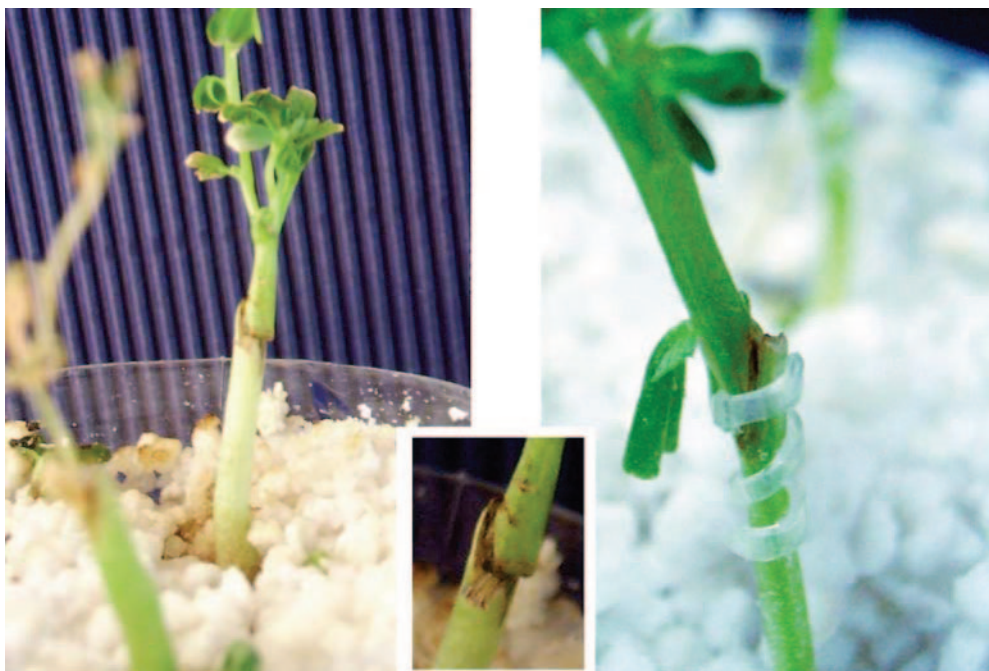


Figure 4 Grafting of lentil shoots [Reproduced from Kamci (2004)]

of graft success. Besides these factors, efficiency of chickpea as root stock was evaluated and found to be inefficient.

In a recent study, Newell *et al.* (2006) examined *in vitro* rooting of lentil nodal segments to test the conclusion of Fratini and Ruiz (2003), which states that shoot orientation has an effect on rooting. In the study, it was reported that when the proximal end was in agar (a hypotonic environment) the rooting percentage was low (9–25%) even when the orientation of the microcutting was altered by inverting the culture tube. In contrast, when the proximal end of the microcutting was in an aerobic environment (from the shoot being placed upside down in agar medium or placed normally or upside down in an aerated medium), rooting percentages were higher (62–100%). With these results Newell *et al.* (2006) concluded that the medium aeration at the proximal end of the microcutting is more important than shoot orientation for *in vitro* rooting of lentil microcuttings. Also when the very low rooting frequencies of nodal segments isolated from seedlings older than several days taken into consideration, grafting of regenerated shoots into root stocks seems to be the most reliable rooting procedure developed so far.

3. FUTURE ROAD MAP

Although lentil's contribution to human nourishment is of vital importance especially in some areas like South Asia, Africa, and Middle East, it is still considered as an underexploited crop on which little research has been done. The first priority on lentil improvement is the establishment of a reliable and reproducible transformation technique, which is followed by an efficient plant regeneration system.

Although lentil is a nutritious food legume with rich protein content averaging 26%, like other grain legumes, there is a shortage of amino acids methionine and cysteine. Engineering lentil for the production of methionine and cysteine can make the plant complete in terms of essential amino acids for human diet.

The plant is characterized by poor yield; it is highly susceptible to insect pests as well as fungi and considered to be very sensitive to wide range of herbicides. Weeds are important limiting factors for the yield of food legumes, which slow initial growth rates. If not adequately controlled, weed infestations can reduce yields by as much as 75%. In the case of parasitic weed *Orobanche crenata*,

complete crop loss can be the result (Knott and Halila, 1992).

Although lentil is moderately resistant to drought, it is known to be severely effected by salt. Especially in well-irrigated areas, soil salinity becomes a major constraint in obtaining good yields.

Lentil requires improvement for both its agronomical properties described above and its nutritional characteristics, and this field is totally open for new research.

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Tepary Bean

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1. INTRODUCTION

Within the genus *Phaseolus*, *Phaseolus vulgaris* or the common bean is the most important grain legume for direct food use (Broughton *et al.*, 2003). It produces seeds that are consumed worldwide as dietary protein source by millions of people, mainly in South America, India, and China. Four other *Phaseolus* species are also cultivated, although at a lower scale, namely *P. lunatus* (lima bean), *P. coccinus* (scarlet runner bean), *P. polyanthus* (year-long bean), and *P. acutifolius*, the tepary bean; and the tepary bean is the focus of this chapter.

The tepary bean is seen by many as one of the most drought tolerant annual legume in the world. The tepary bean is considered as a minor subsistence crop and, hence, is not very important from an economic point of view, as it is not widely cultivated and consumed. Nevertheless, it has some interesting characteristics. On the one hand, its tolerance to drought, high temperatures, and common bacterial blight has inspired breeders to explore the possibilities of crosses with the common bean, *P. vulgaris*, for its improvement (Muñoz *et al.*, 2004). On the other hand, the tepary bean is the only *Phaseolus* species that can easily be transformed with an *Agrobacterium*-based system, and for which several classes of transgenic plants have been described, and hence its coverage in this volume. The developed transgenics, however, are mainly designed for addressing research questions

and to our knowledge, there are no transgenic *P. acutifolius* plants beyond the research phase, neither in field trial nor in commercialization.

1.1 History, Origin, and Distribution

Archaeological findings have shown that the tepary bean was grown already in ancient times by the Indians in the southeastern United States, where it was probably introduced from Mexico 1200 years ago (Debouck, 1994). It has been recently suggested that one of two Mexican states, Sinaloa or Jalisco, could have been the center of domestication (Muñoz *et al.*, 2006).

The tepary bean is highly heat and drought resistant, and has been grown since long in Mesoamerica, mainly as a vegetable crop. It grows well in desert zones or areas with a long dry period. The cultivated form of the tepary bean is found up to an altitude of 2000 m. They can produce a good harvest with a single rain in otherwise dry conditions. When irrigated, however, it produces higher yields only up to a certain point, after which excess moisture becomes a detriment and leads to overproduction of foliage and low bean production. It appears that moisture stress is even a necessity to trigger fruiting. Thus, part of the tepary bean's secret to success in dry areas is to grow quickly when water is available. It can grow in sandy, muddy, sometimes organic soils with an optimum pH of 6.7–7.1.

P. acutifolius grows in the wild in the Sonoran Desert, with local populations on Isla Tiburon in the Sea of Cortez and in the Santa Maria mountains of Arizona (Nabhan, 1985). The present distribution of the cultivated form of *P. acutifolius* is limited and extends from Arizona and New Mexico, over Nicaragua to Costa Rica.

1.2 Botanical Description

1.2.1 Taxonomy

Kingdom: Plantae
Division: Magnoliophyta (Flowering plant)
Class: Magnoliopsida (Dicotyledons)
Order: Fabales
Family: Fabaceae (Pea family or Leguminosae)
Tribe: Phaseoleae
Genus: Phaseolus
Species: *Phaseolus acutifolius*
Variety: *Phaseolus acutifolius* Gray var. *acutifolius*

Common names: tepary bean (English), frijol tepari (Spanish), escomite (Nahuatl), bawi or bavi (O'odham/Pima), xmauym or xmayum (Mayan), tsatasi mori (Hopi) frijol piñuelero (Costa Rica) (Debouck, 1994; Nabhan and Rood, 2004).

1.2.2 Morphology

Wild tepary beans are climbers, growing up to 3 meter long, enabling it to climb desert shrubs. The cultivated varieties are more bushy types, or sometimes semiviny, growing to an average height of about 30 in. Leaves are pointed and trifoliate. The crop has a short cycle, flowering 30–40 days after germination. Autogamy appears to be dominant. The pods take about 2 months to ripen; they are short, about 3 in. long. Pods are initially green, later they dry to a light straw color and the plants completely wither. Usually there are five or six seeds per pod. The average weight of 100 cultivated tepary bean seeds are between 10 and 20 g (Zambre *et al.*, 2005) and between 2 and 5 g for the wild form (Debouck, 1994). Tepary beans (TB1 genotype) are shown in Figure 1.

P. acutifolius can be distinguished from other species of beans by its epigeal germination, sessile primary leaves, acute rhomboid folioles, pseudoracemes, two to four fruit-bearing stems, small pink or white flowers, with very small triangular bracteoles and pods that have sutures marked with five to ten ovules (Debouck, 1994).

P. acutifolius is a true diploid with a $2n$ chromosome number of 22. The 1C (or haploid) content of *P. acutifolius*, which reflects the genome size, is 0.75 pg (picogram) (Bennett and Leitch, 2005). In comparison, *P. vulgaris* has an 1C value



Figure 1 Close-up of seeds of tepary bean TB1

of 0.59 pg and its genome size has been estimated at 500–650 Mb (Broughton *et al.*, 2003).

1.3 Economic Importance

The total annual production of *Phaseolus* is around 24 million metric tons (Popelka *et al.*, 2004), but no data are found on the production of *P. acutifolius* individually. Nowadays, *P. acutifolius* is mainly grown as a minor subsistence crop in the southwest United States and adjacent Mexico. The cultivated genetic variability does not seem to have been very extensive, as judged from the few domesticated populations. This could be because the initial genetic base was already reduced at the time of its domestication.

It is likely that rural communities have conserved *P. acutifolius* because of its low agricultural requirements and short life cycle. It is mainly sown at the border of maize fields, although in southeastern United States it is also sown in small fields. Yields are estimated to be 200–900 kg ha⁻¹, with wide variations depending on sowing density and rainfall. About 1000–2000 kg ha⁻¹ is obtained with fertilizer, with harvests of up to 4 t ha⁻¹ under optimized conditions. It is noteworthy that the tepary bean can produce good yields without supplemental irrigation in the arid areas where it is cultivated, whereas most conventional beans would not even survive.

The main product of the tepary bean is dry seed, which is eaten because of its high protein (up to 30%) and carbohydrate content. In fact, the tepary bean has a slight superior nutritional value as compared to some other common beans. Besides the high protein content, it has higher levels of oil, calcium, iron, magnesium, zinc, phosphorus, and potassium. In addition, tepary beans are lower in polyunsaturated fat and in the antienzymatic compounds, which make common beans hard to digest (Hamama and Bhardwaj, 2002). Tepary dry beans are cooked like other dry beans after soaking. Sometimes dry seeds are toasted and then ground to the flour, which is mixed with water before eating. Tepary bean can also be consumed as a young tender string bean and used as fodder after harvesting.

There could be a renewed economic interest in the tepary beans for several reasons. They are promoted by specific gourmet retailers and restaur-

rants because of their nutty-tasting superior flavor, as well as a much creamier texture. In addition, organizations of native farmers who maintain traditional agriculture also promote the consumption of the tepary bean (examples are the Tohono O'odham Community, www.tocaonline.org and www.nativeseeds.org). Last but not the least, one of the main reasons for promoting cultivation of the tepary bean is to limit the use of water in subdesert areas. Its potential for introduction into desert areas (such as the American tropics, the Sahel, the Near East, India) is considerable but it has not been exploited. Tepary bean cultivation is being explored in dry areas of Africa due to its low rainfall requirements (Shisanya, 2002), Puerto Rico and Honduras (Miklas *et al.*, 1994) and is being revived in the southern states of the United States, like Arizona, where it was quite common as recently as 70 years ago. Researchers in Virginia have demonstrated that tepary beans can also produce well in the southeastern of the United States if planted in late spring, when moisture is lower than in early spring and temperatures are higher (Hamama and Bhardwaj, 2002).

1.4 Traditional Breeding

Due to the high variability within the *Phaseolus* family, genetic improvement by classical breeding of *P. vulgaris* has been quite successful (Singh, 1999). The tepary bean is usually seen as a secondary gene pool for improving the common bean, but to our knowledge, no specific programs have been carried out for improving the tepary bean itself. Debouck (1994) has suggested that *P. acutifolius* breeding could be done to improve the small seed size and the variability in colors, to decrease the susceptibility to rust, mildew, root rot, leaf miners, bruchids, and leafhoppers and to enhance tolerance to acidity of the soil and aluminum toxicity.

Thus, for *P. acutifolius*, the breeding research is still wide open and should consist of an increase in the germplasm collection, data on the biodiversity of the species, an understanding of the inheritance patterns of some of the traits of interest, and finally the setting up of tepary bean improvement programs and the distribution of tepary beans to farmers, giving them information

on the production potential of the tepary bean in dry zones.

To support cultivation and consumption of the tepary bean, research should be done to develop novel food technologies suited to leguminous vegetables (for example, industrial processing of bean proteins), which would free the farmer from market constraints. Also distributing information on the methods of consumption of tepary bean could be an option, in order to promote the use of this native legume.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

As is the case for most crops, the main targets for *Phaseolus* improvement are to breed varieties with higher yield, resistance, or at least tolerance to biotic and abiotic stresses such as diseases, pests, salinity, and drought. Other more specific legume-related breeding objectives include better taste, higher nutritional value of the seeds, reduced content of antinutritional factors, and flatulence factors, improved cooking qualities, and the prevention of early pod shattering as well as a pod distribution that would allow for easy, preferentially mechanical harvesting.

Biotechnology and the production of transgenic plants come in when a desired trait cannot be found within the germplasm of the species itself. But even if the desired trait is present in a more distant wild relative of the common bean, it is not always straightforward to introduce it through classical breeding into a bean elite cultivar, at which point transgenic approaches could also be considered. It should be emphasized in this context that classical breeding in *Phaseolus* has been quite successful, although interspecific crosses, like between *P. vulgaris* and *P. acutifolius* have been proved difficult (Mejía-Jiménez *et al.*, 1994; Muñoz *et al.*, 2004).

Most legumes suffer from insects that attack both the leaves and the pods, and during growth as well as postharvest, and few legume genes have been found that could confer resistance but often providing a limited tolerance to the pest. *Phaseolus* also suffers from pre and postharvest insects such as bean pod borers, bruchids, and weevils, which can be very devastating for economic value

(Singh, 1999). The expression of non-native arcelin genes, isolated from wild *P. vulgaris* varieties, and linked to resistance against Mexican bean weevil, *Zabrotes subfasciatus* (Boheman), has been evaluated with regard to insect resistance. For this, *P. acutifolius* lines have been obtained with high levels of arcelin expression, but these only show a marginal lower resistance toward the bean bruchid, *Z. subfasciatus* (see below; Goossens *et al.*, 2000; Zambre *et al.*, 2005). Another approach will be to use genes encoding insecticidal proteins. Such genes have been extensively used in transgenic plants, such as maize and cotton, but only to a limited extent in legumes (Shade *et al.*, 1994). The genes used are mainly isolated from bacteria *Bacillus thuringiensis*, and also from higher plants. The latter includes genes for lectins, proteases, protease inhibitors, and α -amylase inhibitors.

A particular application that has been extensively studied in legumes, in general, is the improvement of the nutritional quality of the seeds, especially to increase the methionine content (Wang *et al.*, 2003). A low seed-methionine content is a common problem for all legumes and classical breeding has not been very effective in this field. The heterologous expression of methionine-rich proteins has been shown to be possible (Molvig *et al.*, 1997; Dinkins *et al.*, 2001; De Clercq *et al.*, submitted). However, care should be taken so that these proteins will not cause allergic reactions as has been shown for the methionine-rich 2S albumin from Brazil nut (Nordlee *et al.*, 1996). The potential of such approaches is still largely untapped but should yield seeds with enhanced protein quality for the future, mainly in animal feed applications (White *et al.*, 2000).

Another target for legume improvement through transgenics is virus resistance. Two widely spread viruses affect bean production, namely the bean golden mosaic virus and the bean yellow mosaic virus. Virus resistance has been shown to be a trait that can be introduced through genetic transformation. The strategy is based on the expression of virus related sequences that trigger the virus induced silencing system of the plant (Tenllado *et al.*, 2004). This has been used for beans by the Brazilian research center Embrapa to obtain transgenic plants tolerant to bean golden mosaic virus (Aragão *et al.*, 1998).

2. DEVELOPMENT OF TRANSGENIC TEPARY BEAN

Transformation of *P. acutifolius* is still in its early development, and has until now mainly been focused on research applications. The only transgenic plants described in literature have been generated in the group at Ghent University, Belgium. Work is also ongoing in other groups (see “transformation group” on the website of the Phaseomics Initiative: <http://www.phaseolus.net>).

Transformation of *P. acutifolius* is stable and consistently reproducible (Dillen *et al.*, 1997; De Clercq *et al.*, 2002; Zambre *et al.*, 2005; De Clercq *et al.*, submitted). The protocol described by this group is based on the generation of callus tissue from either floral buds (Dillen *et al.*, 1996) or the cotyledonary node region (Figure 2) (Zambre *et al.*, 1998). This callus is co-cultivated with *Agrobacterium tumefaciens* for one week, followed by selection on antibiotic containing medium. β -glucuronidase (GUS) positive callus is transferred on shoot induction medium, *in vitro* developed shoots are either grafted (Figure 3) or allowed to root and then transferred to soil for further establishment in the greenhouse (Dillen *et al.*, 1997; De Clercq *et al.*, 2002; Zambre *et al.*, 2005). For details on the protocol with steps described in details consult Zambre *et al.* (2006). Using this protocol, dozens of transgenic lines have been generated, aimed at either insect resistance (Zambre *et al.*, 2005) or increased methionine content (De Clercq *et al.*, submitted). In addition, *P. acutifolius* has been evaluated as a potential crop for molecular farming (De Jaeger *et al.*, 2002).

2.1 Donor Gene

For the different transgenics that have been published, three classes of transgenes can be described.

A first category concerns the use of arcelin genes. Arcelins are seed proteins found in some wild *P. vulgaris* genotypes that are resistant to the bruchid beetle *Z. subfasciatus* (Mexican bean weevil). At least seven arcelin variants exist, each consisting of different isoforms. Resistance



Figure 2 Graft of a transgenic tepary bean shoot into a wild-type seedling

to *Z. subfasciatus* is genetically linked to the arcelin locus (Osborn *et al.*, 1988; Cardona *et al.*, 1989), and arcelin proteins have been regarded as its causal factors (Osborn *et al.*, 1988; Sales *et al.*, 2000), although direct evidence that the arcelin genes themselves are involved in resistance is lacking. To test this hypothesis, transgenic lines of *P. acutifolius* (genotypes NI576 and TB1) expressing arcelin genes (*arc1* and *arc5*) at high levels were evaluated for insect resistance.



Figure 3 Calluspiece (left) that was derived from callus forming on the cotyledonary node of a tepary bean seedling (right), as part of the transformation protocol

The results indicated that these lines were not significantly protected against *Z. subfasciatus* (Goossens *et al.*, 2000; Zambre *et al.*, 2005).

For this analysis, *A. tumefaciens* strain C58C1Rif^R containing pMP90 (Koncz and Schell 1986), a nopaline-type nononcogenic Ti plasmid, and harboring a binary plasmid vector—pATARC3 (Goossens *et al.*, 1999) or derivatives were used in all experiments. These binary vectors contained the neomycin phosphotransferase II (*nptII*) gene under control of the nopaline synthase promoter and the β -glucuronidase (GUS)-encoding gene (*uidA*) (Jefferson *et al.*, 1987) with an intron under control of the cauliflower mosaic virus (CaMV 35S) promoter. In addition, the vectors carried gene sequences from arcelin genes encoding for arcelin proteins, namely Arcelin 5a, Arcelin 5b, and Arcelin 5c as well as from Arcelin 1 (Anthony *et al.*, 1991; Goossens *et al.*, 1994). All arcelin coding sequences were under the control of the seed-specific promoter region and the 3' flanking sequence from the arcelin gene (*arc5-I*) that is encoding Arcelin 5a (Goossens *et al.*, 1999). Transgenic beans from one wild, and two different cultivated *P. acutifolius* genotypes (TB1 and PI440795), all showing high accumulation of arcelin levels were tested for their resistance to *Z. subfasciatus*, but they were only marginally less susceptible to infestation than control or

nontransgenic *P. acutifolius*. Hence, the arcelin genes tested are likely not major determinants of resistance against *Z. subfasciatus*.

A second category of transgenic plants was developed using the arcelin genes as described above but with modifications allowing them to express a modified arcelin protein with a higher number of methionines. For that purpose modified *arc5-I* genes (encoding for Arcelin 5a) were created with up to 12 additional methionine codons and were introduced into the *P. acutifolius* genotype TB1. The constructs used again were based on the same pATARC3 binary vector as described above and 5' and 3' sequences were from the *arc5-I* gene. Proteins accumulated at high levels in seeds of several independent transgenic *P. acutifolius* TB1 lines. This high accumulation level in turn resulted in some lines in a significant increase of the methionine content in the seeds of these transgenic beans (De Clercq *et al.*, submitted).

Finally, to evaluate the potential of tepary bean for molecular farming in the pharmaceutical sector, a gene encoding an antibody fragment has been expressed (De Jaeger *et al.*, 2002). These constructs were also made in the backbone of the same binary vector family, pATAG4 (Goossens *et al.*, 1999). The construct contained the neomycin phosphotransferase II (*nptII*) gene under control of the nopaline synthase promoter, with a 3' end of the octopine synthase gene. The gene of interest, in

this case the coding sequence of the murine single chain Fv binding dihydroflavonol-4-reductase of *Petunia hybrida* (De Jaeger *et al.*, 1999) was cloned under the control of the *arc5-I* promoter (4600 bp) and the first 13 bp of the 5'UTR of the *arc5-I* gene and a signal peptide of the 2S2 seed storage protein gene of *Arabidopsis thaliana* (Krebbers *et al.*, 1988). It is terminated by a KDEL (ER retention signal) and the 3' end of *arc5-I* (3900 bp).

2.2 Transformation Methods

The protocol that is described is an *Agrobacterium*-based method, whereby green nodular callus (Figure 2) is co-cultivated with a low dense *A. tumefaciens* strain C58C1 Rif^R culture (Zambre *et al.*, 2006). Among three nononcogenic Ti plasmids that were tested—the nopaline-type pMP90 (Koncz and Schell 1986), the octopine-type pGV2260 (Deblaere *et al.*, 1985), and the agropine/succinamopine-type pEHA101 (Hood *et al.*, 1986)—the first one has been shown to be most effective (De Clercq *et al.*, 2002).

Particle bombardment could be another method, although in some cases less preferred (Altpeter *et al.*, 2005) for genetic transformation. It has to our knowledge not been explored for the tepary bean transformation, although it has been successfully used to achieve genetic transformation of *P. vulgaris* (Aragão, 1998, 2002).

2.3 Selection of Transformed Tissue

After co-cultivation, transgenic calli are selected through four or five passages on media containing the antibiotic geneticin. Resistance to geneticin is conferred by the *nptII* gene that is present on the transfer DNA (T-DNA). This lengthy callus phase ensures the strict selection of transgenic callus, although the selective agent is omitted in the subsequent plant regeneration steps, essentially all obtained plants are truly transgenic.

To monitor transformation efficiency, GUS expression is checked on small parts of nice green, growing callus pieces and GUS positive calli are transferred to shoot induction medium (Zambre *et al.*, 2005, 2006).

2.4 Regeneration of Whole Plant

Shoots appearing on the shoot induction medium will in many cases form roots spontaneously. Shoots that are not rooting spontaneously are grafted (Figure 3). For this, the base of apical or nodal segments (approximately 5 mm) of 2–4 cm shoots is trimmed to obtain a wedge shape. The hypocotyls of wild type seedlings as root stocks are cut about 1.5 cm above the root by a transverse cut in the epicotyl region to remove the plumule. The scion base is inserted into a vertical hole, made with a needle in the epicotyl region of the root stock. The resulting grafts are cultured on Murashige and Skoog (MS) medium, and incubated in a growth chamber for 3 weeks. *In vitro*-established grafted shoots and spontaneously rooted shoots are transplanted to soil in 4.51 pots after washing the roots thoroughly with running tap water. Initially the plants are covered with transparent plastic to maintain humidity; thereafter, the plants are gradually acclimatized by cutting holes in the plastic to lower the humidity.

2.5 Follow-up Testings

All transgenic plants that are obtained with the above-described procedure are fertile and appear to be morphologically normal over at least four generations. Stable integration of the transgenes and transmission to the progeny in a Mendelian fashion has been demonstrated by assaying GUS expression on small pieces of the harvested dry seeds. Leaves of mature plants have been used to extract DNA and perform southern blots with different probes related to the construct that has been inserted. As ultimate proof the expression of the gene of interest is shown by western blot analysis.

2.6 Specific Regulatory Measures

As *P. acutifolius* is not a widely cultivated and consumed crop, there is not so much specific to be found in regulatory guidelines. As for the movement of legumes in general, the International guidelines for the movement of legumes has been agreed and published as “FAO/IBPGR Technical guidelines for the safe movement of

legume germplasm” (<http://www.ipgri.cgiar.org/publications/pdf/96.pdf>). A special emphasis for *P. acutifolius* is given for its seed transmission of bean common mosaic virus.

3. FUTURE ROAD MAP

3.1 Expected Products

Because of the limited scale of consumption of *P. acutifolius* not much effort has been put in attractive traits that could be introduced through genetic engineering. One area where this crop could be relevant is within the field of molecular farming. *P. acutifolius* seems to be able to express heterologous proteins at a high level, and moreover it cannot cross in the field with the more commonly consumed *P. vulgaris*, which could be perceived as an advantage by regulators in view of the prevention of transgene spread to important food crops.

It has been observed by breeders that there is a pronounced heterotic effect in tepary bean and thus a transgenic approach to create male sterile tepary beans could be an interesting and straightforward project. However, no cytoplasmic male sterility has been described for *P. acutifolius*. Transgenic approaches to introduce male sterility have been well described and consist of the expression of a cytotoxic ribonuclease, called barnase, under the control of a tapetum specific promoter (De Block *et al.*, 1997).

3.2 Addressing Risks and Concerns

As a minor subsistence crop, *P. acutifolius* is a legume that could well be more promoted, especially in drought prone areas. It is, however, not expected that the introduction of a genetically modified tepary bean, even with improved agricultural qualities, will give rise to big shifts in its use and thus in agriculture or food production. They will likely just replace other tepary bean varieties, or *P. vulgaris* or other legumes. Since tepary beans are highly adapted to adverse environmental conditions, one expects that transgenic varieties will improve economies in developing countries while maintaining traditional agricultural practices.

It must be clear that any new transgenic lines that will be developed, either with improved agricultural properties or for industrial applications, will undergo trials and safety evaluations, and when approved by the respective authorities for commercialization will be safe for human health and environment.

3.3 Expected Technologies

Major improvements are still needed for transgenic applications in *Phaseolus*, and for the complete grain legume family in general, first in relation to the transformation efficiency and secondly in relation to specific promoters that could be used.

For the first, improvement of culture-based transformation protocols is surely possible and will include testing various culture conditions, media compositions, hormones, and selection systems. However, this is a quite time-consuming approach and, to our knowledge, not many research laboratories are currently active on improving *Phaseolus* transformation protocols.

More scientific insights on tissue culture-based plant regeneration systems might also allow the development of alternative approaches to improve legume transformation. Examples are genes known to be involved in embryogenesis or cell cycle regulation, that have been shown to increase transformation frequency in maize (Gordon-Kamm *et al.*, 2002) or have this potential (Boutilier *et al.*, 2002; Zuo *et al.*, 2002; DeYoung *et al.*, 2006). Alternatively new developments in systems biology, that combine genomics, proteomics, and metabolomics, could allow for a better understanding of the *Agrobacterium* transformation process. This could lead to the identification of genes with a positive effect on transformation related steps that could be very useful in the development of *Agrobacterium* transformation systems for recalcitrant species and varieties, among which the very important *P. vulgaris*. It would be a breakthrough if *Agrobacterium* could be “engineered” in such a way that it becomes a perfect gene delivery tool for all plant systems.

For the second aspect, to obtain more specific promoters it is worthwhile to mention that an international *Phaseolus* genome initiative has been undertaken, named Phaseomics (Broughton *et al.*, 2003; <http://www.phaseolus.net>). The focus of

this program is to physically map the genome (Blair *et al.*, 2003) with the ultimate goal of sequencing it. This should deliver many more gene sequences that could be studied for their expression and function, which could eventually lead to new tissue specific or condition-induced promoters.

As already mentioned, the improvement of tepary beans by genetic engineering is at its birth. No commercial genetically modified (GM) variety is in the pipeline. The development of such varieties shall, therefore, follow the trend for safer markers and exclusion of antibiotic resistance markers. It is, however, important to stress that there is no experimental evidence of gene transfer to the bacterial gut. Nevertheless, several National Academies of Sciences have recommended the phasing out of antibiotic markers because alternative strategies are available that avoid unnecessary concerns on GM crops.

3.4 Specific Details for Intellectual Property Rights (IPR), Public Perceptions, Industrial Perspectives, Political and Economic Consequences

From an industry perspective, *P. acutifolius* might hold promises for the production of proteins of industrial or pharmacological interest, as mentioned above, as it seems to be able to express heterologous proteins at a high level in the seeds, which can also benefit from relative straightforward and well established extraction procedures. *P. acutifolius* cannot cross in the field with the more commonly consumed *P. vulgaris*, thus preventing transgene spread to this food crop. Measures should, however, be taken to avoid gene flow to wild tepary bean varieties. The above mentioned male sterility strategy could be a good option.

In addition, in view of its drought tolerant behavior, the tepary bean could become a more important crop, as water shortages become a reality in increasing regions of the world. Its use in molecular farming can be extremely beneficial for developing countries, since it will add high value to a minor local crop production. With this in mind, *P. acutifolius* should be considered as a legume crop with potential for the future and genetic improvement programs, either through classical breeding, molecular markers-assisted breeding, or

newer genetic engineering approaches, should be encouraged.

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Asiatic Beans

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1. INTRODUCTION

The genus *Vigna*, subgenus *Ceratotropis* consists of 16–17 recognized species (Tateishi, 1996), which are distributed across Asia. Asian *Vigna* species of the subgenus *Ceratotropis* constitute of an economically important group of cultivated and wild species, of which a rich diversity occurs in the Southeast Asia. *Vigna* species contribute a vital protein component to the vegetarian diet of the resource-poor populace of the underdeveloped and developing countries. The production of these species is severely limited by numerous diseases and environmental stresses. Research efforts have been initiated to increase their yield by elevating and/or imparting resistance for diseases, insect pests and rescuing from natural genetic inadequacies. In the last few decades, production of these species has been maintained mainly due to the high rate of chemicals applied (fertilizers, herbicides, pesticides, or insecticides), mechanization, as well as amelioration practices. However, these practices have resulted in severe economic and ecological implications in the developing countries. Progress in genetic improvement by conventional breeding is hampered due to the lack of sufficient and satisfactory level of genetic variability within the germplasm. Because of the lengthy process of breeding and recurrent selection, the genetic diversity of *Vigna* species has been eroded and became very narrow. Further, breeding methods became limited by low recombination potential

as an outcome of self-pollination, low inheritance capacity of some important characteristics (total yield and yield components), and embryo abortion in some interspecific hybrids. The immense potential of biotechnological tools to supplement breeding programs in developing high yielding, disease resistant, and stress tolerant *Vigna* species is being realized. Progress has been made for *in vitro* regeneration of sexually mature plants, in majority of these recalcitrant species, from various tissues. Commendable progress has been made with regard to development of robust genetic transformation systems in *Vigna* species. An overview of the *in vitro* regeneration and critical analysis of the genetic transformation studies in major *Vigna* species are presented. The bottlenecks to overcome their recalcitrance *vis-à-vis* the advantages and limitations of all those transformation techniques applicable, along with future directions in research are discussed.

1.1 Cultivation and Production

Mungbean has been widely cultivated in the Indian subcontinent and adjacent regions for several thousand years and is known to have spread to other Asian countries and northern Africa. The widespread distribution in the tropics and subtropics of Africa, West Indies, North America, and Australia is relatively recent. Presently, it is the most important grain legume in Thailand

and the Philippines; it ranks second in Sri Lanka and third in India, Burma, Bangladesh, and Indonesia. It is a minor crop in Australia, China, Iran, Kenya, Korea, Malaysia, the Middle East, Peru, Taiwan, and the United States. In India, mungbean is cultivated either as summer/spring season or rainy season crop. It is also grown in winter in South India. Recent estimates of dry bean production (includes *Phaseolus* and other *Vigna* species) in India are 7 million tons (<http://www.grainlegumes.com>).

Urdbean is an important pulse crop in India, Burma, Bangladesh, Pakistan, and Thailand, and of minor importance in Sri Lanka. It is grown in a limited scale in other parts of Southeast Asia, Australia, and Fiji. India is the leading producer of urdbean in the world contributing around 1.5 million tons, around 10% of the total quantity of pulses produced in India. Myanmar and Thailand follow India in production, though Myanmar produces mainly for export.

Azukibean is an important grain legume in East Asia especially in Japan, China, and Korea. It ranks second after soybean in Japan, the main producing district being Hokkaido. Azukibean is also grown to a greater extent in Nepal and Bhutan. In Japan, 70 000–90 000 metric tons were produced annually between 1996 and 2000. In China, azukibean is cultivated on 47 000 ha and may have an annual production of about 700 000 metric ton (Vaughan *et al.*, 2005).

1.2 Economic Importance

Asiatic beans, mungbean, and urdbean are primarily grown for its protein-rich dry edible seeds and fresh sprouts. The dried seeds are used as whole or split in making *dal*, soups, and curries and ground into flour for noodles, bread, and biscuits. The seeds have high protein (18–36%) that is easily digestible, are easy to cook and lack flatulence factors (lowest level of oligosaccharides of raffinose family—reffinose, stachyose, and verbascose shown to be responsible for flatulence) in contrast to other legumes. Seed protein is deficient in sulfur amino acids, particularly methionine and tryptophan. In contrast, the lysine content is high. Therefore, combination of mungbean/urdbean and cereal in diet provide a more balanced amino acid profile. The seeds are also rich in calcium,

phosphorous, and certain vitamins (thiamine, riboflavin, and niacin). Beside protein, mungbean also has relatively high iron content. A new variety Pusa Bold-1 (Pusa Vishal) contains 6 mg of iron per 100 g raw seeds compared to 3.3 mg or 3.5 mg in traditional varieties (Vijayalakshmi *et al.*, 2003). The iron in mungbean is made more biologically available when seeds are cooked together with certain vegetables such as tomato, mustard greens, and cabbage (Bains *et al.*, 2003). Such consumption can substantially increase the iron store of anemic school children (Weinberger, 2002). Mungbean seeds and soup are also a rich source of alkaloids, coumarin, and phytosterin that play an important role in promoting the physiological metabolism of human beings and animals. In traditional Chinese medicines, part of mungbean plants are used for treating various ailments including hepatitis, gastritis, uraemia, toxicosis, red dysentery, cholera, corneal opacity, and macula (Zheng *et al.*, 2002). The other health promoting substance, isoflavones are present at levels of 0.19 mg 100 g⁻¹ in mungbean (Yu and Mc Gonigle, 2005). Mungbean seeds are free from antinutritional factors such as trypsin inhibitor, phytohemagglutinins, and tannin (Liener, 1975; Price *et al.*, 1980).

The sprouts of both mungbeans and urdbeans are used as a green vegetable, when other vegetables are scarce. One kilogram of dry seeds of mungbean can yield 8–9 kg sprouts. With sprouting, there is an increase in thiamine, riboflavin, niacin, and ascorbic acid concentrations (Abdullah and Baldwin, 1984). Mungbean sprouts are favored throughout Asia especially in Chinese cooking, Europe, and North America where they are used as a garnish in mixed diets or used as a valuable source of protein and vitamins in vegetarian diets. The young pods are also used as a vegetable. An increased consumption of mungbean sprouts, particularly by people consuming western-type diets, could have a significant effect on cardiovascular disease, which is a major problem associated with such diet.

Besides human food, they also serves as nutritive fodder for cattle, and used as manure. All *Vigna* species are annual, nitrogen fixing, generally short-duration plants, and therefore widely used as mixed intercrop or in rotation to improve nitrogen status of the soil, and to break disease/pest cycles.

1.3 Constraints in Production

The major limitations in mungbean (*Vigna radiata*) and urdbean (*Vigna mungo*) yield are:

1. Mungbean yellow mosaic virus disease (MYMV), *Cercospora* leaf spot (*Cercospora canescens*), powdery mildew (*Erysiphe polygoni*), bacterial leaf spot (*Xanthomonas campestris* pv. *phaseoli*), anthracnose (*Colletotrichum lindemuthianum*), and *Sclerotinia* blight caused by *Sclerotinia sclerotiorum*.
2. Major insects that include whitefly (*Bemisia tabaci*), bean thrips (*Megalurothrips distalis*), gram pod borer (*Helicoverpa armigera*), and legume pod borer (*Maruca vitrata*).
3. Storage pests mainly bruchid, *Callosobruchus* species.
4. Major abiotic stresses include salinity, drought, heat and cold stress.
5. Indeterminate growth habit, defective plant type, excessive vegetative growth, low harvest index, sensitivity to photoperiod, asynchronous flowering, and shattering of pods.
6. Sprouting of seeds *in situ* following rains.

The major constraints in azukibean (*Vigna angularis*) production are postharvest damage by bruchids, azukibean weevil (*Callosobruchus chinensis*), and the cowpea weevil (*Callosobruchus maculatus*); bacterial infection; brown stem rot caused by a fungus, *Fusarium oxysporum*.

1.4 Taxonomy

Vigna cultigens and soybean are warm weather and tropical legumes of subtribe Phaseolineae of tribe Phaseoleae of subfamily Papilionoideae (Fabaceae). The genus *Vigna* savi of subtribe Phaseolineae includes seven subgenera (Marechal *et al.*, 1978) of which three subgenera, *Ceratotropis*, *Plectotropis*, and *Vigna* include cultigens. Recently, the taxonomy of the subgenus *Ceratotropis* has been revised (Doi *et al.*, 2002; Tateishi and Maxted, 2002; Tomooka *et al.*, 2002a, b). In the new system, the subgenus has been divided into three sections, *Angulares*, *Ceratotropis*, and *Aconitifolia*, consisting of 12, 4, and 5 species, respectively. Of the 21 species of subgenus *Ceratotropis*, eight are cultivated (Tomooka *et al.*, 2002b) (Table 1). Among these the widely grown are mungbean

(*V. radiata*), urdbean (*V. mungo*), and ricebean (*V. umbellata*) in the tropical countries of South and Southeast Asia and azukibean (*V. angularis*) in the East Asia.

V. radiata along with its allied species, *V. mungo*, *V. grandiflora*, and *V. subramaniana* has been placed within the subgenus *Ceratotropis* of section *Ceratotropis* as classified by molecular analyses and taxonomic studies (Doi *et al.*, 2002; Tomooka *et al.*, 2002c). These species shared several morphological traits, such as epigeal germination and elliptic to ovate first leaves without a petiole. However, cultivated and wild species of *V. radiata* are distinct from *V. mungo* with broader stipules, pale yellow flowers, more ovules per pod, spreading pods with short brown hairs, and nonarillate hilum and also in chemical and molecular characters. *V. radiata* var. *sublobata*, the wild progenitor of mungbean is distributed across tropical Africa, Oman, South and Southeast Asia, Papua New Guinea, and Australia (Tomooka *et al.*, 2004). India is considered the center of diversity of the mungbean weed (Paroda and Thomas, 1988) and wild forms (Lawn, 1995), which are found in hilly tracks of northern Terai region and Western Ghats (Arora and Nayar, 1984; Saravankumar *et al.*, 2003). India is likely the place of origin and domestication (Tomooka *et al.*, 2004) as remains of *Vigna* seeds have been reported at Indian archaeological sites dating back to 3000–3500 BC and 2500 and 3000 BC (Jain and Mehra, 1980). Indian mungbean landraces have the most diverse protein and plant growth types (Tomooka *et al.*, 1991, 1992). The mungbean landraces of Western Asia (Afghanistan–Iran–Iraq) region are considered primitive (small and variously colored seeds, plants with many laterals) with diverse protein types while those of Southeast Asia have large shiny green seeds, tall plant with thick main stem, late maturing, and simple protein types. The mungbean landraces of East Asia include growth type diversity that is intermediate between the types from Western Asia and Southeast Asian (Tomooka *et al.*, 2004).

V. angularis, which belongs to section *Angulares* of the subgenus *Ceratotropis*, is characterized by hypogeal germination, cordate first and second leaves with petioles, well-developed keel pocket in flower, and beaked style. The putative wild progenitor, *V. angularis* var. *nipponensis*, and weedy

Table 1 The cultivated and domesticated *Vigna* species

Subgenus sections	Cultigen species (common name)	Distribution	Ploidy level	Chromosome number	1C DNA content (Mb)
<i>Vigna</i>					
<i>Catjang</i>	<i>V. unguiculata</i> subsp. <i>unguiculata</i> var. <i>unguiculata</i> (cowpea)				
<i>Vigna</i>	<i>V. subterranean</i> (L.) (bambara ground nut)		2	11	880
	<i>V. luteola</i> (jacq) Benth		—	—	—
	<i>V. marina</i> (Burm.) Merrill		—	—	—
<i>Plectotropis</i>	<i>V. vexillata</i> (L.) A. Richard		—	—	—
<i>Ceratotropis</i>					
<i>Angulares</i>	<i>V. angularis</i> var. <i>angularis</i> (Azuki bean)	Worldwide	2	11	540
	<i>V. angularis</i> var. <i>nipponensis</i> (wild)	Bhutan, China, Indian Himalaya, Japan, Korea, Myanmar, Nepal			
	<i>V. reflexo-pilosa</i> var. <i>glabra</i>		—	—	—
	<i>V. trinervia</i> (Heyne ex Wight and Arnoh)		—	—	—
	<i>V. umbellata</i> (Thumb.) Ohwi and Ohashi (rice bean)	Southeast and East Asia	2	11	570
<i>Ceratotropis</i>	<i>V. mungo</i> (L.) Hepper var. <i>mungo</i> (urdbean)	South Asia	2	11	540
	<i>V. mungo</i> var. <i>silvestris</i> (wild)	India, Bangladesh, Myanmar			
	<i>V. radiata</i> (L.) Wilczek var. <i>radiata</i> (mungbean)	Worldwide	2	11	520
	<i>V. radiata</i> var. <i>sublobata</i> (wild)	Asia, Australia, Equatorial Africa, Middle East Asia			
<i>Aconitifoliae</i>	<i>V. aconitifolia</i> (jacquin) Marechal (mothbean)	South Asia cultivated/wild India	2	11	1110
	<i>V. trilobata</i> (L.) Vercourt (junglebean)		—	—	—

forms, which show an intermediate phenotype between cultivated and wild bean, are distributed throughout Japan that is considered to be a center of diversity for azukibean (Vaughan *et al.*, 2005).

Recent studies suggest that there are four different groups of azukibean germplasm according to their geographic origin, namely, China, Korea, Japan, and the Himalayan region. Among these, Himalayan germplasm is well differentiated from other groups (Zong *et al.*, 2003). The results suggest that azukibean was probably domesticated independently in the Himalayan region and East Asia.

1.5 Conventional Breeding

The general objectives of breeding Asiatic *Vigna* beans are to develop varieties possessing

1. high yield, early and uniform maturity (55–60 days);
2. shining green and bold seeds;
3. resistance to pathogens (that cause MYMV, *Cercospora* leaf spot, and powdery mildew) and insect pests (bruchids, thrips, and pod borer);
4. resistance to abiotic stresses (salinity, alkalinity, heat, etc.);
5. resistance to preharvest sprouting; and
6. improved nutrient content such as sulfur-containing amino acids in proteins and altered seed starch composition for use of mungbean in different foods.

Some of the sources of cultivated and wild germplasm for these traits have been identified or improved (see Jaiwal and Gulati, 1995; Srinives, 1996; Tomooka *et al.*, 2004) but durable and broad resistance to biotic and abiotic stresses are difficult to achieve through conventional breeding.

2. GENETIC TRANSFORMATION OF *VIGNA* SPECIES

Production of Asiatic *Vigna* species is severely limited by a number of biotic and abiotic stresses (Sahoo *et al.*, 2003). Genetic improvement of these bean species through conventional breeding relies on the utilization of domestic cultivars and related genera as the source of genes. Genes conferring resistance to biotic and abiotic stresses have been found in many wild or related species (Sahoo *et al.*, 2003). However, the major impediments in using the wild species as a source of resistance are cross-incompatibility with the cultivated ones, and linkage drag (Watanasit and Pichitporn, 1996). The resistance in cultivated gene pool in subgenus *Ceratotropis* is rare when compared to the wild species (Tomooka *et al.*, 2002b). Genetic barriers existing in the native *Vigna* species further prevent the transfer of useful traits to the related species (Palmer *et al.*, 2002).

Genetic engineering has opened up new avenues to modify crops providing viable solutions to alleviate the constraints in productivity (Estruch *et al.*, 1997). The process offers plant breeders access to an infinitely wide array of novel genes and traits across the organisms, which can be introduced through a single event into high-yielding and locally adapted cultivars through the conceptual framework and technical approaches of plant tissue culture and genetic transformation. Further, it is possible to control the timing, tissue specificity, and expression level of transferred genes for their optimal function. The powerful combination of genetic engineering and conventional breeding programs permits useful traits encoded by transgenes to be introduced into *Vigna* species within an economically viable time frame, saving precious time required for introgression of desired traits from the wild relatives by conventional practices. Enormous potential for genetic manipulation of these species lies in increasing resistance to diseases, pests, and abiotic stress, and qualitative change in their seed composition. Moreover, with the establishment and expansion of genomics programs, a much broader range of genes with potential for crop improvement are being identified and, in some cases, tailored and/or redesigned for further enhancement of their properties within specific crops. This has further intensified the interest

in developing efficient plant transformation technologies to be able to concurrently test and capture the value of these genes in *Vigna* species. Beyond crop improvement, the ability to engineer *Vigna* species holds a powerful and informative means for studying gene function and the regulation of physiological and developmental processes.

Development of efficient and routine genetic transformation systems in grain legumes, the *Vigna* species in particular, poses a formidable challenge in the generation of stable transgenic plants. Advances in tissue culture, combined with improvements in transformation technology, have resulted in increased transformation efficiencies in many grain legumes (Somers *et al.*, 2003). However, Asiatic *Vigna* species have still remained recalcitrant to tissue culture and are stubbornly resistant to the overtures of genetic engineering (Chandra and Pental, 2003). Further, advances in molecular genetics, e.g., gene overexpression, gene suppression, promoter analysis, and transfer DNA (T-DNA) tagging, require efficient transformation systems (Somers *et al.*, 2003).

Gene transfer to these species demands development of robust plant regeneration systems from tissues amenable to routine genetic transformation methods, efficient selection system for recovery of viable and fertile transgenic plants from transformed explants at a reasonable frequency, and a time frame in culture to avoid somaclonal variation and possible sterility (Sahoo *et al.*, 2003).

Whilst different approaches have been attempted for transformation of *Vigna* species, *Agrobacterium tumefaciens*-mediated gene transfer has been the most successful in generation of transgenic plants besides considerable progress made with regard to particle bombardment. The *Agrobacterium*-mediated genetic transformation is considered so far advantageous over direct DNA delivery techniques in its capacity for high frequency of stable genetic integration, the transfer of relatively large segments of DNA, and insertion of single/low copy numbers of gene(s) (McCormac *et al.*, 1998). The relative ease with which *Agrobacterium* may transform is likely due to the wound response these species exhibit (Potrykus, 1990); however competence of *Agrobacterium* to access the cells capable of dedifferentiation, followed by regeneration limits its success. Thus, combining transformation

competence with totipotency is imperative in this species (Birch, 1997).

Considerable progress has been made in *A. tumefaciens*-mediated genetic transformation of Asiatic *Vigna* species. A summary of the key transformation events is presented in Table 2. Severe host-genotype specificity, lack of wounding response, the choice of suitable explant source potentially competent for both regeneration and *Agrobacterium* infection have so far limited the success of the *Agrobacterium*-mediated transformation. The ultimate choice is to target as many competent cells as possible; moreover, to develop ways to maximize the number of cells (Christou, 1995).

The ability to engineer organized tissue, comprising different cell populations that are developmentally and positionally distinct, considerably limits the time duration of introduction of foreign genes into elite germplasm. Regeneration systems based on pre-existing meristems, precisely shoot apex, are promising as they develop directly into various tissues and organs of a mature plant including flowers (Ritala *et al.*, 1994; Schoneberg *et al.*, 1994; Aragao and Rech, 1997) without an intervening callus phase (Babaoglu *et al.*, 2000). The avoidance of a callus phase, followed by regeneration to shoots or somatic embryos ensures a low incidence of somaclonal variation. Particle bombardment, which relies upon the acceleration of DNA-coated particles into target cells, is much preferred option for gene delivery to recalcitrant grain legumes, circumventing the host specificity as well as genotype dependence by *Agrobacterium* (Christou, 1994, 1995, 1997). Shoot apex with its very high regeneration potential, offers an ideal target for bombardment, overcoming many of the regeneration problems (Potrykus, 1990; Kim and Minamikawa, 1996; Aragao *et al.*, 2000). Although transformation based on such tissue carries the risk of producing chimeric plants containing both transformed and untransformed sectors, regardless of whether regeneration is achieved through axillary shoot growth of organogenesis (Pigaire *et al.*, 1997), production of chimeric transformants is not always undesirable as there is evidence to indicate that wholly transformed plants can be recovered from chimeric shoots (McHughen and Jordan, 1989). The genetic transformation study in *Vigna* species is presented in Table 2.

2.1 *Vigna radiata*

Direct organogenesis pathway from cotyledon, cotyledonary nodes, and primary leaf explants has been adapted for *Agrobacterium*-mediated transformation of *V. radiata*. The axillary meristems at the junction of the cotyledon and cotyledonary node explants contain cells most responsive for regeneration through multiple shoot induction and hence, offers suitable target for gene delivery.

Pal *et al.* (1991) employed a protocol based on shoot organogenesis from cotyledons of *V. radiata* cvs. B1 and T44 for infection with *A. tumefaciens* strains, LBA4404 harboring binary vector pBI121, and GV2260 carrying a cointegrate vector pGV2260 pSGluc1, and recovered primary transformants on kanamycin selection. Approximately 4–5% of the shoots produced on the kanamycin selection were transformed as confirmed by dot blot assay. However, large number of escapes on kanamycin selection, lack of evidence for stabilization, and inheritance of transgenes ruled out the applicability of such transformation system.

Transgenic calli were generated from primary leaf explants of *V. radiata* co-cultured with *A. tumefaciens* strain LBA4404 pKIWI105 (Phogat *et al.*, 1999). The transformed calli selected on kanamycin exhibited β -glucuronidase activity. The integration of *nptII* gene was confirmed by junction fragment analysis by Southern hybridization. However, the calli failed to regenerate to plants under tested culture conditions.

In *V. radiata*, nearly 80–100% transformation frequency of cotyledon and hypocotyls explants has been observed with *Agrobacterium rhizogenes* strain LBA9402 (Jaiwal *et al.*, 1998), but transformed tissue gave rise to roots only.

Transgenic plants of *V. radiata* cv. K-851 were successfully generated from cotyledonary nodes co-cultured with *A. tumefaciens* strain LBA4404 pTOK233 at an overall efficiency of 0.9% (Jaiwal *et al.*, 2001). The plants selected on kanamycin-containing medium were raised to maturity. Stable β -glucuronidase (GUS) expression was detected in stamens, pollen grains, and seeds of the T₀ plants. Molecular analysis of T₀ transformants revealed the integration and expression of transgenes.

Transgenic calli were recovered from primary leaves and hypocotyls explants of *V. radiata* at a transformation frequency of up to 50% using *A.*

Table 2 Current status of genetic transformation of *Vigna* species

Species and cultivars	Explant and mode of regeneration	DNA delivery method	Vector/construct	Selection	Frequency of transformation	Transgenic plants and progeny analysis	References
<i>Vigna radiata</i> (B1 and T44)	Cotyledon, shoot organogenesis	At LBA4404 GV2260	pBI121 (<i>uidA</i> and <i>nptII</i>) pGV2260	Kanamycin (300 mg l ⁻¹)	Not reported	T ₀ plants, confirmed by dot blot assay	Pal <i>et al.</i> , 1991
<i>Vigna radiata</i> (ML-5 and K-851)	Shoot regeneration from mature germinating embryos	PB	pBI221 (<i>gus</i> and <i>nptII</i>)	Kanamycin	Not reported	Putative transformed shoots rooted on kanamycin, no molecular analysis	Bhargava and Smigocki, 1994
<i>Vigna radiata</i>	Callus induction from primary leaf explants	At LBA4404	pKIWI105 (<i>uidA</i> and <i>nptII</i>)	Kanamycin	Not reported	Transgenic calli, confirmed by southern hybridization	Phogat <i>et al.</i> , 1999
<i>Vigna radiata</i> (K-851)	Induction of roots and callus from hypocotyl and primary leaves	At LBA9402	–	Kanamycin	80–100%	Transgenic roots and calli	Jaiwal <i>et al.</i> , 1998
<i>Vigna radiata</i> (K-851)	Shoot regeneration from cotyledonary nodes callus regeneration from cotyledon and hypocotyl	At LBA4404 C58C1 EHA105 At EHA105	pTOK233 pIG121Hm pBin9GusInt pBin9GusInt	Kanamycin	0.9% 50%	T ₀ plants confirmed by Southern analysis Transgenic calli confirmed by Southern analysis, failed to regenerate to shoots	Jaiwal <i>et al.</i> , 2001
<i>Vigna radiata</i> (K-851)	Adventitious regeneration of shoots from primary leaves	At C58	pCAMBIA1301	Hygromycin	2% (mean)	Stable integration of the marker gene in the T ₀ transgenics and its inheritance in the T ₁ transgenics confirmed through molecular analysis	Mahalakshmi <i>et al.</i> , 2006
<i>Vigna radiata</i> (Pusa 105)	Shoot regeneration from cotyledonary nodes	At EHA105	pKSB (<i>bar</i> and <i>α-ai1</i>)	Phosphinothricin	1.51%	Integration <i>α-ai1</i> gene confirmed by Southern analysis PCR analysis revealed inheritance of both the transgenes	Sonia <i>et al.</i> , 2007

(Continued)

Table 2 Current status of genetic transformation of *Vigna* species (Continued)

Species and cultivars	Explant and mode of regeneration	DNA delivery method	Vector/construct	Selection	Frequency of transformation	Transgenic plants and progeny analysis	References
<i>V. angularis</i> (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At LBA4404	pBin19 (<i>nrpII</i> , <i>gus</i> and <i>α-<i>ail</i></i>)	Kanamycin	Not reported	Presence of <i>αAI</i> in seeds of T ₀ and T ₁ plants confirmed by immunoblotting Transgenic seeds showed resistance to bruchids Transgenic plants Molecular analysis confirmed the stable integration and expression of foreign genes, GFP expression detected in progenies Genomic Southern analysis confirmed stable integration of transgene, RT-PCR indicated the transcription of D6-fatty-acid desaturase gene in transgenic lines, the whole lipids extracted from the leaves and seeds of the T ₀ transgenic plants and the leaves of their T ₁ progenies were identified using GC-MS	Ishimoto <i>et al.</i> , 1996
<i>V. angularis</i> (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At EHA105	pIG121 (<i>gus</i> and <i>nrpII</i>) pSG65T (<i>gfp</i> , <i>nrpII</i> and <i>gus</i>)	Kanamycin	2.0%	Transgenic seeds showed resistance to bruchids Transgenic plants Molecular analysis confirmed the stable integration and expression of foreign genes, GFP expression detected in progenies Genomic Southern analysis confirmed stable integration of transgene, RT-PCR indicated the transcription of D6-fatty-acid desaturase gene in transgenic lines, the whole lipids extracted from the leaves and seeds of the T ₀ transgenic plants and the leaves of their T ₁ progenies were identified using GC-MS	Yamada <i>et al.</i> , 2001
<i>V. angularis</i> (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At EHA105	pSPB559 (<i>nrpII</i> and Δ 6-fatty-acid desaturase gene)	Kanamycin	Not reported	Transgenic seeds showed resistance to bruchids Transgenic plants Molecular analysis confirmed the stable integration and expression of foreign genes, GFP expression detected in progenies Genomic Southern analysis confirmed stable integration of transgene, RT-PCR indicated the transcription of D6-fatty-acid desaturase gene in transgenic lines, the whole lipids extracted from the leaves and seeds of the T ₀ transgenic plants and the leaves of their T ₁ progenies were identified using GC-MS	Chen <i>et al.</i> , 2005
<i>V. angularis</i> (cv. Beni-dainagon)	Adventitious shoot regeneration from epicotyl	At EHA105	pBI221 (<i>αAI-Pa1</i> or <i>αAI-Pa2</i>)	Kanamycin	Not reported	Transgenic seeds showed resistance to bruchids Transgenic plants Molecular analysis confirmed the stable integration of transgene, RT-PCR indicated the transcription of D6-fatty-acid desaturase gene in transgenic lines, the whole lipids extracted from the leaves and seeds of the T ₀ transgenic plants and the leaves of their T ₁ progenies were identified using GC-MS	Yamada <i>et al.</i> , 2005

<i>V. mungo</i> (cvs. T-9 and RV-19)	Shoot regeneration from mature germinating embryos	PB	pBI221 (<i>gus</i> and <i>nptII</i>)	Kanamycin	Not reported	Putative transformed shoots rooted on kanamycin, no molecular analysis	Bhargava and Smigocki, 1994
<i>V. mungo</i> (cvs. T-9 and RV-19)	Induction of callus from segments of primary leaves	At LBA4404 EHA105	pGA472 (<i>nptII</i>)	Kanamycin	23% (LBA4404) 10% (EHA105)	Integration and expression of <i>nptII</i> in transformed calli confirmed by Southern analysis and neomycin phosphotransferase assay, respectively	Karthikeyan <i>et al.</i> , 1996
<i>V. mungo</i> Pusa-2	Shoot regeneration from cotyledonary nodes	At LBA4404	pCambia2301 (<i>nptII</i> and <i>gus</i>)	Kanamycin	1%	Stable integration and expression of transgenes in T ₀ , inheritance to T ₁ progeny demonstrated	Saini <i>et al.</i> , 2003
<i>V. mungo</i> Pusa-2	Shoot regeneration from shoot apices	At EHA105	pCambia2301 (<i>nptII</i> and <i>gus</i>)	Kanamycin	1–6.5%	Stable integration and expression of transgenes in T ₀ , inheritance to T ₁ progeny demonstrated	Saini and Jaiwal, 2005
<i>V. mungo</i> Pusa-2	Shoot regeneration from cotyledonary nodes	At EHA105	pCambia2301 (<i>nptII</i> and <i>gus</i>)	Kanamycin	4.31%	Stable integration and expression of transgenes in T ₀ , inheritance to T ₁ progeny demonstrated	Saini <i>et al.</i> , 2007

tumefaciens strain EHA105 harboring pBingusint (Jaiwal *et al.*, 2001). Integration of foreign genes was confirmed by Southern analysis. However, the callus failed to regenerate to shoots.

Mahalakshmi *et al.* (2006) recovered transgenic plants of *V. radiata* cv. K-851 from primary leaf explants of 10-day-old seedlings, co-cultured with *A. tumefaciens* strain C58 pCAMBIA1301, on hygromycin selection medium. Molecular analysis confirmed the stable integration and inheritance of the *hpt* gene. However, absence of prolific regeneration from primary leaf explants and generation of escapes on hygromycin-containing medium may restrict the use of these explants for routine introduction of desirable genes to *V. radiata*.

Sonia *et al.* (2007) generated fertile transgenic plants of *V. radiata* from cotyledonary node explants co-cultured with *A. tumefaciens* strain EHA105 harboring a binary vector pKSB that carried bialaphos resistance (*bar*) gene and *Phaseolus vulgaris* α -amylase inhibitor-1 (α AI-1) gene. Transformed shoots were regenerated and rooted on phosphinothricin (PPT)-containing medium. Preculture and wounding of the explants, presence of acetosyringone, and PPT-based selection of transformants played significant role in enhancing transformation frequency. Expression of the *bar* gene in primary transformants were confirmed by polymerase chain reaction (PCR) analysis and PPT leaf paint assay, respectively. Integration of the α AI-1 gene was confirmed by Southern hybridization. Inheritance of both transgenes was confirmed by PCR analysis. Transgenic plants were recovered after 8–10 weeks of co-cultivation with *Agrobacterium* with a frequency of 1.51%.

Bhargava and Smigocki (1994) observed transient expression of *gus* gene in the germinated embryos of *Vigna aconitifolia*, *V. mungo*, and *V. radiata*, 18–24 h after particle bombardment. Bombarding embryos two to three times increased the level of expression up to fourfold in the explants. The plantlets were rooted on a kanamycin selection medium. However, molecular analysis was not carried out to confirm the stable expression of *gus*.

2.2 *Vigna angularis*

Reproducible transformation in azukibean (*V. angularis*) was reported using *A. tumefaciens*-

mediated gene transfer to epicotyl explants (Yamada *et al.*, 2001). Binary vectors containing the neomycin phosphotransferase II gene (*nptII*), and *gus* or the modified green fluorescent protein (sGFP(S65T)) gene were used for transformation. The combined action of acetosyringone and high concentration of benzyladenine (BA) in co-cultivation medium played important roles in improving transformation efficiency in epicotyl explants of azukibean. Further, the *Agrobacterium* strain was found to influence the transformation efficiency. AGL1 and EHA105 showed higher transformation than the strain LBA4404. AGL1 and EHA105 harbor a hypervirulent Ti plasmid and were developed to exhibit a broad host range and high transformation frequency. Molecular analysis confirmed the stable integration and expression of foreign genes. The T₀ plants produced viable seeds.

Ishimoto *et al.* (1996) introduced bean α -amylase inhibitor gene to azukibean (*V. angularis*) and generated transgenic plants resistant to the storage pest, bruchid. The bean α -amylase inhibitor gene was driven by the promoter of phytohaemagglutinin leading to high level accumulation of α -amylase inhibitor proteins in the transgenic seeds and subsequent complete block of bruchid development. The system relied on *Agrobacterium* infection of epicotyl explant and subsequent selection on kanamycin-containing medium.

The D6-fatty-acid desaturase gene isolated from *Mortierella alpina* IS-4 was introduced into *V. angularis* by *Agrobacterium*-mediated transformation and constitutively expressed (Chen *et al.*, 2005). The γ -linolenic acid (GLA) accumulated at levels of up to 0.44% and 1.26% of the total fatty acids in the leaves and seeds, respectively, of a transgenic *V. angularis* line and at 0.69% in the leaves of its progeny. These findings reveal that the modification of the fatty-acid biosynthetic pathway by genetic manipulation in order to produce specific polyunsaturated fatty acids in grain legume crops is a promising technique. The seed germination, hypocotyl culture, and genetic transformation procedures were performed using a modified version of the method of Yamada *et al.* (2001). The *A. tumefaciens* EHA105/pSPB559 vector was used for this transformation. Kanamycin-resistance callus lines were obtained on kanamycin selection after 2 months, which regenerated plantlets. The

adventitious shoots that formed on the induced calli were transferred to a rooting medium with kanamycin. The T₀ transgenic plants and their T₁ progenies grew, flowered, and produced seeds normally. Genomic Southern hybridization analysis confirmed the stable integration of transgene. The D6-fatty-acid desaturase gene was inserted multiple times into the transgenic plant genomes. The RT-PCR results indicated the transcription of D6-fatty-acid desaturase gene in transgenic lines. The fatty-acid compositions of the whole lipids extracted from the leaves and seeds of the T₀ transgenic plants and the leaves of their T₁ progenies were detected by using GC-MS. These findings reveal that modification of the fatty-acid biosynthetic pathway by genetic manipulation in order to produce specific polyunsaturated fatty acids in grain legume crops is a promising technique.

Yamada *et al.* (2005) isolated cDNAs encoding for two isoforms of α -amylase inhibitor, i.e., α AI-Pa1 and α AI-Pa2 from tepary bean (*Phaseolus acutifolius*) and expressed these cDNAs in *V. angularis*, which revealed that active forms of the inhibitor proteins accumulated in seeds and exhibited specificities for insect α -amylases identical to those of the native proteins purified from tepary bean seeds. Seeds transgenic for *ai-Pa1* thus inhibited the larval α -amylase activity of *C. chinensis* but not that of *Zabrotes subfasciatus*, whereas *ai-Pa2* transgenic seeds inhibited the larval α -amylases of both of these insects. Seed extracts of both types of transgenic plants inhibited the larval α -amylases of the cereal storage pests *Tenebrio molitor* and *Tribolium confusum* but had no effect on porcine pancreatic α -amylase. The inhibitory specificities of the transgenic azukibean seeds thus further confirmed that *ai-Pa1* and *ai-Pa2* encode α AI-Pa1 and α AI-Pa2, respectively. The active form of α AI-Pa1 in transgenic azuki bean was a single polypeptide with a size similar to that expected for the full-length encoded protein. These results suggest that α AI-Pa1 defines a new type of bean α AI that is structurally related to lectins and is not activated by proteolytic processing.

2.3 *Vigna mungo*

Urdbean production is severely limited due to its prominent susceptibility to MYMV, fungal

pathogens, and insect pests (Sahoo *et al.*, 2003). MYMV is the most devastating and widespread viral disease, which can cause complete damage to plants when infected at the seedling stage (Varma *et al.*, 1998). None of the known cultivars of *V. mungo* is fully resistant to MYMV. Genetic engineering of *V. mungo* for MYMV resistance is one of the major research mandates.

Karthikeyan *et al.* (1996) obtained transformed calli of *V. mungo* by co-cultivating segments of primary leaves with *Agrobacterium* strains LBA4404 and EHA105 carrying binary vector pGA472. Transformation frequency with LBA4404 and EHA105 was 23% and 10%, respectively. The stable integration of *nptII* gene in transformed calli was confirmed by Southern blot analysis and the expression was demonstrated by neomycin phosphotransferase assay.

The amenability of various seedling explants of *V. mungo* to *Agrobacterium* infection has been evaluated in preliminary transformation studies. Saini and Jaiwal (2002) demonstrated the competence of seedling epicotyl explants for *Agrobacterium*-mediated transformation. A highly site-specific direct shoot regeneration system was developed from the apical end of these nonmeristematic explants.

Saini *et al.* (2003) generated fertile transgenic plants from cotyledonary node explants inoculated with *A. tumefaciens* carrying binary vector pCambia2301 at a frequency of 1%. Mechanical wounding of the explants prior to inoculation with *Agrobacterium*, time lag in regeneration due to removal of the cotyledons from explants, and a second round of selection on kanamycin at the rooting stage were found to be critical for transformation. Southern analysis of T₀ plants showed the integration of *uidA* into the plant genome. Stable GUS activity was detected in leaves, roots, flowers, anthers, and pollen grains. The progenies revealed a Mendelian pattern of transgene inheritance. However, the efficiency of transformation of meristematic cells in the axil of the cotyledonary node regions was low (1%), which may be attributed to the presence of limited number of regenerable cells whose capacity for regeneration was short lived, and inefficient T-DNA delivery to the regenerable cells (Saini and Jaiwal, 2005). An *Agrobacterium*-mediated transformation of shoot apex explants was developed to overcome these limitations (Saini and Jaiwal, 2005). A significant increase in production

of transgenic plants was observed from an average of 1–6.5% when shoot apices were preconditioned with BAP, and wounded prior to their inoculation with *A. tumefaciens* strain EHA105 carrying the binary vector pCAMBIA2301. GUS activity was detected in T₀ shoots and T₁ seedlings. Southern analysis of T₁ plants showed integration of *nptII* into the plant genome. Majority of the T₀ plants transmitted transgenes in Mendelian fashion. Shoot apex is preferred because of the high regeneration potential of the pre-existing meristems, which require minimal tissue culture manipulations for rapid and direct multiple shoot induction in a less genotype independent fashion. *A. tumefaciens* strain EHA105 carrying a binary vector pCAMBIA2301, which contains a neomycin phosphotransferase gene (*nptII*) and a β -glucuronidase (GUS) gene (*uidA*) interrupted with an intron, was used for transformation of *V. mungo* cotyledonary node explants. Various factors such as preculture and wounding of explants, manipulations in inoculation and co-cultivation conditions were found to play a significant role in influencing tissue competence, *Agrobacterium* virulence and compatibility of both, for achieving the maximum transformation frequencies. The stable transformation with 4.31% efficiency was achieved using the optimized conditions. The transformed green shoots that were selected and rooted on medium containing kanamycin and tested positive for *nptII* gene by PCR were established in soil to collect seeds. GUS activity was detected in leaves, roots, pollen grains, and T₁ seedlings. Southern analysis of T₀ plants showed the integration of *nptII* into the plant genome. Saini and Jaiwal (2007) systematically optimized the conditions favorable for enhanced transformation of cotyledonary node explants and generated stable transgenic plants with an overall transformation efficiency of 4.31%. These efficient transformation systems would enable the transfer of desirable genes in future.

2.4 Assessment of Gene Delivery in *Vigna* Species

Agrobacterium-mediated transformation is more directed and stable method for introduction of a transgene due to the preferential mechanism of T-DNA insertion (Zupan and Zambryski,

1997). Owing to the natural recalcitrance exhibited by *Vigna* species to *Agrobacterium* infection, compatibility of plant regeneration system to *Agrobacterium* infection decides the success or failure in transformation.

Majority of the transformation systems developed in these species target cells of the seedling explants, which permit either adventitious regeneration or axillary shoot proliferation. They are more promisingly used for *Agrobacterium*-mediated transformation with a high degree of success and in more or less variety independent manner. The rapid regeneration of fertile plant from this tissue avoiding intermediate callus phase considerably shortens the time required to generate transgenic plants.

The system for *Agrobacterium*-mediated genetic transformation of *V. radiata* and *V. mungo* using cotyledonary node explants from young seedling has been perfected with success rate ranging from low to moderate transformation frequency. Extensive work needs to be initiated for incorporation of genes encoding desirable traits in these two Asiatic *Vigna* species using the perfected transformation system. Similar genetic transformation system targeting pre-existing meristems of young seedling of *V. aconitifolia* may lead to recovery of fertile transgenic plants.

While chances of recovery of true transgenics from dedifferentiated tissue, with a *de novo* regeneration mode, is very high; such tissue neither has been targeted nor adequately applied for *Agrobacterium*-mediated transformation except in *V. angularis* (Ishimoto *et al.*, 1996; Yamada *et al.*, 2001).

Owing to the natural resistance of many plants including legumes to antibiotics (Dekeyser *et al.*, 1990), appropriate antibiotic and its effective concentration for selection of transformants for particular *Vigna* species can only be determined empirically (Babaoglu *et al.*, 2000). The dose of selective agent (lethal or sublethal), time of its application (i.e., more precisely immediately after co-culture or at a much delayed stage), and increments (gradual increasing or decreasing increments) need to be carefully assessed for a particular species and a variety of explants. Judicious choice of selective levels has remained as an important criterion for the recovery of transformed cells at initial stage of screening (Sharma and Ortiz, 2000).

Comparison of various analogs of antibiotics for their ability to select transformants without interference in regeneration may help in deciding the suitable antibiotic for selection of transformants in *Vigna* species. The herbicide-based selection has been efficiently used both at shoot and induction level for recovery of transformants in *V. radiata* (Sonia *et al.*, 2007). Such herbicide-based selection may find useful in providing an efficient selection and enhancement of transformation frequency in related *Vigna* species.

In the case of *Agrobacterium*-mediated transformation, some strains are more effective with a particular species than others (Christou, 1994). Evaluation of strain–cultivar compatibility is an important step in the establishment of a transformation protocol. While the natural virulence of *Agrobacterium* varies and hence their ability to infect *Vigna* species, the virulence of some strains can be improved by the introduction of supervirulent plasmid carrying extra copies of key virulence genes, alongside the binary vector. Use of *Agrobacterium* strain with super binary vectors or binary vector with additional copies of *vir* genes is an important consideration in the transformation of least amenable *Vigna* species.

Vir gene activity and transformation have been shown to be affected by diverse conditions during the infection and co-cultivation stage (Stachel *et al.*, 1986; Vernade *et al.*, 1988; Godwin *et al.*, 1991; Holford *et al.*, 1992; Dillen *et al.*, 1997). These parameters are vital for achieving optimal T-DNA transfer to cotyledonary node explants in *Vigna* species (Jaiwal *et al.*, 2001; Saini and Jaiwal, 2007) although these effects may not consistent across explants and species.

The beneficial effect of preculture/preconditioning of explants on transformation has been reported in *Vigna* species (Sonia *et al.*, 2007) although it is not clear why regeneration of transgenic shoots requires a preculture period, especially in the light of the fact that no preculture period is required for cellular transformation (McHughen *et al.*, 1989). The insertion of T-DNA does not interfere with regeneration because regeneration from transformed cells does occur from precultured explant. During the preculture time, the tissues undergo a physiological and developmental shift to become competent to regenerate; when the T-DNA is inserted after such a period, the recipient cells are already

on the developmental pathway for regeneration. In such cases, if preculture is not allowed, the presence of bacteria of the selection agent perhaps interferes with the expected physiological shift in the cultured cells from nonregenerable to competent for regeneration and leads to failure of transformation.

Direct DNA delivery by particle bombardment is more promising as no specific DNA regions are required for the DNA integration, as in the case of *Agrobacterium*, thus allowing the introduction into the plant genome of only the target genes without any extra redundant plasmid DNA (Fu *et al.*, 2000). The high degree of specificity (severe species specific and frequently cultivar specific) reflected in *Agrobacterium*-mediated transformation has precluded the use of this method. Thus, particle bombardment may be the preferred option for gene introduction into grain legumes, *Vigna* species in particular, circumventing the host specificity of these species to *Agrobacterium* infection.

Shoot apical meristems of mature seeds or whole embryos have been used extensively as target tissues for particle bombardment in legumes (Kim and Minamikawa, 1996, 1997; Aragao *et al.*, 2000) and, with limited success in *V. radiata* (Bhargava and Smigocki, 1994). In majority of the cases, explants from near the shoot apex or the apex itself, have been the targets of choice (Christou, 1997) as apical meristems permit rapid regeneration of complete fertile plant with minimum culture manipulation in a variety-independent manner.

The major problem encountered with organized tissue, shoot apex in particular, is that transformed cells permit proliferation of nontransformed tissue in their vicinity by effectively detoxifying the selective agent. This results in the creation of chimeric tissue, which subsequently gives rise to both transformed and nontransformed plants, the latter being of the overwhelming majority. Although regeneration of intact plants from transformed tissue is not always an easy task, the problem can be overcome by using a highly specific selective agent that effectively mobilize to apical tissues, making selection of these remote tissues much efficient. Using imazapyr for selection of transformed shoots in combination with *ahas* gene, transgenic plants have been successfully recovered from bombarded shoot apices in common bean (Aragao *et al.*, 2000).

Although the use of particle bombardment for transformation of *Vigna* species is still at an infant stage, mostly restricted to transient GUS expression in bombarded tissue (Bhargava and Smigocki, 1994), success in generating transgenics using shoot apex-particle bombardment with subsequent highly specific selection system seems to be practical.

Electroporation-mediated gene transfer system described by Chowira *et al.* (1995, 1996) uses intact nodal meristems and relies on the plant to develop its reproductive structures from the treated apices in a more or less normal fashion. Transformation of intact embryonic explants by electroporation (Dillen *et al.*, 1995) obtained high frequency of transformation with wider applicability to diverse legume species. *In planta* techniques that does not rely on shoot production from tissues cultured *in vitro* are potentially more rapid, avoiding the complexities of cell and tissue culture, and reduce the likelihood of somaclonal variation. *In planta* techniques in conjunction with electroporation may perhaps circumvent the high recalcitrance of *Vigna* species exhibited to transformation.

3. CONCLUSION AND FUTURE PROSPECTS

The importance of *Vigna* species as a cheap source of protein is an important consideration in developing countries as the people in this region derive most of their nutritional calories from grain legumes. Although these crops occupy unique position with regard to their total area of cultivation as well as production, the yield is severely limited due to diseases (viral, fungal, insects, and storage pests) besides considerable yield loss due to drought and salinity.

In view of the severity of insect-pest related grain yield losses and the expectation of only a slow rate of progress by routine conventional breeding, transfer of ideal genes coding for the insect-specific toxins of *Bacillus thuringiensis* (*Bt*), α -amylase inhibitor for storage pests resistance (Chrispeels, 1997), chitinase, glucanase, RIP (ribosome inactivating protein) for resistance to fungal pathogens can check the damage. In the quest for resistant cultivars to multiple stresses, combining several biotechnological approaches such as transgenesis or mutagenesis and marker-

assisted selection (MAS) to pyramid multiple resistance genes appears to be a powerful strategy (Dita *et al.*, 2006). Such an approach was recently achieved in soybean to manage insect resistance, resulting in the enhancement of resistance levels to corn earworm (*Helicoverpa zea*) and soybean looper (*Pseudoplusia includens*) in eight soybean lines in which two major insect-resistance quantitative trait loci (QTLs) and a synthetic *Bt* gene (*cry1Ac*) were combined (Walker *et al.*, 2004).

Drought and salinity prevalent in Southeast Asia considerably limits the yield potential of *Vigna* species. Abiotic stresses generally involve perturbation of various cellular functions and activation of complex metabolic pathways, and are conferred by polygenic traits (Kassem *et al.*, 2004; Lee *et al.*, 2004; Popelka *et al.*, 2004). Polygenic nature together with the lack of good sources of natural tolerance makes conventional breeding strategies difficult to tackle these constraints. In plants, there is a poor understanding of most abiotic stress responses. Hence, successful use of genetic transformation strategies requires a better understanding of physiological and molecular mechanisms associated with these stresses. Recent progress achieved in nonlegume plants supports the potential use of transgenic approaches to produce stress tolerant varieties in *Vigna* (Kasuga *et al.*, 1999; Jiang *et al.*, 2004; Shou *et al.*, 2004; Umezawa *et al.*, 2004). The use of transgenic, mutagenic, and genetic approaches strongly improved the understanding of the genetic and molecular mechanisms of salinity tolerance in plants, and this will help to develop legumes including *Vigna*, with improved tolerance (reviewed in Hasegawa *et al.*, 2000; Apse and Blumwald, 2002). It was found that overexpression of a single-gene controlling vacuolar or plasma membrane Na^+/H^+ antiport protein, in transgenic *Arabidopsis*, tomato, and rapeseed provided them with a high level of salt tolerance under greenhouse conditions (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001; Shi *et al.*, 2003). Similarly, manipulating expression of pea DNA Helicase45 or the glyoxalate pathways confers high salinity tolerance in tobacco (Singla-Pareek *et al.*, 2003; Sanan-Mishra *et al.*, 2005). Although transgenic plants are yet to be examined for salt tolerance in the field, recent genetic advances suggest that there are good prospects for developing transgenic

legumes with enhanced salt tolerance in the near future (Sharma and Lavanya, 2002).

Although *Vigna* species are consumed by millions of people, little research has been carried out on improving their nutritional value. Priority should be given to screening lines for high essential amino acid content, particularly methionine. Strains should be identified that contain lower levels of antinutritional factors and selections should be made to reduce their flatus potential. Quality improvement of these crops with enhanced protein production as well as correct nutritional balance is to be addressed and necessitates transfer of genes encoding methionine-rich proteins.

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Pigeonpea

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1. INTRODUCTION

Pigeonpea is one of the major grain legume crops grown in the tropics and subtropics. It is usually grown in rainfed areas that are prone to drought. Because of its high protein content, pigeonpea forms a significant component of the diet of the vegetarians in the semi-arid tropics (SAT). Genetic improvement of pigeonpea has received considerable attention over the years from plant breeders with the aim of increasing the grain yield and to minimize crop losses due to unfavorable environmental conditions, and attack by various pests and pathogens. Conventional plant breeding coupled with improved farm management practices have led to a significant increase in world pigeonpea production. Conventional breeding of pigeonpea has succeeded in producing short duration varieties and better cultivars with yield improvement, which are being grown in different parts of the world. Pigeonpea shows considerable yield losses due to various biotic and abiotic constraints. Though the wild accessions of pigeonpea are rich source of resistance to these constraints, the introgression of genes conferring resistance or tolerance to these stresses into cultivars is difficult due to cross-incompatibility. Linkage drag of desirable genes with undesirable

genes also complicates such breeding programs. Biotechnological approaches, such as gene transfer for enhanced disease and pest resistance offer opportunities for rapid improvement of pigeonpea. In recent years, biotechnology has emerged as one of the important tools for agricultural research. In concert with traditional plant breeding practices, biotechnology is contributing toward the development of novel methods to genetically alter and control plant development, plant performance, and plant products. Genetic engineering offers a possible solution by lowering the farm level production costs through making plants resistant to various abiotic and biotic stresses and by enhancing the product quality (i.e., by increasing the appearance of end product, nutritional content, or processing or storage characteristics). Therefore, pigeonpea improvement efforts have focused on raising the yield potential, quality characteristics, and resistance to biotic and abiotic stresses depending on the regional requirements of the crop through biotechnological approaches. This review deals with the recent advancements in pigeonpea breeding and emerging transgenic innovations that would play a significant role in the future pigeonpea improvement programs and offer many new opportunities to develop it as a new generation legume food crop.

1.1 History, Origin, and Distribution

There has been a major dispute on the possible origin of pigeonpea. Several conclusions have been made in favor of India given the presence of several wild relatives, the large diversity of the gene pool, ample linguistic evidences, a few archaeological remains, and the wide usage in daily cuisine (Van der Maesen, 1983). Pigeonpea now acclimatized in several tropical countries was cultivated in ancient Egypt, Africa, and Asia since prehistoric times. The name pigeonpea was first reported from plants used in Barbados where it was used as pigeon feed which led to the name “pigeonpea” in 1692 (Van der Maesen, 1986). The major producer of pigeonpea is India with over 100 cultivars, 2.4 million hectares cultivated and 90% of world production.

1.2 Botanical Description

1.2.1 Taxonomy

Pigeonpea, *Cajanus cajan* (L.) Millsp., belongs to the subtribe Cajaninae of the agriculturally most important tribe Phaseoleae under the subfamily Papilionoideae of the family Leguminosae and is the only cultivated food crop of the Cajaninae subtribe (Purseglove, 1988). Lackey reviewed the Phaseoleae as a group and realigned Bentham's classical classification (Bentham, 1837; Bentham and Hooker, 1865) taking into account the genera described since the last century. The tribe Phaseoleae also includes many important crops such as soybean (*Glycine max* L.), common bean (*Phaseolus vulgaris* L.), and mungbean (*Vigna radiata* L. Wilczek) (Young *et al.*, 2003). Pigeonpea has a diploid genome comprising 11 pairs of chromosomes ($2n = 22$) (Greilhuber and Obermayer, 1998). Karyotype analysis of both mitotic and meiotic chromosomes of pigeonpea, various wild species, and their interspecific hybrids indicated high homology and almost complete meiotic pairing.

Among the members of Phaseoleae, Cajaninae is well distinguished by the presence of vesicular glands on the leaves, calyx, and pods. Currently, 11 genera remain under Cajaninae, including *Rhynchosia* Lour, *Eriosema* DC., *G. Don*, *Dunbaria*, *W. & A.* and *Flemingia* Roxb. ex Aiton. The mem-

bers of the earlier genus *Atylosia* closely resemble the genus *Cajanus* in vegetative and reproductive characters. However, they were relegated to two separate genera mainly on the basis of the presence or absence of a seed strophiole. In 1986, Van der Maesen revised the taxonomy of *Cajanus* and merged the two genera under *Cajanus* following systematic analysis of morphological, cytological, and chemotaxonomical data, which indicated the congenicity of the two genera. *Cajanus* is now recognized as having 32 species most of which are found in India and Australia. Wild *Cajanus* species exist mostly as remnants of cultivations and persist in forests in several places. The closest wild relative, *Atylosia cajanifolia* Haines, has been found in some localities in East India and most other *Atylosias* are found scattered throughout India. In Africa, *Cajanus kerstingii* grows in the drier belts of Senegal, Ghana, Togo, and Nigeria. Pigeonpeas occur throughout the tropical and subtropical regions, as well as the warmer temperate regions (as North Carolina) from 30°N to 30°S (Duke, 1981).

Pigeonpea is divided into two botanical varieties “var. *flavus*” and “var. *bicolor*”. The cultivars of var. *flavus* are earlier maturing, have shorter plants with yellow standards, and green glabrous pods, which are light colored when ripe, and are usually three seeded. These are the *tur* cultivars of India, where they are extensively cultivated in the Peninsula. The cultivars of var. *bicolor* are perennial, late maturing, large, bushy plants, with dorsal side of standard red or purple or streaked with these colors, and hairy pods blotched with maroon or dark colored, with 4–5 seeds, which are darker colored or speckled when ripe. These are the *arhar* cultivars of India, which are more extensively cultivated in the north of the country (Purseglove, 1988).

1.2.2 Plant habit and morphology

Among the grain legumes only the pigeonpea has not been subjected to a radical change in life form. Pigeonpea is an erect perennial legume shrub often grown as an annual, attaining height of up to 5 m. Pigeonpea is almost exclusively grown as an annual, in rows and/or mixed with any other crops such as cotton, sorghum, millets, and groundnut, which are harvested several months

prior to pigeonpea. The pigeonpea plants are cut down when most of their pods have ripened, often when green leaves are still present. Spreading forms are preferred for filling the gaps formed by earlier harvested intercrop. Erect cultivars may be useful for intercropping with other crop species of similar duration, but have not proved better than spreading ones.

Pigeonpea leaves are trifoliate, green, and pubescent above and silvery grayish-green with longer hairs on the underside and spirally arranged on the stem. While the pigeonpea seedlings emerge 2–3 weeks after sowing (Duke, 1981), the vegetative growth begins slowly and accelerates at 2–3 months. Flowers occur in terminal or axillary racemes and are 2–3 cm long (Purseglove, 1968), usually yellow, but can be flocked or streaked with purple or red. Pods are flat, usually green in color, sometimes hairy, sometimes streaked, or colored dark purple, with 2–9 seeds/pod. Seeds are, widely variable in color, 6–9 mm in diameter, and weigh 4–25 g/100 seed (Sheldrake, 1984). Roots are thin with a deep-rooting taproot reaching up to 6 ft. (2 m) in depth. This deep-rooting system helps to improve water infiltration into the soil.

1.2.3 Climatic and soil requirements

Pigeonpea needs moist and warm weather during germination (30–35 °C), 20–25 °C during active vegetative growth, and about 15–18 °C during flowering and pod setting; however, at maturity it needs higher temperature of around 35–40 °C. Waterlogging, heavy rains, frost are very harmful for the crop (Chauhan, 1987). Hailstorm or rain at maturity damages the entire crop. The crop may be grown on any type of soil but sandy-loam to clayey-loam soils are supposed to be best. Soil must be very deep, well drained, and free from soluble salts.

1.2.4 Ecology

Pigeonpea is hardy, widely adaptable, and more tolerant of drought and high temperatures than most other crops. It grows best at a soil pH of 5.0–7.0, but tolerates a wider range (4.5–8.4). It grows on acid sands in the Sahel and alkali clays

in India. Ranging from warm temperate moist to wet through tropical desert to wet forest life zones, pigeonpea has been reported to tolerate annual precipitation of 5.3–40.3 dm (mean of 60 cases 14.5 dm), annual mean temperature of 15.8–27.8 °C (mean of 60 cases = 24.4 °C), and pH of 4.5–8.4 (Duke, 1981). The traditional varieties of pigeonpeas grown by farmers in India in the early 1970s were photoperiod sensitive medium- to long-season types.

1.3 Economic Importance

Pigeonpea plays an important role in food security, balanced diet, and alleviation of poverty because it can be used in diverse ways. Pigeonpea seed contains 20–30% protein, is rich in essential amino acids, carbohydrates, and minerals (Faris *et al.*, 1987; Saxena *et al.*, 2002), and is the principal source of dietary protein for an estimated 1.1 billion people, most of whom are vegetarian and poor (Jones *et al.*, 2004). Pigeonpea contains approximately 57.3% carbohydrates in dried seed. The protein and carbohydrate composition of Indian split dal is 22.3% and 57.2%, respectively (Purseglove, 1988). The crop can be described as unique because it is a legume and a woody shrub. Pigeonpea is grown for its seed for human consumption and for income generation by trading surpluses in local and commercial markets. Besides its main use as dal (dry, dehulled, split seed used for cooking), the green seeds are cooked as a vegetable in Africa, Central America, and the states of Gujarat and Karnataka in India, tender pods are cooked whole in Brazil, Thailand, and the eastern islands of Indonesia. Green peas are processed for canning and freezing in Central America and India for export to North America. The seed husks, pod walls, and green leaves are commonly fed to cattle. Branches and stems can be used for making baskets and as fuel. Pigeonpea is also used as a shade crop and wind break (young coffee, forest seedlings), cover crop, or as support (vanilla) (Duke, 1981). It has an inherent ability to withstand environmental stresses (especially drought) due to the fast growing, deep, extensive root system making it one of the most sought after crops in plant introduction trials aimed at bringing new areas under cultivation (Okiror, 1986). The slow growth of the plant above ground during

its early phase offers very little competition to other crops and allows productive intercropping with virtually any crop. It is grown as a sole crop or as an intercrop mixed with cereals (maize, sorghum, pearl millet, finger millet), fiber, and other legume crops (groundnut, soybean) under wide climatic conditions in rainfed low-input agricultural systems. Pigeonpea adapts to different climates and soils except those that are excessively wet or experience frost (Troedson *et al.*, 1990). The effect of pigeonpea on soil fertility has been studied in detail (Ong and Daniel, 1990). It contributes to the C, N, and P economy of the soil (Kumar Rao *et al.*, 1987; Rego and Nageswara Rao, 2000; Fujita *et al.*, 2004), enhancing its performance even under marginal input. Pigeonpea is tolerant to low P supply and acid soils as well as having a high capacity for incorporation of external P into organic P (Fujita *et al.*, 2004). Its critical requirement of P concentration for dry matter production is low compared to other major protein crops like soybean (Adu-Gyamfi *et al.*, 1990). This benefits both the pigeonpea crop and subsequent crops in rotation, thus contributing to increased productivity and soil amelioration (Duke, 1981; Ae *et al.*, 1990). Pigeonpea is also used to restore soil fertility and to prevent soil erosion (Al-Nahidi *et al.*, 2001; Xuxiao *et al.*, 2002). Morton (1976) listed many folk medicinal uses of pigeonpea. Dry roots, leaves, flowers, and seeds are used in different countries to treat a wide range of ailments of the skin, liver, lungs, and kidney. Because pigeonpea is a low-input rainfed crop with characteristics that provide economic returns from each and every part of the plant, its cultivation has a direct bearing on the overall economic and financial well being, and on the nutritional status of subsistence farmers in the Asian subcontinent.

A good crop of 1ha gives about 25–30 quintals of grains and about 50–60 quintals of sticks and 10 quintals of dried leaves in the form of straw. In India, pigeonpea is mainly cultivated by small and marginal farmers, accounting for 85–90% of the world's area under pigeonpea cultivation. Although, in India, there has been a considerable increase in the area under pigeonpea cultivation from 2.18 to 3.82 mha⁻¹, and the production from 1.72 to 2.88 million tons between 1950–1951 and 1996–1997, there was a significant drop in productivity from 780 to 753 kg ha⁻¹ during the same period (AICPIP, 1999). Andhra Pradesh

accounts for 10.2% of area and 4.26% of the pigeonpea production in the country. Globally, pigeonpea has recorded a 43% increase in area since 1970. It is currently grown on 4.3 m ha⁻¹. India is the largest producer with 3.2 m ha, followed by Myanmar (580 000 ha), China (60 000 ha), and Nepal (28 000 ha). In Asia, between 1972 and 2003, pigeonpea recorded 57% increase in area (2.44–3.81 m ha⁻¹) and 61% increase in production (1.72 to 2.77 million tons). In Africa between 1972 and 2003, pigeonpea recorded 66% increase in area (0.26–0.42 m ha⁻¹) and 96% increase in production (0.13 to 0.26 million tons) (<http://www.icrisat.org/PigeonPea/PigeonPea.htm>). Pigeonpea is now reported to be grown in 50 countries of Asia, Africa, and the Caribbean. The current global annual production of pigeonpea is valued at more than US\$ 1700 m (FAOSTAT, 2005).

1.4 Constraints to Pigeonpea Productivity

The production of pigeonpea has remained static over the last several years (Souframanien *et al.*, 2003). The yield on farmers' fields is low due to a number of factors. A large variation is seen in productivity across years as farmers continue to grow their traditional landraces that frequently suffer from several biotic and abiotic stresses due to lack of quality seed. Poor production practices such as low plant densities, low soil fertility, insufficient weeding, and insufficient/inappropriate use of fungicides and herbicides are other constraints. Environmental (frequent droughts, easily erodible soils with poor waterholding capacity) and socio-economic (lack of roads, marketing infrastructure, and exploitation by middlemen) factors also affect productivity. Apart from these, biotic and abiotic are the most important constraints for pigeonpea production and are listed below.

1.4.1 Biotic factors

Biotic stresses due to fungus, bacterial, and viral diseases, and insects pests cause heavy losses in yield of pigeonpea. Fungal diseases involving 45 pathogens are known in pigeonpea and the most important and widespread disease is wilt (*Fusarium udum*), favored by soil temperatures

of 17–20°C. It affects the plants at all stages of its development and in India it causes a loss of 5–20% and in severe case upto 50% loss is observed. *Fusarium* wilt is especially prevalent in India and East Africa, where field losses of over 50% are common (Marley and Hillocks, 1996). Other fungi include: *Cercospora indica* (leaf spot), *Colletotrichum cajanae*, *Corticium solani*, *Diplodia cajani* (stem canker), *Leveillula taurica*, *Macrophomina phaseoli*, *Phaeolus manihotis*, *Phoma cajani*, *Phyllosticta cajani*, *Phytophthora cajani* (stem blight), *Rhizoctonia bataticola*, *Rosellinia* spp., *Sclerotium rolfsii*, and *Uredo cajani* (rust). So far, economic damages by these have been small or negligible, but rust is locally of some importance. Pigeonpea is also attacked by the bacterium *Xanthomonas cajani* that causes leaf spot and stem canker diseases (Kay, 1979). The sterility mosaic disease caused by sterility mosaic virus is being recognized as a serious economic threat as it can cause complete crop failure (Kay, 1979).

Nematodes that are of minor importance are *Helicotylenchus cavevessi*, *H. dihytera*, *H. microcephalus*, *H. pseudorobustus*, *Heterodera* spp., *H. cajani*, *H. trifolii*, *Hoplolaimus indicus*, *Meloidogyne hapla*, *M. incognita acrita*, *M. javanica*, *M. javanica bauruensi*, *Pratylenchus* spp., *Radopholus similis*, *Rotylenchulus reniformis*, *Scutellonema bradys*, *Scutellonema clathricaudatum*, *Trichodorus mirzai*, *Tylenchorhynchus brassicae*, *T. indicus*, *Xiphinema campinense*, and *X. ifacolum*. Damage caused by insect pests is a major constraint on yield in most areas. The podborer, *Helicoverpa armigera*, is commonly regarded as the key pest throughout Africa and Asia. It is particularly damaging on early formed pods. In many parts of India, the podfly, *Melanagromyza obtusa*, takes over as the dominant pest later in the season. In some areas, a newly recognized hymenopteran pest, *Tanaostigmodes*, can also cause extensive pod damage late in the season. Pests, which can be locally or seasonally important, are plume moth (*Exelastis atomosa*), blue butterfly (*Euchrysops cnejus*), leaf tier (*Eucosma critica*), bud weevil (*Ceutorhynchus aspurulus*), spotted pod borer (*Maruca testulalis*), pea pod borer (*Etiella zinckenella*), and bugs (*Clavigralla* spp.). A blister beetle (*Mylabris pustulata*), which destroys flowers, can be a spectacular but localized pest (Kay, 1979).

Thrips (*Frankliniella schultzei*, *Megalurothrips usitatus*) may cause premature flower drop. In general, the determinate (clustering) plants lose more to lepidopterous borers while podfly causes more damage to the later indeterminate cultivars.

1.4.2 Abiotic stresses

Drought, cold heat, and salinity are the abiotic stresses that affect the pigeonpea yield. In India pigeonpea is grown predominantly in the states of Uttar Pradesh, Gujarat, and Maharashtra, which together contribute about 85% of the total growing area and production of India (Muller *et al.*, 1990). More than 51% of the saline soils in India are located in these states (Agarwal *et al.*, 1976). Among cultivated legumes, pigeonpea is classified as moderately sensitive to salinity (Keating and Fisher, 1985). Waterlogging, heavy rains, frost are very harmful for the crop. Hailstorm or rain at maturity damages the entire crop.

1.5 Pigeonpea Breeding

Many traditional breeding tools including selection, hybridization, mutation, and polyploidy, have been employed in pigeonpea improvement and some of them have been successful in improving the crop. Development of extra-early varieties and resistance to drought and waterlogging were identified as important breeding targets. Pigeonpea produced by resource-poor farmers is more vulnerable to attack by disease and insect pests and to abiotic stresses. To combat these stresses usage of pesticides, fertilizers, and irrigation are a common practice. Utilization of such inputs, however, can seriously reduce profitability and threaten the environment, and many pests are not effectively controlled with chemicals. Thus, across farming systems, biotic and abiotic stresses continue to represent the major constraints on subsistence production and economic yield of pigeonpea. Development of cultivars with improved resistance to biotic and abiotic stresses is a primary goal of pigeonpea breeding programs throughout the world. Some of the conventional breeding methods employed in pigeonpea improvement are delineated below.

1.5.1 Wide hybridization

In any crop improvement program, the parents used in the hybridization are generally different varieties of the same species. Hybrids produced from intervarietal crosses possess maximum fitness value and are favored under both nature and domestication. But, in many cases, it may be desirable or even necessary to cross individuals belonging to two different species or genera. Wide crosses or more precisely interspecific and intergeneric hybrids do not occur naturally and are eliminated by the natural forces because of nonviability and/or sterility of the hybrids. In certain crops, plant breeders in the 20th century have increasingly used interspecific hybridization for transfer of genes from a noncultivated plant species to a crop variety in related species. Distant hybridization is mostly aimed at introducing new genetic variability or to achieve a new genomic constitution in such a way that the characters of the parental species are recombined effectively. These possibilities are directly related to the degree of genetic relatedness between the parents, i.e., the closer the genome relationship between the cultivated and the wild species the greater the amount of genetic recombination, and consequently variability. Valuable characters present in wild species that can be utilized in improvement of pigeonpea cultivars and the attempts made to transfer them to cultivars are reviewed hereunder.

Interspecific hybridization in *Cajanus* species dates back to 1956 when Deodikar and Thakur (1956) made the first cross between *C. cajan* with *C. lineatus* and obtained fertile hybrids. Roy and De (1967) obtained a hybrid between *C. cajan* and *C. scarabaeoides*. The prospects of potential gene transfer for pod borer resistance, drought resistance, high fruit set, higher seed protein content, early maturity, etc., from some *Atylosia* and *Cajanus* species have been observed (Pundir and Singh, 1987).

Pundir and Singh (1987) obtained fertile hybrid from the crossings of *Atylosia cajanifolius* with the cultivated varieties, Pant A2 and UPAS 120. Considering the value of the traits, namely, leaf spot resistance, pod fly resistance, seed size, and high methionine content possessed by *Cajanus cajanifolius*, crosses involving this species might throw useful segregants in advanced generations.

Similarly, *Cajanus scarabaeoides* another species of the secondary gene pool, tolerates drought and exhibits mechanical resistance and antibiosis to *Helicoverpa* in its pods. This species can easily be crossed with pigeonpea. The high protein content (28.3%) in some accessions of this species has been transferred to pigeonpea (Saxena *et al.*, 1997). Studies carried out by Dodia *et al.* (1996) revealed that larval and pupal mass of *Helicoverpa* fed on wild pigeonpea flowers and F₁ hybrids of *C. scarabaeoides* and cultivated *C. cajan* were significantly lower than those for larvae fed on the cultivated pigeonpea indicating the scope of transferring of resistance from this wild species to the cultivated pigeonpea. Unfortunately, there has not been much success in crosses between cultivated *C. cajan* and *Cajanus sericeus*. *C. sericeus* is known for high fruit set, 5–6 seeds per pod, drought tolerance, pod borer resistance, and high seed protein content. Ariyanayagam *et al.* (1993) noted poor success when *C. sericeus* was taken as maternal parent of crosses with pigeonpea as it produced male sterile lines having mitochondria of the *C. sericeus*. Another species of secondary gene pool having salinity tolerance is *Cajanus albicans* (Subbarao, 1988). Kumar *et al.* (1985) obtained 7% success, when *C. cajan* was taken as a female in crosses with *C. albicans*. Whereas, Ariyanayagam *et al.* (1993) noted that crosses of *C. albicans* with pigeonpea with latter as male parent resulted in high level of male sterility in F₁ generation (Table 1).

Cajanus acutifolius, a species of Australian native, belongs to the secondary gene pool. It is drought tolerant and crossable with pigeonpea. Fertile seeds were not formed in hybrids when *C. acutifolius* was used as female parent (Ariyanayagam *et al.*, 1993). *Cajanus platycarpus* is the only species of tertiary gene pool that has a great potential donor traits including resistance to *Fusarium* wilt (*F. udum*) and *Phytophthora* blight (*Phytophthora drechsleri* f. sp. *cajani*) diseases, high pod set and large seed size (Pundir and Singh, 1987), and salinity tolerance (Subbarao, 1988). Ariyanayagam and Spence (1978) reported successful hybrids between *Atylosia platycarpus* and pigeonpea, and suggested that genes for earliness and insensitivity to day length could be transferred from *A. platycarpus* in to pigeonpea.

Table 1 Wide hybridization and important traits in wild species of pigeonpea

Wild species	Useful characters	Remarks	References
<i>C. scaraboides</i>	Drought tolerant, mechanical resistance, and antibiosis to <i>Helicoverpa</i> , high protein content (28.3%)	High protein content transferred to pigeonpea, larval and pupal mass reduced when fed on F ₁ hybrids	Dodia <i>et al.</i> , 1996
<i>C. sericeus</i>	High fruit set, 5–6 seeds/pod, drought resistant, pod borer resistant, high protein content (28.6%)	Male sterile hybrids with <i>C. sericeus</i> as maternal parent	Ariyanayagam <i>et al.</i> , 1978; Wanjari, 1998
<i>C. albicans</i>	Sterility mosaic resistant, high seed protein content (28.7%), salinity tolerance	7% success in crosses with <i>C. cajan</i> as female parent, male sterility in F ₁ hybrids with <i>C. cajanus</i> as male parent	Kumar <i>et al.</i> , 1985; Subbarao, 1988; Ariyanayagam <i>et al.</i> , 1978
<i>C. acutifolius</i>	Drought tolerant, sterility mosaic resistant, high seed protein content (28.7%)	Male sterile F ₁ hybrids with both <i>C. cajanus</i> as female and male parent	Ariyanayagam <i>et al.</i> , 1978
<i>C. platycarpus</i>	Resistant to <i>Fusarium</i> wilt and <i>Phytophthora</i> blight, early maturity and annuality, high pod set and large seed size, high protein content (29.3%) salinity tolerance	F ₁ hybrids were produced, F ₁ hybrid with under developed seeds, reciprocal pollination using <i>C. platycarpus</i> as male parent was unsuccessful	Ariyanayagam and Spence, 1978; Saxena <i>et al.</i> , 1996
<i>C. cajanifolius</i>	Leaf spot and pod fly resistant, high methionine content, high protein content (29.2%)	Fertile F ₁ hybrids, semi-fertile F ₁ hybrids with forage potential, high protein content in F ₁ hybrids	Pundir and Singh, 1987

1.5.2 Heterosis breeding

Heterosis is the superiority in the performance of hybrid over both the parents. Commercial exploitation of heterosis in crop plants is regarded as a major breakthrough in the realm of plant breeding. It has led to considerable yield improvement of several cereals and other crops (Rai, 1979). Exploitation of “hybrid vigor” or “heterosis” showed a quantum jump in yields in some cereals and vegetable crops in the past. In legumes, this system could not be exploited for enhancing the productivity, primarily due to their cleistogamous nature of flowers that do not permit economical mass pollen transfer, necessary for large-scale hybrid seed production (Saxena *et al.*, 1997). Pigeonpea, however, is an exception where insect-mediated natural outcrossing up to 70% has been reported (Saxena *et al.*, 1990). Solomon *et al.* (1957) were the first to report hybrid vigor in pigeonpea for yield as 24.5%. The availability

of cytoplasmic male sterility (CMS) in this crop (Tikka *et al.*, 1997; Wanjari *et al.*, 2001; Saxena and Kumar, 2003) has opened up the possibilities of developing commercial hybrids. The discovery of stable male sterility systems, availability of natural outcrossing, and evidence of yield advantage has set a perfect stage for increasing yield through developing high yielding widely adapted hybrids to break the persisting yield plateau in pigeonpea.

Male sterility is the inability of a bisexual flower to produce functional male gamete or viable zygote attributed to nuclear genomic origin (genomic male sterility) (g mst; Ariyanayagam *et al.*, 1993) or to nuclear and cytoplasm factors (cytoplasmic-genetic male sterility, g-c mst). Reddy *et al.* (1978) made the first serious attempt at ICRISAT to search a male sterile system that could be used in hybrid production technology. In pigeonpea, two types of g mst, namely, ms1 (Reddy *et al.*, 1978) and ms2 (Saxena *et al.*, 1983) were found and are governed by single

recessive genes. The inheritance of g-c mst is non-Mendelian, as the transfer of cytoplasm is through the maternal parent. In pigeonpea, natural outcrossing was noticed as early as 1919 (Howard *et al.*, 1919) but its utilization in commercial hybrid breeding program was ruled out (Singh *et al.*, 1974) mainly due to the nonavailability of male sterility. With the identification of g mst and g-c mst commercial utilization of the heterosis in pigeonpea was possible. In pigeonpea, male sterility can be induced through crosses, through chemicals such as streptomycin sulfate (SS), sodium azide (SA), Terramycin, ethylmethane sulfonate (EMS), through γ -rays, and through wide hybridization.

Hybrids produced using genetic male sterile lines were not successful on a commercial scale because of high labor costs and skill requirements among seed producers. This drawback was overcome by CMS-based hybrid production introduced by ICRISAT. The CMS technology involves crossing the wild relative of pigeonpea with the cultivated variety and producing cytoplasmic male sterile plant through backcrosses. The male sterile progeny resulting from this cross is then crossed with other fertile restorer lines, resulting in all fertile offspring. CMS-based hybrids produce 30% more root mass than other varieties, which was a significant achievement in case of pigeonpea as it is a semi-arid crop (Saxena *et al.*, 2005).

Experimental hybrids developed by using CMS lines have demonstrated a yield advantage of over 25% (Saxena, 2004). It is a known fact that for a long-term commercially viable hybrid breeding program both genetic as well as cytoplasmic diversity are essential. For utilizing this new CMS source in a practical hybrid pigeonpea breeding program, the male sterility maintainers need to be identified among the cultivated types and this can be achieved by crossing a number of genetically diverse pigeonpea lines with the male sterile genotypes that should be followed by backcrossing and selection.

The magnitude of heterosis in pigeonpea is more or less similar to those of other crops such as maize, cotton, rice, millet, and sorghum (Saxena, 2004). Heterosis in pigeonpea could be exploited commercially if a grower-friendly mass-scale hybrid seed production technology is developed. So far, four wild relatives of pigeonpea have been successfully used to breed CMS systems

for developing commercial hybrid pigeonpea breeding technology. These are: *C. sericeus* (Benth. ex Bak.) van der Maesen comb. nov. (Saxena *et al.*, 2002), *C. scarabaeoides* (L.) Thou. (Tikka *et al.*, 1997; Saxena and Kumar, 2003), *Cajanus volubilis* Blanco (Wanjari *et al.*, 2001), and *C. cajanifolius* (Haines) Van der Maesen comb. nov. (Saxena, 2004). Very recently, Saxena *et al.* (2005) reported a new source of CMS developed by using the cultivated pigeonpea as the female parent and one of its wild relative *C. acutifolius* as the pollen donor and it is the first report in pigeonpea where CMS has been developed using the cytoplasm of cultivated pigeonpea.

So far four pigeonpea hybrids based on genetic male sterility were released in India and they are as follows:

ICPH 8: The world's first pigeonpea hybrid ICPH 8 (MS Prabhat DT \times ICPL 169) was released by ICRISAT and ICAR in 1991. ICPH 8 was superior to controls UPAS 120 and Manak by 30.5% and 34.2%, respectively.

PPH 4: It was released in 1993 by Punjab Agricultural University (PAU), Ludhiana (Verma and Sindhu, 1995). PPH 4 (MS Prabhat DT \times AL 688) recorded 32.1% higher yield than the best national check, UPAS 120. These early maturing pigeonpea hybrids with high yield potential are highly suitable for pigeonpea-wheat cropping system in the irrigated areas of northern India.

COPH 1 and COPH 2: In 1994, a short-duration hybrid IPH 732 (MST-21 \times ICPL 87109) was released by Tamil Nadu Agricultural University (TNAU), Coimabtoire as COPH 1 that recorded 32% higher yield over control VBN 1 (Murugarajendran *et al.*, 1995). In 1997, TNAU released another pigeonpea hybrid COPH2 (Ms CO 5 \times ICPL 83027), which outyielded COPH 1 and CO 5 by 13% and 35%, respectively.

AKPH 4104 and AKPH 2022: They were released by Punjabrao Krishi Vidhyapeeth, Akola. AKPH 4104, released for central zone, is a short-duration hybrid gave 64% higher yield than control UPAS 120. AKPH 2022 is a medium-duration hybrid released for Maharastra state that recorded 34.9%, 28.2%, and 25.2% more yields than controls BDN 2, C 11, and ICPL 87119, respectively.

At ICRISAT, three CMS lines are being maintained. They are CMS 85010, a short-duration line with determinate growth habit; CMS 88034 is a nondeterminate short-duration type while CMS 13092 has genome from African germplasm and it belongs to long-duration group.

1.5.3 Embryo rescue

Wild relatives of crop plants comprise an important germplasm resource for plant improvement (Davey *et al.*, 1994). Crosses between distantly related plants are generally unfruitful because of the abortion of embryos on the mother plant. These embryos can be precociously excised and cultured *in vitro* (Monnier, 1990). By using this technique, a large number of hybrid plants have been obtained and several genetic characteristics have been transferred in grain legumes, such as hybrids produced in *Arachis* (Bajaj, 1990) and *Glycine* (Grant, 1990).

Mallikarjuna *et al.* (2006) reported successful generation of backcross progeny by the use of *in vitro* techniques and conventional backcross program. This is the first report in pigeonpea where an incompatible wild species from tertiary gene pool, such as *C. platycarpus*, has been successfully crossed with cultivated pigeonpea and fertile hybrids and backcross progeny obtained. When using wild species from the tertiary gene pool, it is usually necessary to use embryo rescue techniques at least once to obtain hybrid plants. But in pigeonpea using embryo rescue method twice, and increasing the ploidy ($2n = 44$) hybrids were produced between the incompatible cross of *C. platycarpus* with cultivated *C. cajan*. The study shows that it is possible to transfer important traits such as resistance to phytophthora blight from *C. platycarpus*, although it is distantly related to cultivated pigeonpea.

2. TRANSGENIC PIGEONPEA

The genetic transformation of pigeonpea plants involves the stable introduction of functional genes into the nuclear genome of cells capable of giving rise to a whole plant. Despite significant advances over the past decade, the development of efficient transformation methods can take many

years of painstaking research. Transformation efficiencies, frequently, are directly related to the tissue culture responses and therefore, highly regenerative cultures are often transformation competent. Direct regeneration is preferred to indirect regeneration as the length of callus phase is negatively correlated with regeneration ability, where the somaclonal variation can also influence phenotype of the regenerated shoots (Fontanna *et al.*, 1993). In pigeonpea only few reports are available on genetic transformation and they are reviewed here.

2.1 Donor Genes

Gene cloning is the process of isolation and multiplication of an individual gene sequence by insertion of that sequence into a bacterium where, it can be replicated. Vectors used in cloning have been specially developed by adding certain features like: reduction in size of vector to a minimum; introduction of selectable markers and synthetic cloning or polycloning sites; incorporation of axillary sequences, etc. The genetic transformation generally involves two genes, namely, transgene that should be integrated in plant genome and expressed in the transgenic plant and the other is selectable marker gene. Each of the two transgenes should thus have their own promoter and termination sequences.

For producing pigeonpea transgenics for insect resistance, Lawrence and Koundal (2001) used the binary vector construct (pCPI) cloned in Bin19 having the cowpea protease inhibitor gene. Similarly, transgenic pigeonpea plants for oral vaccines were produced by using the recombinant binary vector pBI H carrying the *hemagglutinin* gene (*H*) for rinderpest virus and peste des petits ruminants virus (PPRV) under the control of a cauliflower mosaic virus (CaMV 35S) promoter and *nos* polyadenylation sequence, and *nptII* as a marker gene under the control of the *nos* promoter and polyadenylation sequences (Satyavathi *et al.*, 2003; Prasad *et al.*, 2004). Here, the *H* gene was cloned initially in pBI 121 by replacing the *uidA* gene and mobilized to EHA 105 *Agrobacterium* strain. Pigeonpea transformation for disease resistance has been carried out with a disarmed C-58 strain of *Agrobacterium tumefaciens* harboring a binary plasmid pCambia 1302: *RChit* (Kumar

et al., 2004b). Here, a rice chitinase (*Rchit*) gene driven by CaMV 35S promoter and a CaMV 35S poly-A sequence was subcloned into pCAMBIA 1302 vector to produce a pCAMBIA 1302:*Rchit* binary plasmid. It also contained the hygromycin phosphotransferase (*hpt*) gene (used as a selectable marker) and the green fluorescent protein (*gfp*) gene (used as reporter gene) under the control of CaMV 35S promoter and CaMV 35S poly-A sequences.

Surekha *et al.* (2005) used the hybrid endotoxin CryIE-C by replacing 530–587 amino acid residues of CryIEa protein with 70 amino acid region of CryICa in domain III for pigeonpea transformation. The binary vector pPK 202 carried the *cryIE-C* and *nptII* genes driven by CaMV 35S promoter and *nos* polyadenylation sequence. Very recently, Sharma *et al.* (2006a) also reported pigeonpea transformation using *Bt cryIAb* gene cloned into the binary vector pHS723 by blunt end ligation, where CaMV 35S promoter and polyadenylation sequence had driven the *cryIAb* gene. The plasmid also contained fused *uidA* and *nptII* genes as reporter and marker genes, respectively. These genes were driven by double enhanced CaMV 35S promoter and poly-A sequence.

2.2 Methods of Genetic Transformation

Transformation of plants involves the stable introduction of DNA sequences usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. Transformation without regeneration and regeneration without transformation are of limited value. Transformation in pigeonpea has been feasible using both *Agrobacterium* and biolistics mode of transformations but transgenic plant targeting a trait has been produced only using *Agrobacterium* mode of transformation. *A. tumefaciens* is a soil-borne bacterium that has been implicated in gall formation at the wounded sites of many dicotyledonous plants. To date, numerous strains of *A. tumefaciens* have been isolated and characterized, but only a few of these have been modified for use in the transformation of higher plants (Muthukumar *et al.*, 1996; Jaiwal *et al.*, 1998; Yan *et al.*, 2000; Krishnamurthy *et al.*, 2000).

The recent developments in genetic transformation in pigeonpea have emboldened researchers to

pursue the development of transgenic pigeonpea plants resistant to various diseases, and insect pests (Satyavathi *et al.*, 2003; Kumar *et al.*, 2004b; Surekha *et al.*, 2005; Sharma *et al.*, 2006a). In most of the available reports in transformation of pigeonpea (Arundhati, 1999; Geetha *et al.*, 1999; Satyavathi *et al.*, 2003; Kumar *et al.*, 2004a, b), co-cultivation for 48 h in nonselective shoot regeneration medium proved good. However, Sharma *et al.* (2006a) reported that co-cultivation of the axillary meristem explants for 72 h was useful in obtaining a high frequency of stable transformants. The *Agrobacterium* strains that have been successfully used for pigeonpea transformation are based on chromosomal backgrounds of strains LBA4404 and C58 with a wide range of Ti and binary plasmids. Strains such as LBA4404, C58, EHA105, GV3101, and GV2260 and binary vectors like pBI121, pCAMBIA1301, pBAL2, and pHS723 were used in *Agrobacterium*-mediated transformation of pigeonpea (Arundhati, 1999; Geetha *et al.*, 1999; Lawrence and Koundal, 2001; Satyavathi *et al.*, 2003; Prasad *et al.*, 2004; Kumar *et al.*, 2004a, b; Singh *et al.*, 2004; Sharma *et al.*, 2006a, b).

Factors like growth phase of the *Agrobacterium* culture, co-cultivation medium, time period of co-cultivation, temperature during co-cultivation, addition of *vir*-inducing chemicals in the medium, promoters, and explants used etc. effects the *Agrobacterium*-mediated genetic transformation in pigeonpea. Addition of chemicals that induce *vir* genes in co-cultivation media is common practice in genetic transformation of many crop species especially monocots, however, the liberation of phenolic compounds by the explant itself is enough to favor the *Agrobacterium* infection in pigeonpea (Sharma *et al.*, 2006a).

Although, pigeonpea was considered to be recalcitrant for long, recent reports on its genetic transformation with convincing molecular evidence indicates that the *Agrobacterium*-mediated genetic transformation is feasible in pigeonpea. Lawrence and Koundal (2001) have used *Agrobacterium* strain GV2260 carrying the *nptII* and cowpea protease inhibitor genes on a binary vector to infect the embryonal axes of pigeonpea and obtained multiple shoots following callus proliferation on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) (2 mg l⁻¹) and indole 3-acetic acid (IAA)

(0.2 mg l⁻¹) using this method a transformation frequency of less than 1% was obtained. Following this, Satyavathi *et al.* (2003) reported the successful recovery of pigeonpea transgenics for protective *H*-antigen gene (Haemagglutinin gene) through direct regeneration of cotyledonary node and embryonic axis after infecting with the virulent strain EHA105 of *A. tumefaciens* that harbored the binary plasmid pBI121 carrying the *H* antigen of rinderpest virus and *nptII* genes. The bacterial culture at late log phase with an O.D. 0.6 at 260 nm and a co-cultivation period of 48 h was used in this study. Similarly, Prasad *et al.* (2004) produced transgenics expressing the HN protein of PPRV through *Agrobacterium*-mediated genetic transformation of cotyledonary node explants of pigeonpea. Kumar *et al.* (2004a) reported stable transformation of pigeonpea by using cotyledonary nodes from *in vitro*-grown seedlings of pigeonpea. Co-cultivation of the explants with strain C58 of *A. tumefaciens* harboring the binary plasmid pCambia1301 carrying *uidA* and *nptII* as reporter and selectable marker genes, respectively and rice chitinase gene as the candidate gene at late log phase with an O.D. of 0.6 and co-cultivation for 48 h resulted in stable transformation with 45% transformation efficiency. Pigeonpea transgenics resistant to *Spodoptera litura* were developed by Surekha *et al.* (2005) through direct regeneration of embryonal axis after infecting with the virulent strain GV2260 harboring a modified binary vector pPK202 carrying a synthetic *cryIE-C* gene under a constitutive 35S promoter and the marker gene neomycin phosphotransferase II (*nptII*). A co-cultivation period of 72 h was reported for transformation with *Agrobacterium* and obtained a transformation efficiency of 15%. More recently, Sharma *et al.* (2006a) reported the recovery of pigeonpea transgenics for insect resistance through direct organogenesis of axillary bud following 72 h co-cultivation with *A. tumefaciens* strain C58 harboring the binary plasmid pHS 723 having codon-optimized *cryIAb* and fused *nptII* and *uidA* genes.

2.3 Selection of the Transformed Tissues

A successful gene transfer does not guarantee stable integration and expression of the foreign gene, even by using signals for the regulation of

transgene expression. Antibiotic resistance genes allow the transformed cells expressing them to be selected from the populations of nontransformed cells. The population of cells that has been transformed and expresses a resistance gene is able to neutralize the toxic effect of the selective agent, either by detoxification of the antibiotic through enzymatic modification (Evans *et al.*, 1996; Joersbo *et al.*, 1998; Wang *et al.*, 2000; Jaiwal *et al.*, 2002) or by evasion of the antibiotic through alteration of the target (Jaiwal *et al.*, 2002). Effectiveness of a particular antibiotic resistance system depends mainly on elements such as, selective agent, explant used, and selectable marker genes. Amongst the most widely used antibiotic resistance genes as selectable markers, neomycin phosphotransferase II (*nptII*; Bevan *et al.*, 1983; Herrera-Estrella *et al.*, 1983) and hygromycin phosphotransferase (*hpt*; Van den Elzen *et al.*, 1985; Waldron *et al.*, 1985) are most common. Other selectable marker genes like gentamycin acetyltransferase (*accC3*) resistance, bleomycin and phleomycin resistance have also been employed, but are not used for routine transformations (Roa-Rodriguez and Nottenburg, 2003).

In pigeonpea so far only *nptII* and *hpt* genes have been employed for selecting the transgenic plants from untransformed ones. Concentration of the kanamycin used for selection of the transformants varied based on the explant used and mode of regeneration. For effective selection of transformants 50 mg l⁻¹ kanamycin in the medium was reported by Lawrence and Koundal (2001) and Satyavathi *et al.* (2003). Whereas, 75 and 125 mg l⁻¹ kanamycin concentrations were reported by Surekha *et al.* (2005) and Sharma *et al.* (2006a), respectively. Similarly, 5 mg l⁻¹ hygromycin as selection pressure for selecting the transgenics was reported by Kumar *et al.* (2004b).

2.4 Regeneration of Whole Plants

Recent advances in plant tissue culture techniques have been exploited *in vitro* regeneration of pigeonpea plants. Like any other crop, genetic engineering of pigeonpea requires reproducible tissue culture protocols. Though pigeonpea is a recalcitrant crop, ample reports on its regeneration through tissue culture are available (Kumar *et al.*,

1983, 1984; Shivaparakash *et al.*, 1994; Mohan and Krishnamurthy, 1998; Geetha *et al.*, 1999; Singh *et al.*, 2002; Dayal *et al.*, 2003; Thu *et al.*, 2003; Sharma *et al.*, 2006a). Regeneration in pigeonpea is by development of shoot buds by organogenesis from areas surrounding a meristem such as cotyledonary nodal meristem (Mehta and Mohan Ram, 1980; George and Eapen, 1994; Geetha *et al.*, 1998) and rarely through somatic embryogenesis (Patel *et al.*, 1994). However, only a few protocols could be successfully utilized in genetic transformation studies (Lawrence and Koundal, 2001; Satyavathi *et al.*, 2003; Kumar *et al.*, 2004b; Surekha *et al.*, 2005; Sharma *et al.*, 2006a, b).

Direct regeneration systems have advantages, due to the rapidity of morphogenesis and no requirement of frequent subculture. Besides, *de novo* production of shoot primordial is extremely rapid and initially synchronous with the period of cellular differentiation. Such a regeneration system favors easy accessibility for *Agrobacterium*-mediated genetic transformation in pigeonpea. Lawrence and Koundal (2001) reported indirect regeneration from callus obtained from the embryonal axes when cultured on MS medium fortified with 2 mg l⁻¹ BAP. This callus was used for infecting with *Agrobacterium* strain GV2260 containing the *CPTI* gene. For shoot initiation and development, the infected calluses were transferred to MS basal medium with 2 mg l⁻¹ BAP, 0.2 mg l⁻¹ IAA, and 50 mg l⁻¹ kanamycin. Rooting of the regenerated shoots was accomplished on half-strength MS medium containing 2% sucrose, 0.7% agar, and 0.5 mg l⁻¹ IBA (indole 3-butyric acid). However, this system resulted in a transformation efficiency of less than 1%.

Direct organogenesis from cotyledonary node and embryonic axes was obtained by culturing on MS media fortified with 8.87 µM BAP for initiation of shoot buds and 2.22 µM BAP for shoot elongation (Satyavathi *et al.*, 2003). And for rooting MS media supplemented with 1.48 µM IBA was used. Selection was applied after two days of co-cultivation with the *Agrobacterium* culture containing the *H*-gene. Using this method they obtained transformation frequency of 67% in cotyledonary node explants and 51% in embryonal axes, which was calculated as the number of explants survived the selection pressure. The same protocol was used by Prasad *et al.* (2004)

in producing pigeonpea transgenics expressing heamagglutinin- neuraminidase gene.

Kumar *et al.* (2004a) reported direct regeneration from cotyledonary nodal explants on MS medium supplemented with 2 mg l⁻¹ BAP. Further elongation of these shoots was obtained by culturing on MS medium supplemented with 0.5 mg l⁻¹ GA₃ (gibberellic acid) and rooted on root induction medium consisting of MS medium with 1 mg l⁻¹ IBA. Rooted plants were transferred to pots containing a 1:1 mixture of sand and soil and incubated for 1 week for acclimatization (by covering with a plastic bag initially and gradually exposing the plant to the open environment) prior to transfer to a glasshouse, thus obtaining a transformation frequency of 42.5%. Surekha *et al.* (2005) achieved regeneration from embryonal segments when cultured on MS medium fortified with 2 mg l⁻¹ BAP. The same medium was used for further development of shoots from embryonal axes after co-cultivation with *Agrobacterium* containing the *cryIE-C* gene. Elongation of the developed shoots was obtained by culturing on MS medium fortified with 1 mg l⁻¹ BAP, 3 mg l⁻¹ GA₃, and 0.1 mg l⁻¹ NAA (α-naphthaleneacetic acid). Elongated kanamycin (75 mg l⁻¹) resistant shoots were subsequently rooted on MS medium supplemented with 1.0 mg l⁻¹ NAA and later transferred to sterile vermiculite for hardening followed by transfer to the transgenic green house.

Recently, a highly efficient and genotype-independent direct organogenesis from the meristematic tissue developed from the axillary bud region of *in vitro*-grown seedlings of pigeonpea has been reported by Sharma *et al.* (2006a) by culturing the meristematic tissue on MS medium supplemented with 22.0 µM BAP for induction and development of shoot buds. Selection and enrichment of the transformed cells was initiated by applying a selection pressure of 125 mg l⁻¹ kanamycin in the media after 2 weeks of infection with *Agrobacterium*. For elongation developed shoots were transferred to Shoot elongation medium containing MS with 0.5 µM GA₃. Rooting of the elongated shoots were obtained by giving pulse treatment with 25 µM IAA and culturing on plane MS medium with 1% sucrose (Figure 1a–k). Using this method 60% transformation efficiency was obtained that was calculated based on the positive gene integration.



Figure 1 Regeneration of multiple shoots from leaf explants derived from *in vitro* germinated seedlings of pigeonpea, *Cajanus cajan* L. (a) Seeds showing germination on MS medium after 7 days of culture, (b) five-day-old leaf explants on MS medium supplemented with 5.0 μM BAP and 5.0 μM kinetin (shoot induction medium (SIM)) after carefully cutting and removing the preformed meristematic region, (c) swelling of the petiolar region after 5 days resulting in the induction of adventitious shoot buds, (d, e) leaf explant with half-cut lamina showing multiple shoot initiation and development on reduced SIM (MS+ 2.5 μM BA+ 2.5 μM kinetin), (f) multiple shoot formation after 21 days of culture from the petiolar cut end, (g) explant with elongated shoot in shoot elongation medium (SEM) containing MS supplemented with 0.58 μM GA₃, (h) elongated shoot after pulse treatment with IAA (11.2 μM) showing rooting on root induction medium (RIM), (i) well-developed plantlet just before its transfer to small pots, and (j, k) fully established healthy seedlings transferred into bigger pots (13 in.) containing sand:soil mixture.

2.5 Testing of Transgenic Plants

Selection and growth of plant cells on selective media provide initial phenotypic evidence for transformation. After selection, the putative transgenic shoot is propagated *in vitro* followed by rooting and transfer to the containment glasshouse for further evaluation and production of seeds from subsequent sexual generations. Molecular evidences are essential to further confirm the integration and expression of transferred genes followed by genetic characterization (Birch, 1997).

Transgenes are expected to behave as dominant genes due to their hemizygous state in recipient genome and thus segregate as dominant loci in a typical 3:1 Mendelian ratio (Campbell *et al.*, 2000). Successful genetic transformation of any plant involves not only the production of primary transformants showing stable expression of inserted gene but also the inheritance of introduced trait. Skewed segregation of the introduced genes, during meiosis leading to non-Mendelian inheritance may be caused due to various reasons, such as linkage to a recessive lethal gene, mutational effect of transfer DNA (T-DNA) insertion, and chromosomal rearrangement.

Satyavathi *et al.* (2003) were successful in producing oral vaccines in pigeonpea for rinderpest virus (RPV) disease in cattle by using surface glycoprotein *H*-gene of RPV. Presence and the expression of the candidate and marker genes were confirmed by polymerase chain reaction (PCR) analysis and reverse transcriptase-PCR (RT-PCR) analysis, respectively. Southern hybridization proved the integration of the genes with one to more than three copies of the gene. Expression at the protein level was confirmed by Western analysis and enzyme-linked immunosorbent assay (ELISA) for H-protein and was reported as 0.12–0.49% of the total soluble leaf protein in pigeonpea. Transgenic plants were analyzed till T₁ generation and observed 3:1 ratio of segregation of the genes. Oral vaccines were also produced in pigeonpea for another cattle disease, Peste des petits ruminants (PPR) by Prasad *et al.* (2004) using *HN* gene. Presence and integration of the transgene was confirmed by PCR and southern analysis and the protein expression through Western blot analysis.

Kumar *et al.* (2004b) produced pigeonpea transgenics with rice chitinase gene. The plants

were characterized by PCR and RT-PCR analysis for the confirmation of presence and expression of the gene. Plant DNA isolated was also subjected to southern hybridization for copy number and it varied from one to four. Plants were advanced to T₁ generation and the inheritance pattern followed Mendelian segregation.

Surekha *et al.* (2005) reported the molecular characterization of the produced pigeonpea transgenics using PCR and Southern blot analysis for *cryIE-C* gene. Protein analysis was carried out by Western blot analysis and the inheritance pattern followed Mendelian inheritance in T₁ and T₂ generations. Bioassays were performed using first and second instars of *S. litura* in T₁ and T₂ generations, which showed a varied response (60–80% mortality) for resistance to *Spodoptera*. Besides, Sharma *et al.* (2006a) produced pigeonpea transgenic plants resistant to *H. armigera* using *cryIAb* gene. PCR, Southern, and RT-PCR analysis were used for confirming the presence, integration, and expression at RNA level. Southern hybridization data had shown one to two copies of the integrated gene in the transgenic plants. All plant parts were used for ELISA analysis to check the expression of the *cryIAb* gene. The transgene protein level reported was as high as 0.1% in flowers and as low as 0.025% in leaves. The segregation of the genes were tested till T₃ generation and showed Mendelian inheritance (3:1). In all the available reports of pigeonpea transgenics (Table 2) there are no indication of adverse effects of genetic transformation methods on the growth, yield, and quality of the transgenic plants.

2.6 Regulatory Measures

In pigeonpea, no reports are so far available on commercially grown transgenics. At ICRISAT, transgenic pigeonpea plants carrying either the *cryIAb* (unpublished data) or the *cryIAc* (Sreelatha, 2006) genes were evaluated under contained field conditions with the approval of the Institutional Biosafety Committee (IBSC) of ICRISAT and the Department of Biotechnology, Government of India. However, these transgenic events are still under evaluation and validation.

Table 2 List of transgenic plants produced in pigeonpea

Explant	Mode of regeneration	Genes used	Mode of transformation	<i>Agrobacterium</i> strain used	References
Leaf disks	Direct organogenesis	<i>nptII</i> and <i>uidA</i>	<i>Agrobacterium</i>	LBA 4404	Arundhati, 1999
Shoot apices and cotyledonary node	Direct organogenesis	<i>nptII</i> and <i>uidA</i>	<i>Agrobacterium</i>	LBA 4404	Geetha <i>et al.</i> , 1999
Embryonic axes	Callus	<i>CPTI</i> , <i>nptII</i>	<i>Agrobacterium</i>	GV 2660	Lawrence and Koundal, 2001
Embryonic axes and cotyledonary node	Direct organogenesis	<i>H</i> -gene and <i>nptII</i>	<i>Agrobacterium</i>	EHA 105	Satyavathi <i>et al.</i> , 2003
Leaf	Direct organogenesis	<i>nptII</i> and <i>uidA</i>	Biolistics	–	Dayal <i>et al.</i> , 2003
Cotyledonary node	Direct organogenesis	<i>nptII</i> and <i>uidA</i>	Biolistics	–	Thu <i>et al.</i> , 2003
		<i>H-N</i> gene and <i>nptII</i>	<i>Agrobacterium</i>	GV3 101	Prasad <i>et al.</i> , 2004
		<i>hpt</i> and <i>uidA</i>	<i>Agrobacterium</i>	C58	Kumar <i>et al.</i> , 2004a
		<i>R chit</i> , <i>nptII</i> and <i>uidA</i>	<i>Agrobacterium</i>	C58	Kumar <i>et al.</i> , 2004b
Shot apices	Direct organogenesis	<i>hpt</i> and <i>uidA</i>	<i>Agrobacterium</i>	C58	Singh <i>et al.</i> , 2004
Embryo axis	Direct organogenesis	<i>cryIE-C</i> and <i>nptII</i>	<i>Agrobacterium</i>	GV 2260	Surekha <i>et al.</i> , 2005
Axillary meristem	Direct organogenesis	<i>cryIAb</i> and <i>nptII</i>	<i>Agrobacterium</i>	C58	Sharma <i>et al.</i> , 2006a

3. FUTURE ROAD MAP

3.1 Expected Products

The majority of protein food in India comes from pulses, grown invariably under unfavorable growing conditions that result in low productivity. Pigeonpea suffers from damage caused by biotic and abiotic stresses and due to these constraints it had a low compound growth rate of 0.8% in production between 1950 and 2004 (Ahlawat *et al.*, 2005). To combat the biotic stresses farmers rely on application of insecticides. Unfortunately, chemical control of insect pests is under increasing pressure due to environmental degradation, adverse effects on human health and other organisms, eradication of beneficial insects, and development of pesticide resistant insects. Improvement of pigeonpea through conventional breeding methods was slow though its wild varieties were rich source for insect resistance. Together with improved techniques for plant genetic analysis and engineering, concepts of exploiting transgenic

plants have gained increasing scientific and economic importance. Even transgenic technology application in improving this crop has started in recent past as it was said to be a recalcitrant crop. Quality of pigeonpea in terms of nutrition, resistance to biotic and abiotic stresses has to be further probed ahead. Modern biotechnological tools in combination with traditional technologies hold great promise for augmenting agricultural productivity in quantity as well as quality. Here under are some of the important traits that can be improved in pigeonpea as well as extended in producing functional recombinant proteins using transgenic technology.

3.1.1 Insect resistance

Insect pest menace is the major factor that destabilizes crop productivity in agricultural ecosystems. A survey conducted among plant breeders, pathologists, and entomologists shows that breeding for resistance to insect pests is at the top of their priority list for many important crops

(Ranjekar *et al.*, 2003). Insect pest management by chemicals has brought about a considerable protection to crop yields over the past five decades. The pod borer, *H. armigera* is commonly regarded as the key pest throughout Africa and Asia on pigeonpea. It is particularly damaging on early formed pods. In many parts of India the podfly, *M. obtuse*, takes over as the dominant pest later in the season. In pigeonpea, the losses due to *Helicoverpa* have been estimated at US\$ 317 million in the SAT, and possibly over US\$ 2 billion on different crops worldwide annually (Sharma *et al.*, 2001). Losses due to pod fly damage have been estimated to be US\$ 256 countries in Asia, Africa, and the Caribbean for food, million annually (ICRISAT, 1992). To overcome these losses, farmers resort to excessive use of pesticides. Crop surveys have indicated that before 1975, only 20% of the pigeonpea farmers were using insecticides, but by 1993, 100% of the farmers have adopted the use of chemicals to control *H. armigera* in India. In pigeonpea, one larva per plant reduces 4.95 green pods, 7.05 dry pods, 18.01 grains, 3.79 g pod weight and 2.05 g grain weight. Wild varieties are a precious source of resistance for insects attack in pigeonpea but genetic improvement of pigeonpea has been restricted due to the nonavailability of better genetic resources and strong sexual barriers with the wild species.

Insect resistant plant varieties, using δ -endotoxins of *Bacillus thuringiensis* (*Bt*), have been produced in several important crops such as tobacco, tomato, cotton, rice, brinjal, maize, broccoli, oilseed rape, soybean, walnut, poplar, sugarcane, apple, potato, groundnut, sweetpotato, chickpea, alfalfa, etc. (Hilder and Boulter, 1999; James, 2002; Sharma *et al.*, 2004). Of the US\$ 10 billion spent annually on insecticides worldwide, it has been estimated nearly US\$ 2.7 billion could be substituted with *Bt*-based biotechnology applications (Krattiger, 1997). There is significant increase in global area under transgenic crops from 1.7 million hectares in 1996 to 90 million hectares in 2005, in which *Bt* crops share was 25% of the total area (James, 2005).

Since the first report on the introduction of *Bt*-derived *cry* genes into tobacco (Barton *et al.*, 1987) and tomato (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987), there has been a rapid increase in the transformation of other crop plants to achieve resistance against insect pests and were successful (Stewart *et al.*, 1996; Nayak *et al.*, 1997; Adamczyk

et al., 2001a, b; Sanyal *et al.*, 2005). At least 10 different genes encoding different *Bt* toxins, namely, *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ba*, *cry1Ca*, *cry1H*, *cry2Aa*, *cry3A*, *cry6A*, and *cry9C* have been engineered into different crop plants (Schuler *et al.*, 1998). All these transgenics showed resistance to the respective pests. These results show that *Bt* gene is an efficient insecticidal gene that can be deployed for producing transgenic pigeonpea plants for pest resistance.

In pigeonpea, though transgenic plants with *Bt* and *CPTI* genes are available to combat the insect pest *H. armigera* more events with high expression need to be produced. Apart from *Bt* and protease inhibitor genes, insecticidal chitinase also been shown to be important in controlling the devastating pest, *Helicoverpa*, by dissolution of the chitin, an insoluble structural polysaccharide that occurs in the exoskeleton and gut lining of insects. Gene pyramiding with two different insecticidal genes and tissue-specific expression to reduce the risk of developing insect resistance are other attractive options to combat this pest and for durable resistance.

3.1.2 Fungal resistance

Fungal diseases involving 45 pathogens are known in pigeonpea and the most important and widespread disease is wilt (*F. udum*), favored by soil temperatures of 17–20 °C. It affects the plants at all stages of its development and in India it causes a loss of 5–20% and in severe case upto 50%. Recombinant-DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant (Keen, 1999) or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms (Bent and Yu, 1999; Rommens and Kishmore, 2000). Transgenic pigeonpea for wilt resistance using rice chitinase gene has developed and are being evaluated for resistance against wilt at ICRISAT. But still much work has to be further done to develop transgenics with high expression of the gene.

3.1.3 Virus resistance

Viral diseases affect worldwide productivity of the economically important crops. In pigeonpea

the sterility mosaic disease considered as “green plague of pigeonpea” caused by sterility mosaic virus (SMD) is being recognized as a serious economic threat as it can cause complete crop failure if occurs early in the season. SMD infection at an early stage (<45 days old plants) results in a 95–100% loss in yield (Kannaiyan *et al.*, 1984; Reddy *et al.*, 1990), while losses from late infection (>45 days old plants) depend on the level of infection (i.e., number of affected branches per plant) and range from 26% to 97% (Kannaiyan *et al.*, 1984). Often the most significant weapons against viral diseases are cultural controls (such as removing diseased plants) and plant varieties bred to be resistant (or tolerant) to the virus, but they may not always be practical or available. There are mainly two approaches for developing genetically engineered resistance depending on the source of the genes used. The genes can be either from the pathogenic virus itself or from any other source. Substantial yield increase that was observed in field trials in some transgenic crop plants has clearly established the reliability of coat protein-mediated resistance (CPMR) as the most favored strategy to engineer resistance against many viruses. Apart from CPMR transgenics with replicase protein-mediated resistance is commonly produced for viral resistance and could be an option to develop pigeonpea transgenics with resistance to SMD.

3.1.4 Abiotic stresses

Drought, cold, heat, and salinity are the abiotic stresses that affect the pigeonpea yield. Besides, waterlogging, heavy rains and frost are very harmful for the crop. Hence, improvement of pigeonpea for tolerance to these abiotic stresses is very important as it increases the harvest index and ultimately the yield.

Though pigeonpea is classified as moderately sensitive to salinity (Keating and Fisher, 1985), development of salt-tolerant variety could be useful for Indian farmers as it is grown predominantly in the states of Uttar Pradesh, Gujarat, and Maharashtra, where more than 51% of the saline soils in India are located (Agarwal *et al.*, 1976). Moreover these states contribute about 85% of the total growing area and production of pigeonpea in India (Muller *et al.*, 1990). Also, pigeonpea is sensitive to photoperiod and temperature. Low temperatures

affect the short duration variety whereas high temperature and photoperiod affects the yield in medium and long-duration variety rendering them to terminal drought. In cool areas, maturity in long-duration pigeonpea is accelerated and severe competition occurs between intercropped maize whose maturity is delayed and pigeonpeas resulting in yield reduction of both crops.

Importance of transgenic technology in improving salinity tolerance has been already proved in *Arabidopsis* by overexpressing *SOS1* gene by limiting Na⁺ accumulation in plant cells (Shi *et al.*, 2003). The overexpression of a *AtNHX1* and *H⁺-PPiase* genes in *Arabidopsis* (Apse *et al.*, 1999; Gaxiola *et al.*, 2001) and *AtNHX1* gene in tomato and canola resulted in transgenic plants that were able to grow in high salt concentrations (Zhang and Blumwald, 2001; Zhang *et al.*, 2001). It is proven that membrane lipids hold the key for improvement of photosynthesis under low temperature and high temperature stress conditions (Grover *et al.*, 2000). Betaine has been shown to accumulate in response to low and high temperature stress in higher plants, where it might play a role in protecting membranes and/or protein complexes (Zhao *et al.*, 1992; Yang *et al.*, 1996). Introduction of choline dehydrogenase (*CDH*) gene that encodes betaine in tobacco resulted in low temperature tolerance a part from salt tolerance. Similarly, catalase in rice (Tanida and Saruyama, 1995; Tanida, 1996), *codA* gene in *Arabidopsis*, cDNA (complementary DNA) of chloroplast enzyme glycerol-3-phosphate acyltransferase in tobacco, and *Arabidopsis* have proven to impart resistance to various abiotic stresses including chilling and high temperatures. However, no transgenics are reported so far in pigeonpea for these traits. These results prove the feasibility of producing transgenics in pigeonpea for various abiotic stresses, which need to be improved for getting high yield varieties.

3.1.5 Biofortification

The efficiency of productivity depends on total nutrient content in the seed that meets the need of the population with minimal waste. Greater attention needs to be given to the pigeonpea to improve amino acids profiles, in particular to improve the level of sulfur-containing amino acids and to eliminate antinutritional factors.

Dissection of biosynthetic pathway and introducing appropriate genes or engineering entire biochemical pathway is generally applied for increasing the nutritional value of the crop. With similar strategies it should be possible to achieve similar increases in pigeonpea plants. Besides enhancing the nutritional value, transgenics produced for micronutrient deficiency such as Vitamin E can also produce antioxidants for industrial applications.

Biotechnology and genetic modification techniques are being optimized for the production and development of healthy foods, and improvement in the levels and activity of biologically active components in food plants (phytochemicals). The production of increased levels of β -carotene (provitamin A) in plants is especially important, as its precursor, lycopene has been shown to have physiological chemopreventive effects with regard to various cancers (Yan and Kerr, 2002). Furthermore, lycopene, commonly found in various carotenoids containing plants, such as tomatoes and carrots, is an essential ingredient in maintaining eye health and vision. β -carotene, α -carotene, β -cryptoxanthin are carotenes that are converted into Vitamin A or retinol in the body. Pigeonpea occupies an important place in human nutrition as a source of dietary proteins in several countries. Work has been initiated at ICRISAT in developing pigeonpea transgenics by using the phytoene synthase gene (*psy1*) that converts geranylgeranyl pyrophosphate to phytene. Increase in the phytene content during the biosynthetic pathway of carotenoids in turn increases the β -carotene level, which is a precursor of Vitamin A. Success in producing transgenic pigeonpea plants with high-level expression of phytene synthase will have much to contribute to the malnourished population. Besides, pigeonpea contains the lowest amount of limiting sulfur amino acids, methionine, and cysteine among all important food legumes, implicating the importance of these amino acids in its protein quality improvement program. The functional importance of dietary methionine and cysteine lies in the intestinal growth and function, beyond its role as a precursor for protein synthesis. Cysteine has a key role in cellular antioxidant function, which is a determinant of cell proliferation and survival. Promoting work in this direction could be helpful in increasing the protein quality of the pigeonpea. With this in view, production

of pigeonpea transgenics with *SSA* (sunflower seed albumin) gene that encodes methionine has been initiated at ICRISAT. Apart from this, considerable attention needs to be given to improve the quality of pigeonpea protein by reducing the polyphenolic compounds that are present in abundance that subsequently affects the activity of digestive enzymes.

3.1.6 Hybrid seed production

For hybrid seed production availability of cytoplasmic male sterile lines and restorer lines are necessary. The development of these lines through conventional breeding is a slow process that minimally requires several years of effort. In pigeonpea, using wide hybridization it was possible to produce CMS line and by screening large accessions, restorer lines were identified and were used to produce hybrids, which performed well in terms of biomass production and abiotic stress tolerance. But hybrid seed production was not to the expected level due to the high cost, poor in-built insect resistance, and management. Development of transgenics for CMS is possible as is the case in tobacco (Mouras *et al.*, 1999) where male sterility was induced by transferring the *u-atp9* (ATPase) gene. Also the development of transgenics by the introduction of transgenes in to the isogenic lines or in to the chloroplast of CMS line already available could reduce the risk of transgenes effect on pollinators such as honeybee and also could restrict the gene flow. Transgenic maintainer lines can be further utilized in enhancement of hybrid seed production with desired characters and also for development of inbred varieties.

Chloroplast engineering has been proposed as a safer approach to the containment of transgenes as chloroplasts are not transmitted in the pollen of flowering plants (Daniell and Khan, 2003; Ruiz and Daniell, 2005).

3.2 Addressing Risks and Concerns

The ethical dilemmas associated with the introducing of transgenic crops in the farming systems have divided both public and private researchers worldwide (Ortiz, 1998). This was not unexpected because the adoption of a new technology has

been always subject to distinct vision and ethical perspectives (Crouch and Ortiz, 2004). In times of severe global decline of biodiversity, proactive precaution is necessary and careful consideration of the likely expected effects of transgenic plants on biodiversity of plants and insects is mandatory (Wolfenbarger and Phifer, 2000; Velkov, 2001, 2003a, b).

Transgenic crops represent a powerful and profitable extension of conventional breeding methods in pigeonpea. However, challenge is to use this technology wisely, as part of a long-term strategy to improve human health, preserve biodiversity, and promote more sustainable agricultural practices in resource-poor countries. Enhancing the productivity of pulse legumes, such as pigeonpea, that are frugal in nature and are also important for nutritional security will be a possible way of addressing the additional problems of water deficits and high input costs in arid and SAT. Traditional cropping systems in pigeonpea include intercropping or mixed cropping (Aiyer, 1949; Acland, 1971; Osiru and Kibira, 1981) and crop rotation schemes for maintaining the soil fertility and minimizing erosion. Pigeonpea that is considered to be frugal in their requirement of water and fertilizers if made resistant to pests and pathogens using transgenic technology, could very well replace the cereals (maize, sorghum, pearl millet, and finger millet), fiber, and other legume crops allowing proper crop rotation and ensuring a diversification. Similarly, transgenic technology can contribute toward enhancing productivity and yield stability of pigeonpea with low-water requirement resulting in a dramatic reduction in the overall dependence on groundwater for irrigation.

However, a major concern of the scientists and the environmentalists is that the transgenes could escape to related species by pollen flow and could convert wild relatives into "super weeds." However, reports on pigeonpea indicate that "genetic pollution" by transgenes escaping into landraces, primitive cultivars, and nontransgenic varieties through pollen dispersal has been recently focused as a major issue in the risk assessment of transgenic plants. However, this risk can be very well addressed by introducing the transgenes into the chloroplast genomes of plants. These organelles are inherited maternally and therefore, pollen-based dispersal is not possible.

Breeding for resistance to pests and pathogens has been a major area of research in pigeonpea breeding. There is a concern that widespread growing of *Bt* transgenics may lead to insects developing resistance to *Bt* proteins, thereby increasing criticisms on the use of one of the most potent but more environmental-friendly pesticidal tool. Most of the pigeonpea transgenics produced so far using *cry1* genes that are insecticidal only to selected groups of insect species (e.g., lepidoptera) and are unlikely to have direct effects on species outside this group. Although the extensive testing on nontarget plant feeding insects and beneficial species that have accompanied the long term and wide scale use of *Bt* plants has not detected significant adverse effects (reviewed by O'Callaghan *et al.*, 2005). However, species representing pollinators (honeybees), natural enemies (including predators and parasitoids), and detritivores are among the insects that should be subjected to elaborate tests with transgenic pigeonpea containing insecticidal genes. However, there is an increasing concern that other transgenic proteins with ranges of activity wider than those of *Bt* may have a greater chance of affecting natural enemies. Transgenic expression of non-native proteins in plants may lead to the concerns on potential for new allergens in genetically modified (GM) crops substantiate the need for the complete risk assessment of transgenic crops before commercialization (Prescott *et al.*, 2005). Hence, an objective assessment of the associated hazards needs to be carried out during feeding trials for testing immunogenic responses of the transgenic foods on the animal model systems before any commercialization. Besides, concerns on the possibilities of breakdown of resistance to pests and pathogens is always there even if the resistance is based on the use of the secondary gene pool or through the use of the tertiary gene pool using conventional breeding methods. Although, this itself can be counteracted by imparting a more durable resistance in crop plants by diversifying the resistance-conferring genes, i.e., using more than one gene in a variety and eventually stacking genes, which confer resistance through diverse mechanisms.

The possible risks posed by cross hybridization with wild relatives have been extensively explored (reviewed in Stewart *et al.*, 2003). However, the Nuffield Council on Bioethics suggests that introgression of genetic material into related

species in centers of crop biodiversity is an insufficient justification to rule out the use of GM crops in the such areas and the developing world (Nuffiel Council on Bioethics (a discussion paper), 1999). Nonetheless, banning of transgenic crops does not appear as a scientifically sound option because of the potential benefits derived from their utilization by farmers, for example, resistant or tolerant crops to abiotic and biotic stresses obtained through genetic engineering (Sharma *et al.*, 2002).

The field evaluation and risk assessment have to be performed according to the biosafety guidelines of the host country under the immediate guidance and supervision of the Institute Biosafety Committee. Assessment procedures are being harmonized internationally by various organizations (Levin and Strauss, 1993). Hence, the release of transgenic materials should take place only after establishing the utility of the material through very transparent and well-documented evaluations. In addition to the measurable parameters such as the crop performance, yield, fitness, invasiveness, rate of hybridization, the expanded risk equation now also includes nonquantifiable terms such as consumer choice, long-term agricultural policy, ethics, and societal responsibility to future generations.

The key to the future of genetically engineered pigeonpeas is firstly the public awareness toward the competent assessed of any risk associated with the transgenics and also that the safety has been ensured. With the development of high-throughput technologies in sequencing, it is now possible to mine genes of high agronomic value from the near and distant relatives of crop plants and to introduce these gene(s) into recipient crop varieties through the techniques of genetic transformation. Understanding the mechanisms of resistance and the mining of alleles that will confer resistance to pests and pathogens will be the most rewarding area for development of resistant pigeonpea cultivars through transgenic approaches

3.3 Expected Technologies

3.3.1 Clean transgenics

The antibiotic resistance genes are often of importance to select for transformants from nontransformants in the process of producing

transgenic plants. But emergence of bacteria that are resistant to multiple or all antibiotics (Levy, 1997; Amtsblatt der Europäischen Gemeinschaften, 1998), transfer of resistance traits into weeds (Dale, 1992; Gressel, 1992) through cross-pollination with the related species and widespread distribution of resistance markers in to food products leading to horizontal transfer of resistance genes to gut bacteria, has increased the concern of antibiotic usage in transgenic plants. As these markers are used only as a tool of selection and as it does not code for any desirable traits the presence of these in transgenic plants is treated as a burden disturbing the genetic constituency of the plant and its wild varieties. Therefore, gene products need to be assessed for safety and environmental impact (Bryant and Leather, 1992; Gressel, 1992). Moreover, it is difficult to introduce a second gene of interest into a transgenic plant that already contains a resistance gene as a selectable marker because of limited availability of marker genes. The presence of multiple homologous sequences in plants enhances the likelihood for homology-dependent gene silencing (Matzke and Matzke, 1991), which could severely limit the reliable long-term use of transgenic crops. Therefore, it is necessary for scientists to look for alternatives for safer marker genes or elimination of the marker genes from transgenic plants to produce environmentally safe transgenic plants and pyramid a number of transgenes by repeated transformation (Yoder and Goldsbrough, 1994).

3.3.2 Marker-free transgenic plants

Marker-free transgenic plants can be obtained by using the site-specific recombinase P1 Cre/lox (Yoder and Goldsbrough, 1994; Vergunst and Hooykaas, 1998; Vergunst *et al.*, 1998; Gleave *et al.*, 1999) system. Marker gene to be introduced into the plant cell if placed between two lox sites will be excised from the plant genome by the expression of *Cre recombinase*. This technology was successful in producing marker-free transgenics in tobacco (Gleave *et al.*, 1999; Jia *et al.*, 2006). Besides cre/loxP, *Zygosaccharomyces rouxii* R/rs (Onouchi *et al.*, 1991; Sugita *et al.*, 1999, 2000) and *Saccharomyces cerevisiae* FLP/frt (Kilby *et al.*, 1995; Lyznik *et al.*, 1996; Davies

et al., 1999; Luo *et al.*, 2000; Gidoni *et al.*, 2001) recombination systems are also in use for producing the marker-free transgenics. Marker-free transgenic plants can also be obtained by using *ipt* gene attached to *Ac* transposable element as a marker. The transgenic plants acquires abnormal phenotype called extreme shooty phenotype and loses its ability to root and therefore can be differentiated visually from nontransgenic plants. As the marker is attached to an *Ac* transposable element during the transposition process the *ipt* gene may transpose or become lost along with the *Ac* element and thus a normal, marker-free transgenic plant can be obtained. Co-transformation of desired gene and the marker gene on separate plasmids within the same *Agrobacterium* strain and selecting the transformants with both the genes unlinked is another option for obtaining marker-free transgenics. The two T-DNA binary vector systems (Komari *et al.*, 1996; Xing *et al.*, 2000; Matthews *et al.*, 1997; McCormac *et al.*, 2001; Miller *et al.*, 2002) represent a useful approach to generate selectable marker-free transgenics by co-transformation of the vector harboring two T-DNAs each bearing a marker gene. This system represents a valuable approach to generate selectable marker-free plants, with a consistent frequency seen among three elite cultivars of rice decreasing the plasmid backbone transfer, lowering the number of T-DNA copy integrations, and avoiding artifacts due to gene silencing (Sharma *et al.*, 2005).

3.3.3 Alternatives to antibiotic resistance markers

It is not possible to remove marker genes once they are integrated into a plant genome unless a particular mechanism for removal is incorporated along with the marker gene and the gene of interest at the time of the transformation. The removal prior to commercialization of marker genes, which are driven by plant promoters and are used for selection of plant cells, has become the aim of both consumers and industry. Markers that confer resistance to chemicals other than antibiotics, such as herbicides, and lethal concentrations of the amino acids lysine and threonine and/or markers that enable the plant cells to grow in the presence of unusual nutrients, including cytokinin,

glucuronides, xylose or mannose, which will not allow nontransformed plant cells to grow can be the alternative selectable markers for plants. But expression of high levels of lysine and threonine causes abnormal growth in plant cells by interfering with amino acid biosynthesis and the presence of herbicide-tolerance markers may be undesirable. The relevant genes are therefore not suitable as marker systems.

Using a scorable marker gene could be an alternate for avoiding the usage of selectable antibiotic marker genes. The reporter genes or scorable markers produce a visible effect, directly or indirectly, due to their activity in the transformed cells. Scorable markers like *uidA* (*gus*) gene, and *cat* gene are commonly used in transformation experiments. However, destructive nature of β -glucuronidase (GUS) assay (Patnaik and Khurana, 2001) and presence of inhibitors of chloramphenicol-acetyltransferase (CAT) activity and endogenous CAT activity hampered the use of these as reporter genes (Patnaik and Khurana, 2001). Thus, to study the fate of introduced transgenes in living cells, vital reporter genes encoding for anthocyanin biosynthesis, green fluorescent protein, and firefly luciferase have been used successfully (Harvey *et al.*, 1999; Jordan, 2000). The delivery of a gene encoding mannose-6-phosphate isomerase and/or xylose isomerase allowing mannose and/or xylose to be metabolized in plant cells and the subsequent cultivation of those cells in a medium containing mannose and/or xylose as the sole source of sugar would allow only those cells that have taken up the gene to grow. Using mannose isomerase successful transformation of sugar beet (Joersbo *et al.*, 1998, 1999, 2000) and maize (Negrotto *et al.*, 2000; Wang *et al.*, 2000) was obtained. Using xylose isomerase, transgenics in potato, tomato, and tobacco were obtained with considerable transformation frequency (Haldrup *et al.*, 1998a, b). Another selectable marker gene that can be used is cyanamide hydratase (*Cah*) gene (Weeks, 2000). The *Cah* gene gives the transformed tissues the ability to grow on cyanamide-containing media by converting cyanamide into urea (which can be used as a fertilizer source).

Most alternatives are still in their development phase, are not widely available and will be difficult to implement in a less developed country. Alternative markers and marker removal systems

are being investigated in response to public concerns and to expand the number of tools available in plant molecular biology. Since the time for development of new alternative methods varies between different crops, it will be necessary to allow for a gradual transition to such technologies. It will also be critical to conduct safety assessments on new systems before they are used in products that are to be commercialized. Replacement of the technology, which makes use of antibiotic resistant marker gene such as *nptII*, will be desirable when the new technologies have ensured at least the same degree of scientific knowledge and confidence regarding their use as *nptII* gene and products containing it.

3.3.4 Choice of promoters

An efficient transformation system, in conjugation with the availability of a range of promoters with varied strengths and tissue specificities is critical to the success of transgenic approaches for crop improvement. An important aspect of transgenic technology is the regulated expression of the transgenes. Variation in transgene expression levels between different species and promoters may be due to different abundance of transcription factors, recognition of promoter sequences or intron splicing sites (Wilmink, 1995), or other factors. Therefore, increasingly, knowledge gained from genomics and postgenomics projects might provide information on designing of new targets for pigeonpea transformation. Establishment of transgenic systems for crops like pigeonpea requires genes of agronomic importance like those for insect resistance, abiotic stress tolerance, nutritional improvement, and male sterility. However, achieving a high expression of the introduced foreign gene in plant cells is still a challenging task. Direct screening of genomic libraries for highly expressed genes is an efficient way to identify promoters that confer high levels of gene expression. Tissue specificity of transgene expression is also an important consideration while deciding on the choice of the promoter, in order to increase the level of expression of the transgene. Thus, the strength of the promoter and the possibility of using stress inducible, developmental-stage- or tissue-specific promoters need to be considered (Bajaj *et al.*, 1999).

Besides, attention also needs to be focused on regulation of expression of plastid genes as well as to isolate target-specific promoters or design promoters with improved potential. Direct isolation of promoters can be done via T-DNA tagging with a promoterless reporter gene, although the most commonly used reporter gene for this kind of tagging has been the *gusA* gene (Casson *et al.*, 2002; Stangeland *et al.*, 2003), the ideal reporter gene should have a sensitive, nondestructive, and nontoxic assay allowing multiple *in vivo* screening rounds to identify simultaneously developmental specific, tissue specific, or stress-responsive patterns of expression.

The recent identification and isolation of a broad range of genes encoding different classes of proteins with activity against phytopathogenic fungi has opened the way to engineer fungus resistance into plants. Transgenic technology for imparting disease resistance requires tissue specific, wound- and pathogen-responsive promoters to express antifungal genes to control several diseases that threaten pigeonpea production. Similarly, for a number of future applications, transgenes will have to be expressed differentially or under specific abiotic (e.g., salt, wounding) stress conditions, which requires the use of a set of specific promoters to drive regulated gene expression. However, relatively few promoters are currently available for a specific or fine regulation of gene expression. It is expected that for these purposes homologous promoters will be more functional than heterologous ones, which should also raise less biosafety concerns.

3.3.5 Pyramiding of genes

Gene stacking is a term that is used in the context of genetically engineered crops, but is not a new idea in plant breeding. Gene stacking is combining desired traits into one line. Plant breeders are always stacking genes by making crosses between parents that each has a desired trait and then identifying offspring that have both of these desired traits. Pigeonpea breeders have been continually developing new varieties that contain the most effective combination of existing characters. A similar trend is expected with the pigeonpea transgenic plants by accumulation of

transgenes that inevitably becomes an increasing feature of new varieties. For example, stacking of different insecticidal genes might be considered as one of the major component of integrated pest management in pigeonpea. Bollgard[®] II developed by Monsanto that has been approved for commercialization in Australia and the United States in 2002 is an example for gene stacking, containing two *Bt* genes; *cry1Ac* and *cry2Ab2*. The proteins produced by these have different mode of action, thus making it very difficult for the pest to develop resistance to both the proteins simultaneously. Similarly, expected technological advancements in pigeonpea include identification and cloning of the genes responsible for traits such as high yield, disease resistance and tolerance to low temperature, to drought or to salt stresses. Although, this is a more difficult task because multiple genes control each trait for abiotic stress tolerance. However, in principle, the major genes involved in each of these traits can be identified first by mapping and then by map-based gene cloning (McCouch and Tanksley, 1991). This might ascertain that different varieties of transgenic pigeonpea plants endowed with a number of the above-mentioned desirable traits, will be grown in the dry and semi-arid regions of the world within the next 10 years.

3.4 Intellectual Property Rights (IPR) Issues

Intensive agriculture requires the use of certified seed (i.e., seed free of pathogens, pests, and weeds) and growers purchase new seed every year as an established practice. Historically, fertility and reproduction of grain and legume crops in Africa, Asia, and parts of the Americas have acquired a deep spiritual significance (Nuffiel Council on Bioethics, 1999). Nevertheless, the application of modern biotechnological techniques to plant species promises production of quality, quantity, and variety of food products. Hence, intellectual property rights are likely to play an important role in securing economic returns for the intellectual and financial investments that make the research and developments possible. The public-sector institutions need to obtain intellectual property rights for their discoveries so that these rights can be used in negotiations with the private sector to produce increased public benefit. Hence,

intellectual property regimes might facilitate the development of beneficial new crop varieties through individual, public, and corporate sources, as well as promote research collaboration.

Most pigeonpea growers prefer plant hybrid varieties that are more uniform and vigorous than ordinary varieties because of heterosis (hybrid vigor) and these advantages are lost when second generation seed is used. However, in many instances, small growers cannot afford to purchase new seed every year, and they wish to maintain their long-standing practice of saving some of the seed from one year's crop in order to plant next year's crop. Although pigeonpea is one of the major grain legume crops grown in the tropics and subtropics, none of the pigeonpea transgenics have been commercialized so far. Therefore, intellectual property rights need to be narrowly tailored to be commensurate with the actual scope of new GM inventions so as not to impede continuing research, innovation, and development of this important pulse legume. IPR issues in pigeonpea needs to be focused on making GM pigeonpeas available to developing countries by establishing a partnership between the research institutions and industries so that the benefits of research, applications, and licensing become much more widely available. Moreover, broad patents should be granted to companies that secure their competitiveness in the market place (<http://www.royalsoc.ac.uk/document.asp?id=1447>). Also, it is important to identify areas of common interest and opportunity between private sector and public-sector institutions so that the generation and use of pigeonpea transgenic not only to help resolve the intellectual property issues involved, but also benefit the poor.

4. CONCLUSIONS

Gene transfer techniques to develop transgenic crops can be seen as a logical extension of the crop plants for millennia displaying considerable potential to benefit both developed and developing countries. Genetic transformation is more expeditious, as the development of new cultivars by classical breeding typically takes from 10 to 15 years. Genetic transformation technology is critical in overcoming the severe bottlenecks associated with conventional

agricultural programs and enhancing their delivery prospects. However, transgenic technology needs to be used as adjunct to and not as substitutes for conventional technologies. Nevertheless, the primary attraction of the gene transfer methods to the plant breeder is the opportunity to tap into a wide gene pool to borrow traits, obviating the constraints of cross-compatible crop species. Most of the developments in plant gene transfer technology and the different strategies to produce improved transgenic plant varieties have been driven by the economic value of the species or the trait. These economic values, in turn, are mainly determined by their importance to agriculture in the developed world, particularly the United States and Western Europe. However, to increase global food production, it is necessary to ensure that this technology is effectively transferred to the developing world and adapted to local crops. The technological challenge here lies in obtaining improvements in agricultural productivity without destroying the global natural resource base. In the present scenario, many new approaches like gene tagging are being used to isolate resistance-conferring genes from resistant germplasm in crop species. Therefore, transgenic approaches can circumvent the difficulties of sterility and linkage drag, which do not allow the successful incorporation of resistance conferring genes from the wild species to crop species. Genetically engineered pigeonpea for virus resistance, insect resistance, and biofortification are good examples of strategies that could potentially benefit a diversity of legume crops. Substantial investments are therefore needed to develop, field test, and commercialize new transgenic pigeonpea varieties expressing insecticidal proteins, or proteins providing tolerance to herbicides or resistance to environmental stresses will revolutionize agriculture especially in arid and semi-arid regions of the world.

In addition, the scientific research aimed at risk analysis, prediction, and prevention, combined with adequate monitoring and stewardship, must continue so that negative ecological impact from GM crops will be kept to a minimum. One must also recognize the potential positive impact of GM crops on the environment, such as decreasing agricultural expansion to preserve wild ecosystems; improving air, soil, and water quality by promoting reduced tillage, reducing chemical

and fuel use; improving biodiversity through resuscitation of older varieties and promotion of beneficial insects; and cleaning up contaminated soil and air through phytoremediation. Since the implications of a risk assessment of GM organisms are dependent on the social context, a participatory approach is needed to determine the balance of benefits to risks. By understanding the nature of genetic modifications and the nature of genomic plasticity in plants, it would be possible to determine an accepted safety baseline, against which the safety of the genetic engineering of plants can be evaluated. To ensure safe crops to humans and the environment, a strong, but not stifling, regulatory system needs to be established and properly implemented. A challenge for the future will be to use this technology wisely, as part of a long-term strategy to improve human health, preserve biodiversity, and promote more sustainable agricultural practices.

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Vetch

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Common vetch, *Vicia sativa* L., is an annual grain legume species and a member of the *Vicieae* tribe (Leguminosae-Papilionoideae) that originated from the arid regions of the Middle East. *Vicia* species have a broad geographical adaptation within Europe, Asia, North America, the temperate zones of South America, and tropical Africa, and more recently the Mediterranean soils and climates of southern Australia. *V. sativa* is a member of the *Vicia* subgenera, which comprises 50 species that are grouped into six sections (*Atossa*, *Hypechusa*, *Peregrinae*, *Vicia*, *Faba*, and *Pseudolathyrus*).

Vetch is traditionally grown as a mix for hay production or for grazing only on land unfavorable for other crop species that are more sensitive to adverse abiotic conditions. The species has considerable potential as a grain and forage legume (Seymour *et al.*, 2002). Vetch is also used as a disease break in rotations with other grain and green manure crops. The seeds contain between 28% and 32% protein and are considered to be a quality feed for ruminant animals, such as sheep and cattle. Vetch seeds have also been used for human consumption, particularly in times of food scarcity (Enneking, 1995). The world's five largest vetch producers are The Russian Federation, Spain, Ukraine, Turkey, and Ethiopia (FAO, 2005).

Common vetch can be grown on a wide range of soil types, from shallow duplex to heavy clays, and across a wide range of soil pH levels (4.5–9.0). Indeed, vetch is more suited to lower pH levels than many other cultivated legume species. However, vetch has poor tolerance to saline soils and soils those are prone to surface sealing, and is best suited to low to medium (<325–450 mm) rainfall areas (Seymour *et al.*, 2003). Vetch seedling development is hypogeal therefore, it is less likely to be killed by frost, wind erosion, or insect attack, as new stems can develop from buds at nodes at or below ground level (Seymour *et al.*, 2003).

V. sativa was introduced to Australia in the 1940s as an alternative leguminous rotation crop to field pea (*Pisum sativum*) due to its greater tolerance to adverse conditions as well as its immunity to pea weevil (*Bruchus pisorum*). Since 1990, the area sown has increased to over 230 000 ha/year primarily in southern Australia where it is grown for green manure, hay, or grazing. Australia ranks as the seventh largest world producer (FAO, 2004) and the majority of breeding efforts have focused on the *V. sativa* varieties Languedoc and Blanche fleur, which possess great potential for improvement in dry matter and seed yield (Seymour *et al.*, 2003).

The Blanche fleur *V. sativa* cultivar is a midmaturing vetch, which originated in France. The grain yields range from 0.5 to 3 tons per hectare. The distinguishing features of Blanche fleur are white flowers, which occur 95–125 days

after sowing as singles or pairs and orange colored cotyledons when the seed is split.

1.2 Neurotoxic Compounds in *V. sativa*

Several *V. sativa* accessions contain the neurotoxic compound β -cyanolalanine and its γ -glutamyl peptide (Ressler *et al.*, 1969). The natural occurrence of β -cyanolalanine was first detected and subsequently isolated and identified in *Vicia angustifolia* and *V. sativa* (Ressler, 1962). Seeds of the Blanche fleur cultivar contain approximately 1.15% of cyanolalanine toxins; i.e., 1.15 g for every 100 g of Blanche fleur seed (Panagiotopoulos, 2004).

The toxicity of these compounds to monogastric animals was demonstrated in several species. When administered to a weanling male rat by stomach tube in a dosage of 15 mg per 100 g, β -cyanolalanine caused hyperactivity, followed by tremors, convulsions, and rigidity, from which the rat recovered after 4 h. When a higher dose (20 mg per 100 g) was injected subcutaneously, it caused convulsions, rigidity, prostration, and death (Ressler, 1962). Poultry that were fed a diet containing 50% Blanche fleur seed had high mortality rates (Ressler *et al.*, 1969). More recently, the feeding of 5 kg per head of vetch hay to cows for a period of 2 weeks was shown to cause sudden death (Suter, 2002). The toxic compounds in vetch are also described as antifeeding factors (Tate, 1996). When consumed, the neurotoxic compound appears to trigger a defense mechanism that subsequently limits the animals food intake and in extreme cases, may result in the animal starving itself to death (Tate, 1996).

Due to the observed neurotoxic effects on monogastric animals, the β -cyanolalanine and γ -glutamyl- β -cyanolalanine compounds are thought to negatively affect humans, particularly in the brain tissue, although this has not yet been proven. Therefore, in order to be safely consumed by humans, vetch seed need to be boiled in water and the broth decanted during cooking. Lengthy steeping in water at room temperature will also effectively leach out the neurotoxins from split vetch seeds. However, these detoxification methods will also remove beneficial water-soluble

compounds such as B-vitamins, proteins, and carbohydrates (Ressler *et al.*, 1997).

1.3 Traditional Breeding and Limitations

Traditional breeding approaches have been applied with an aim to reduce and/or eliminate the neurotoxin compounds from elite Australian vetch accessions. To date, these methods have reduced seed toxin levels from 1.15% to between 0.3% and 0.4% of total seed protein. Two low toxins lines (IR-28 and IR-36) were identified from more than 3000 accessions from the International Center for Agricultural Research in Dry Areas (ICARDA), Iranian, and Russian germplasm collections. The low toxin lines were then crossed with commercial cultivated varieties. However, in order for vetch to be safely used as a fodder crop and moreover, for the seed to be consumed by humans and other monogastric animals, an amount of less than 0.1% of total protein or a zero toxin level is required. Novel and alternative breeding biotechnologies may offer a path toward the production of human-safe vetch. As an initial step toward this ultimate goal, several methodologies are required to be developed, such as reproducible and efficient transformation and regeneration systems, as well as a detailed understanding of the biochemical pathway(s) that produce the neurotoxin compounds. The neurotoxin β -cyanolalanine is synthesized as an intermediate in the assimilation of cyanide in plants by the catalytic enzyme β -cyanolalanine synthase (β -CAS). Another enzyme, γ -glutamyl transferase, functions to catalyze the formation of γ -glutamyl- β -cyanolalanine from β -cyanolalanine. The subsequent catalysis to asparagine is thought to be blocked in *V. sativa* (Fowden and Bell, 1965; Figure 1).

Considering the present knowledge regarding the neurotoxin biosyntheses, in terms of producing a neurotoxin-free plant, there are two possible strategies that can be applied to vetch. These are (1) to insert gene(s) that will activate β -cyanolalanine hydrolase activity, to lead to expression of asparagines, or (2) create a mechanism to suppress the activity of the enzymes that produce the neurotoxins, γ -glutamyl transferase, and/or β -CAS. Both of these strategies require the development of precise, robust, and reproducible

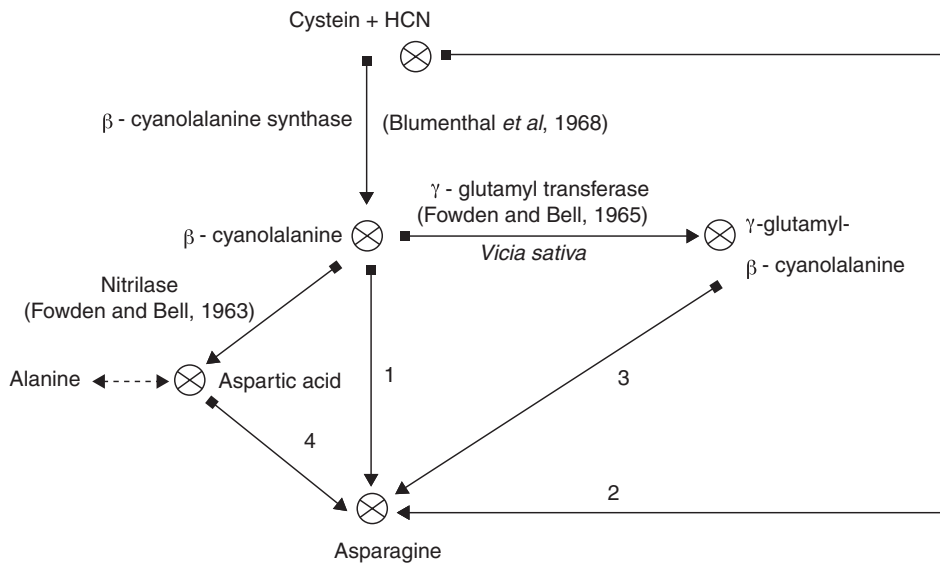


Figure 1 The biosynthetic pathway for β -cyanolalanine and γ -glutamyl- β -cyanolalanine

plant biotechnology techniques such as *in vitro* plant propagation and regeneration, and gene transformation.

2. DEVELOPMENT OF TRANSGENIC VETCH

The application of plant biotechnology to grain legumes has mainly been directed to isolation and characterization of genes that control useful agronomic traits that are qualitatively and quantitatively inherited. This has included genes for herbicide resistance, improved nutritional qualities, elimination of antinutritional factors, and improved features for cultivation and harvesting (Svetleva *et al.*, 2003). Although plant biotechnology offers numerous potential solutions to legume crops' improvement, to date, most studies on grain legume species are still at the stage of finding the best strategies for inserting foreign genes and the production of stable transgenic plants (Somers *et al.*, 2003). Therefore, a large amount of research is currently focused on the development of regeneration protocols from single cells and organized tissues, gene delivery methods, and the discovery of specific genes to be transferred to plant cells or tissues (Christou, 1997).

2.1 A Robust Regeneration System for *V. sativa* cv. Blanch fleur

Species belonging to the family *Leguminosae* are reported to be difficult to regenerate *in vitro* compared to nonlegume species mainly because legume regeneration *in vitro* is slow and is highly genotype specific (Somers *et al.*, 2003). Furthermore, members of the *Viciace* experience inhibition of explant growth in tissue culture from phenolic compounds (Bottinger *et al.*, 2001).

Legume regeneration *in vitro* has been achieved using two different processes, via organogenesis and somatic embryogenesis (Bottinger *et al.*, 2001). Both organogenesis and somatic embryogenesis techniques have been employed to produce regenerated plantlets either directly from an explant or indirectly after a callus phase (Svetleva *et al.*, 2003). Direct somatic embryogenesis occurs when cells undergo none or few mitotic divisions, whereas indirect somatic embryogenesis occurs following many mitotic divisions (Parrott *et al.*, 1995). Successful *in vitro* plant regeneration systems have been developed for legume species, such as peanut (Chengalrayan *et al.*, 2001) and soybean (Hofmann *et al.*, 2004).

In contrast, there has been limited success regarding the *in vitro* culture and/or regeneration of species from the *Viciace* tribe (Christou, 1997).

However, *in vitro* regeneration systems via somatic embryogenesis have previously been developed for recalcitrant related grain legume species and, based on these; two separate systems were recently developed for *V. sativa* (Maddeppungeng, 2006). One system was established from explants derived from etiolated seedlings, adapted from the methods employed for *Vicia narbonensis* (narbon bean; Pickardt *et al.*, 1989) and *Cicer arietinum* (chickpea; Kumar *et al.*, 1994). Another system was established from embryo axes explants and was adapted from the system developed for *Arachis hypogaea* seed (peanut; Baker *et al.*, 1995). Maddeppungeng (2006) determined that the most efficient method for somatic embryo callus production from cv. Blanch fleur was from epicotyl regions of etiolated seedlings (Figure 2a, b). This was far more efficient than using leaf explant material and was most successful when α -naphthaleneacetic acid (NAA) concentrations were higher than 8 μ M and the 6-benzylaminopurine (BAP) concentration was 13 μ M. Increased somatic embryo production was found in the presence of a low 2,4-D concentration (1 μ M). The compound 2,4-D was previously shown to affect somatic embryo production in the related species of narbon bean (Pickardt *et al.*, 1989) and chickpea (Barna and Wakhlu, 1993). The method of Maddeppungeng (2006) produced healthy and rooted regenerated *V. sativa* plantlets (Figure 2c) with a success rate of 12% achieved during the hardening off process.

2.2 A Robust Transformation System for *V. sativa* cv. Blanch fleur

Genetic transformation provides an attractive supplement to conventional breeding approaches

for legume species, particularly when the cultivated species contains extremely narrow genetic variation (Delannay *et al.*, 1983) and desirable genes are not present in the gene pool.

Various methods have previously been employed for delivering foreign genes, within genetic constructs, into the genomes of related legume and grain legume species. A direct gene transfer method was successfully applied to *Vigna aconitifolia* (moth bean). In this method, heat-shocked protoplasts were treated with polyethylene glycol (PEG) and plasmid DNA containing the coding region of the aminoglycoside phosphotransferase gene (*nptII*; Kohler *et al.*, 1987). However, the direct transformation system required protoplasts as the starting material and whole transgenic plants were not recovered (Kohler *et al.*, 1987; Sukhapinda *et al.*, 1987).

The particle gun bombardment or biolistic transformation method has successfully been applied to peanut (Deng *et al.*, 2001), soybean (Ponappa *et al.*, 1999), and *Lathyrus sativus* (Barna and Mehta, 1995). However, due to several drawbacks of this method, such as variable and often low transformation efficiency, variable insert copy numbers, patent issues, and high operation cost, the *Agrobacterium*-mediated transformation method has been more often employed for legumes. Several legume species successfully transformed with *Agrobacterium* include the model legume *Medicago truncatula* (Chabaud *et al.*, 2003), peanut (Sharma and Anjaiah, 2000), chickpea (Kar *et al.*, 1996), *Astragalus sinicus* (Chinese milk vetch; Cho and Widholm, 2002), soybean (Olhoft *et al.*, 2003), *Vigna sesquipedalis* Koern (asparagus bean; Ignacimuthu *et al.*, 1999), *Lupinus luteus* L (yellow lupin; Li *et al.*, 2000),

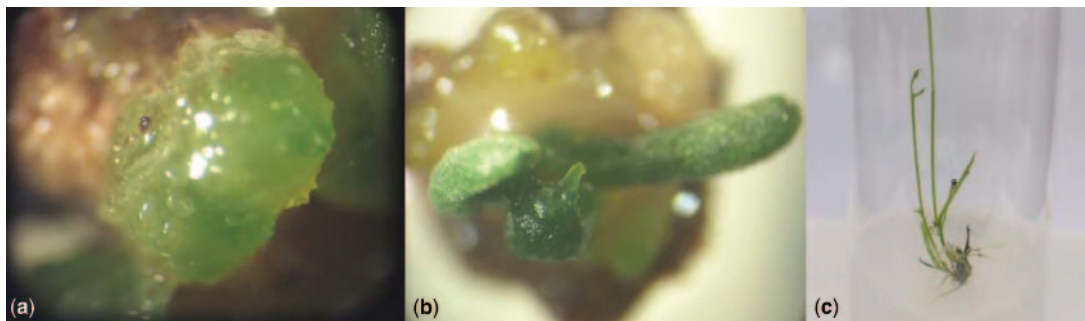


Figure 2 Regeneration of *V. sativa* cv. Blanche fleur via somatic embryogenesis from epicotyl-derived callus. (a) A heart-shaped embryo (magnification $\times 12$), (b) a regenerating plantlet, still attached to a callus clump, and (c) a fully formed plantlet with roots

Vigna mungo (blackgram; Saini *et al.*, 2003), lentil (Sarker *et al.*, 2003), narbon bean (Saalbach *et al.*, 1995), and faba bean (Bottinger *et al.*, 2001).

Recently, Maddeppungeng (2006) developed a transformation protocol for *V. sativa* cv. Blanch fleur using the *Agrobacterium*-mediated approach. For this, the selectable *NptII* gene marker, carried within a variation of the pLMNC95 binary vector (Mankin and Thompson, 2001) was applied and efficiently selected for at 50 mg l⁻¹ Kanamycin. The vector also carried a green fluorescent protein (GFP)-intron containing reporter gene for transformant observation. Experiments were conducted using the *Agrobacterium* strains EHA 101 (Hood *et al.*, 1986), LBA 4404 (Hoekema *et al.*, 1983), and C58 (Koncz and Schell, 1986) to infect epicotyl and leaf explants. Following 3 days of co-cultivation, explant pieces were placed on callus-formation media for up to 11 weeks after which putative regenerating transformant calli were observed microscopically with UV light and two different GFP filters (Figure 3). Since

the calli were resistant to kanamycin selection at 11 weeks after co-cultivation, this may have indicated stable transformation. However, further culture of calli into transgenic plants and Southern hybridization analysis to detect the transgene and copy number is required to confirm this.

Overall GFP expression was higher in epicotyl-derived callus than leaf-derived callus and the callus co-cultivated with the EHA 101 contained most GFP expression (Figure 3). Although GFP expression was observed in one of 22 regenerated putative transformant seedlings, this did not survive to maturity.

3. FUTURE ROAD MAP

3.1 Expected Technology Optimization

Although mature and fertile *V. sativa* cv. Blanche fleur plants have recently been established via somatic embryogenesis (Maddeppungeng, 2006),

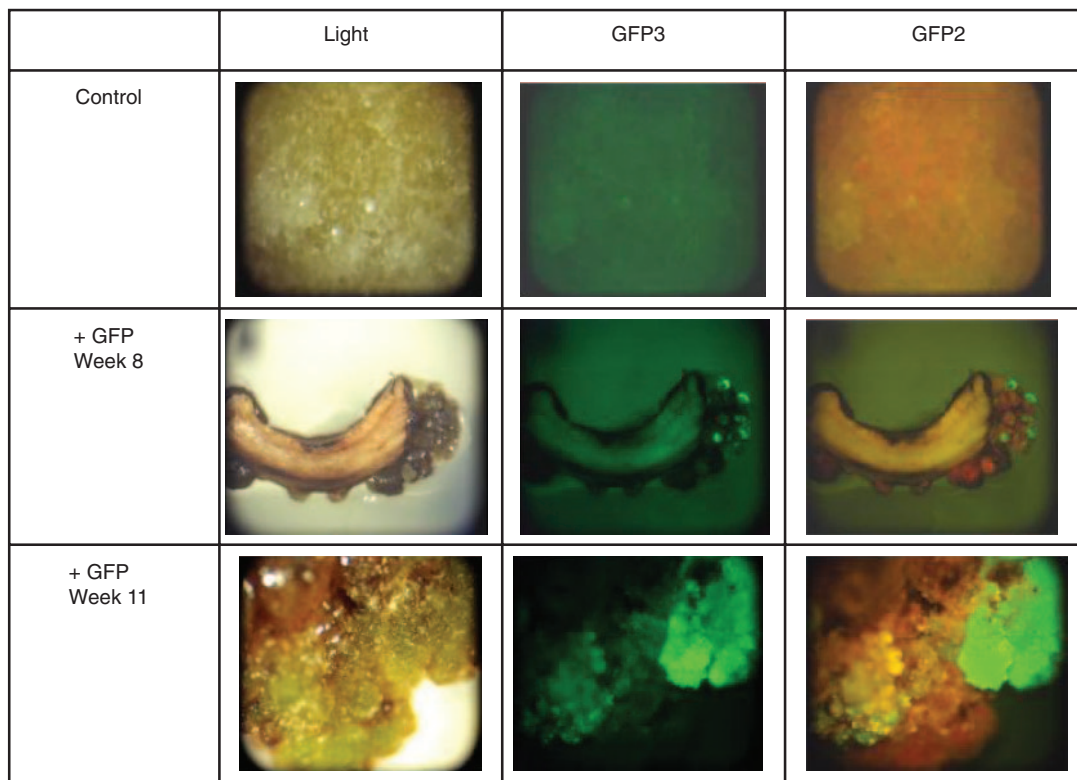


Figure 3 GFP expression in *V. sativa* cv. Blanch fleur callus at 8 and 11 weeks after transformation

further research is required in order to make improvements for reducing the length of culturing time and increasing the regeneration efficiency. This may be achieved through the further investigation of the use of embryo axes as the explant source, a method previously shown to be genotype independent and highly successful in peanut (Baker *et al.*, 1995).

Further research is also required to optimize the stable integration of foreign genes through *Agrobacterium*-mediated transfer. Various tissues, organs, and cell types within a plant may differ in their susceptibility to *Agrobacterium* (Gelvin, 2000). The influence of starting material on transformation efficiency in the study of Maddeppungeng (2006) revealed that GFP expression was higher in epicotyl-originated calli (13.5%) than hypocotyl-originated calli (3.9%). The influence of starting material on the capability of transformant tissues to regenerate *in vitro* has also been observed for *Vigna radiata*. For *V. radiata*, 30–40% of leaf explants co-cultivated with *Agrobacterium*-developed kanamycin resistance and showed a positive expression of the β -glucuronidase (GUS) reporter gene, whereas 53% of hypocotyl explants developed kanamycin resistance and GUS positive calli (Jaiwal *et al.*, 2001). Furthermore, transformation frequency of *L. sativus* was strongly influenced by explant source. The number of transformed explants that expressed the GUS reporter gene was higher for leaf (10.6%) than stem nodal segments (12%) (Barna and Mehta, 1995).

The virulence of a particular *Agrobacterium* strain against a particular plant genotype or species is a key factor for the development of a successful and highly efficient transformation system (Nadolska-Orczyk and Orczyk, 2000). Often one strain is reported to be more efficient than other strains tested and several studies have been aimed at elucidating the efficacies of various *Agrobacterium* strains on transformation of different legume species. In an experiment on cotyledon explants of pea (*P. sativum*), the strain KYRT1 (4.66%) was found to be more efficient than AGL1 (1.59%) (Grant *et al.*, 2003). Whereas, strain EHA 105 was shown to be more virulent and better able to transform pea than the strains LBA4404 and C58 (Nadolska-Orczyk and Orczyk, 2000). Likewise, for the model legume *M. truncatula* cv. Jemalong, the efficiency of transformation using the AGL1 strain was higher

(96%) than using the strain LBA 4404 (56%) (Chabaud *et al.*, 2003).

The length of culture post inoculation may also have a significant influence on the level of GFP positive expression observed. In *A. sinicus* GFP transgenic expression studies, no cotyledon explants fluoresced until at least 4 weeks after transformation, at which point the characteristic green fluorescence was easily detectable in the explants transformed with the *Agrobacterium* strain EHA 105 containing pBINm-gfp5-ER (Cho and Widholm, 2002).

3.2 Expected Products

Once an optimized *in vitro* regeneration system as well as a transformation system for *V. sativa* cv. Blanch fleur has been established, research toward the development of neurotoxin-free genotypes through directed genetic manipulation may begin. However, the gene(s) of interest, to be inserted or down-regulated must first be chosen, dependant on which genes are functional in the biochemical and metabolic pathways responsible for neurotoxin production. These are genomic and functional studies that are yet to be performed. With current knowledge, there are three possible approaches to developing neurotoxin-free vetch using a genetic transformation approach: (1) silence the gene(s) that encode β -cyanoalanine synthase, (2) silence γ -glutamyl transferase, and (3) insert the gene that encodes β -cyanoalanine hydrolase, which appears to be absent. Indeed it may be possible to use the β -cyanoalanine synthase gene previously characterized by Pandian (2002) in a silencing cassette; however, more knowledge regarding the role(s) of this enzyme in *V. sativa* is first required. Particularly since high levels of cyanide accumulated in avocado after this gene was silenced (Yip and Yang, 1998), indicating its potential involvement in cyanide detoxification in *V. sativa*.

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Chickpea

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1. INTRODUCTION

Chickpea (*Cicer arietinum* L.), a self-pollinating diploid annual with $2n = 2x = 16$ chromosomes, is an important food legume crop throughout the world, especially in the developing countries. With over 10 million ha under cultivation in more than 30 countries in arid and semiarid areas of central, south, and southeast Asia, southern Europe, northern and eastern Africa, in the Americas and Australia, chickpea is second only to common bean (*Phaseolus vulgaris*) and third in production among the legumes. Chickpea is the only cultivated species belonging to the *Cicer* genus, which is a member of the Leguminosae family, Cicereae *Alef* tribe (van der Maesen, 1987). Commercially, the species is grouped into *desi* and *kabuli* types: *desi* chickpeas generally have small, colored seeds, whereas *kabulis* produce large, cream colored ones. *Kabulis* are usually utilized as whole grains while *desis* are decorticated and processed into flour. Chickpea is mainly used for human consumption and only a small proportion is used as feed. The chickpea seed is a good source of carbohydrates

and proteins, which together constitute 80% of the total dry seed weight. The crude protein content of chickpea varies from 17–24% containing the essential amino acids like tryptophan, methionine, and cysteine. Chickpea is a cool season annual crop performing optimally in 70–80 °F daytime temperatures and 64–70 °F night temperatures. The crop produces good yields in drier conditions because of the deep tap root system. During 2002–2004, the global chickpea production was 8.0 million tons from an area of 10.1 million ha, giving an average productivity of 786 kg ha⁻¹ (ICRISAT, 2007).

1.1 History, Origin, and Distribution

Chickpea with a moderately sized genome of around 750 Mbp (mega base pair) (Arumuganathan and Earle, 1991) evolved from its wild progenitor *Cicer reticulatum* through natural selection. Chickpea is one of the pulse crops domesticated in the Old World ca 7000 years ago (van der Maesen, 1987). Vavilov (1926) identified

two primary centers of origin, Southwest Asia and the Mediterranean, and one secondary center of origin, Ethiopia. Based on cytogenetical and seed protein analysis, Ladizinsky and Adler (1976) considered *C. reticulatum* as the wild progenitor of chickpea and southern Turkey as the center of origin for the crop. Three wild annual *Cicer* species, *C. bijugum*, *C. echinospermum*, and *C. reticulatum*, closely related to chickpea, co-habit with the cultivar in this area. In general morphology, physiology, and genetics, *C. reticulatum* comes closest to the cultigen, making it a possible contender as the progenitor of chickpea. However, taking into account the polymorphic nature of ancestral populations and complex nature of domestication, one cannot rule out other possibilities, such as *C. reticulatum* and the cultigen sharing a common ancestor or a polyphyletic origin of chickpea. The *Cicer* species occur from sea level (e.g., *C. arietinum*, *C. montbretii*) to over 5000 m (*C. microphyllum*) near glaciers in the Himalayas. Chickpea is the only domesticated species under the genus *Cicer*, which was originally classified in the tribe Viciae of the family Leguminosae and subfamily, Papilionoideae. Based on the pollen morphology and vascular anatomy, *Cicer* is now set aside from the members of Viciae and is classified in its own monogeneric tribe, Cicereae Alef.

1.2 Classification and Crossability

The genus *Cicer* comprises 43 species and is divided into two subgenera. Chromosome number in *Cicer* species can be generalized as $2n = 2x = 16$. The cultivated species, *C. arietinum* is found only in cultivation and cannot colonize successfully without human intervention. The wild species (e.g., *C. reticulatum*, *C. bijugum*) occur in weedy habitats, mountain slopes among rubble (e.g., *C. pungens*, *C. yamashitae*), and on forest soils, in broad-leaf or pine forests (e.g., *C. montbretii*, *C. floribundum*) and can be grouped into annual and perennial forms. Studies on biosystematic relations between chickpea and its wild relatives following interspecific hybridization have been limited to the nine annual species, *C. arietinum*, *C. reticulatum*, *C. echinospermum*, *C. judaicum*, *C. pinnatifidum*, *C. bijugum*, *C. cuneatum*, *C. chorassanicum*, and *C. yamashitae*.

Several groups have studied the genetic diversity and relatedness among annual *Cicer* species by employing hybridization, electrophoresis of seed storage proteins, isozymes, and molecular markers (Ladizinsky and Adler, 1976; van der Maesen, 1987; Kazan *et al.*, 1993; Labadi *et al.*, 1996; Sudupak *et al.*, 2002; Rajesh *et al.*, 2002; Nguyen *et al.*, 2004) leading to the classification of the wild species into three groups. The first group includes the primary and secondary crossability group, chickpea and its closest relatives (*C. reticulatum* and *C. echinospermum*); the second group (the annual tertiary group) consists of *C. pinnatifidum*, *C. judaicum*, and *C. bijugum*; while the third group includes mostly perennial tertiary species as well as two annual species *C. cuneatum* and *C. yamashitae*. Species within the primary gene pool (*C. arietinum*, *C. reticulatum*, and *C. echinospermum*) can be readily crossed usually generating fully fertile progeny; while species within the secondary gene pool (*C. bijugum*, *C. pinnatifidum*, and *C. judaicum*) can be successfully crossed with the cultigen *C. arietinum*, provided hybrid embryos are rescued. However, the progeny of crosses between primary and secondary gene pools are frequently sterile. Species within the tertiary gene pool (*C. cuneatum*, *C. yamashitae*, and others) have not yet been successfully crossed with the cultigen *C. arietinum*.

1.3 Consumers' Preference

During the past 20 years (1985–2004), the global chickpea area increased by 7%, yield by 24%, and production by 33%. Presently, the most important chickpea producing countries are India (64%), Turkey (8%), Pakistan (7%), Iran (3%), Mexico (3%), Myanmar (3%), Ethiopia (2%), Australia (2%), and Canada (1%) (ICRISAT, 2007). Although India produces a large variety of pulses, chickpea alone accounts for 43.2% of the total annual pulse production of 11.79 million tons. Chickpea has one of the highest nutritional compositions of any dry edible legumes and does not contain any specific major antinutritional factors. Chickpea seeds are eaten fresh as green vegetable, parched, fried, roasted, and boiled; as snack food, sweet, and condiments; seeds are ground and the flour can be used as soup, *dal*, and to make bread; prepared with pepper, salt, and lemon it is served as a side dish. *Dal* is the

split chickpea without its seed coat, dried and cooked into a thick soup or ground into flour for snacks and sweetmeats. Sprouted seeds are eaten as a vegetable or added to salads. Young plants and green pods are eaten like spinach. A small proportion of canned chickpea is also used in a few countries, and to produce fermented food. Animal feed is another use of chickpea in many developing countries. On an average, chickpea seed contains 23% protein, 64% total carbohydrates, 47% starch, 5% fat, 6% crude fiber, 6% soluble sugar, and 3% ash (ICRISAT, 2007). Chickpea protein digestibility is the highest among the dry edible legumes. The lipid fraction is high in unsaturated fatty acids, primarily linoleic and oleic acids. Chickpea is also known for its use in herbal medicine and cosmetics. Chickpea meets 80% of its nitrogen requirement from symbiotic nitrogen fixation and can fix up to 140 kg N ha^{-1} from atmosphere. It leaves substantial amount of residual nitrogen behind for subsequent crops and adds much needed organic matter to maintain and improve soil health, long-term fertility and sustainability of the ecosystems.

1.4 Productivity Constraints

Greater and more stable yields are the major goals of plant breeding programs. Chickpea yields are usually an average of $400\text{--}600 \text{ kg ha}^{-1}$, but can potentially surpass 2000 kg ha^{-1} , and in experiments have attained 5200 kg ha^{-1} . Yields from irrigated crops are 20–28% higher than those from rainfed crops. Despite considerable international investment in conventional breeding, productivity of the crop has not yet been significantly improved. Currently, productivity of chickpea is low (world average being $\sim 0.8 \text{ t ha}^{-1}$) (FAOSTAT, 2005) and has stagnated in recent years. Reasons underlying marginal improvements are series of biotic and abiotic stresses that reduce yield and yield stability. Especially *Ascochyta* blight and *Fusarium* wilt, pod borer, drought, and cold are the major constraints of yield improvement. The susceptibility of the plant to a foliar disease, *Ascochyta* blight, caused by the ascomycete *Ascochyta rabiei* and on the Indian subcontinent to the vascular disease *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *ciceri* are the main constraints for increasing

yield. Consequently, chickpea breeding aims at high yielding cultivars that combine long-lasting resistance against *Fusarium* wilt and *Ascochyta* blight with tolerance to abiotic stresses, such as drought and cold. Many other factors also prevent increase in chickpea yield such as, inadequate amount of fertilizers and pesticides, and some of the traditional methods of land preparation and lack of knowledge of elite seed material. Improving resistance to biotic and tolerance to abiotic stresses as well as a general increase in dry matter are major aims of chickpea breeders around the world. Among the abiotic factors, drought stands to be the number one problem in major chickpea growing regions because the crop is grown on residual moisture and the crop is eventually exposed to terminal drought. In West Asia and North African countries, low temperature causing freezing injury or death or delayed onset of podding reduces yield tremendously. Heat and salinity problems are relatively important following drought and cold stresses. In general, estimates of yield losses by individual pests, diseases, or weeds range from 5–10% in temperate regions and 50–100% in tropical regions.

1.5 Rationale for Transgenic Chickpea Breeding

During the past 30 years, the area under cultivation of chickpea has remained stagnant but the production has increased from 6.3 metric tons (during 1975) to 7.4 metric tons (during 2002) because of increase in its productivity from 614 kg ha^{-1} to 735 kg ha^{-1} during this period (ICRISAT, 2007). It is generally accepted that the average yield of chickpea is below its presumed potential, and efforts to improve the productivity of this crop by conventional breeding means have not been very effective. The major reason behind this is lack of sufficient and satisfactory levels of genetic variability within the cultivated chickpea germplasm. Many wild annual *Cicer* species, which possess a wealth of agronomically desirable genes, are sexually incompatible with the cultivated varieties. An effective and alternative approach is, therefore, to transfer genes from sources which are otherwise difficult to introduce through conventional breeding (Meeting Report, 2000). Major yield increases could be achieved

by development and use of cultivars that tolerate/resist abiotic and biotic stresses. In recent years, the wide use of early maturing cultivars that escape drought stress led to significant increase in chickpea productivity. In the Mediterranean region, yield could be increased by shifting the sowing date from spring to winter. However, this is hampered by the sensitivity of the crop to low temperatures and the fungal pathogen *A. rabiei*. Drought, pod borer (*Helicoverpa* spp.) and the fungus *F. oxysporum* additionally reduce harvests there and in other parts of the world. Tolerance to rising salinity will be a future advantage in many regions. Therefore, chickpea breeding focuses on increasing yield by pyramiding genes for resistance/tolerance to the fungi, pod borer, salinity, cold, and drought into the elite germplasm. As chickpea is a self-pollinating crop with a narrow genetic base, there is not much scope for heterosis breeding in this crop. Pure line breeding was recognized as a method of choice for developing new chickpea varieties. Due to limited genetic variability available in this crop, there is not much hope for overall yield increase in chickpea.

Marker-assisted selection (MAS) is another approach for crop improvement where genes of specific agronomic interest are tagged with DNA markers and are transferred to the host plant through conventional breeding. In chickpea, genomic maps have been constructed, genes for resistance to fungi such as *F. oxysporum* and *Ascochyta* blight have been tagged and attempts are being made for MAS as reviewed by Ford and Taylor (2006). However, knowledge of the inheritance of agronomic characters is a basic requirement to identify and integrate interesting genes in linkage maps and to utilize these maps for MAS of these characters to accelerate the development of new cultivars. Nevertheless, most genomic regions harboring genes for important traits are not yet sufficiently saturated with co-dominant markers to apply MAS in chickpea breeding programs. Development of transgenic chickpea is, therefore, identified as an important approach for its improvement.

2. DEVELOPING TRANSGENIC CHICKPEA

Though chickpea is an important grain legume, it suffers from heavy losses due to susceptibility to

insect pests like pod borer, fungal pathogens, and low tolerance to drought and low temperature. As discussed earlier about the difficulty in developing new varieties that are superior to the available ones due to limitations in conventional breeding, it is imperative to develop superior varieties using transgenic technology. For development of transgenic plants, it is necessary to locate the genes those exhibit particular traits from available germplasm, their isolation, making them suitable to transfer into the target plant (modifying them by adding marker gene, promoter sequence, and termination sequence) and its transformation followed by an efficient regeneration protocol.

2.1 Locating and Isolating the Genes of Interest

Since there are significant losses in yield due to attack from viruses, fungi, and insects in chickpea, several attempts are being made to transfer specific coat protein genes and insecticidal protein genes from viruses and bacteria to produce virus resistant and insect resistant chickpea. It is very important to identify a set of germplasm of donor gene pools selected from individual crops for gene transfer studies and crop improvement. The contrasting growing regimes of cultivated chickpea and its wild progenitor may have resulted in a different allelic repertoire at different loci in the wild and cultivated gene pools. In recent years, attempts have been made to use wild *C. reticulatum* as a genetic resource for chickpea improvement (Abbo *et al.*, 2002). The world collection of chickpea germplasm contains genotypes that remain uncultivated because of poor agronomic characteristics, but possess a high level of resistance against *A. rabiei* that is a severe and destructive fungal disease (Collard *et al.*, 2001). Agronomic characters of wild *Cicer* species are presented in Table 1.

Development of gene-based markers to resolve the problems related to limited utilization of wild *Cicer* species by chickpea breeding programs can be used to provide candidate gene markers for mapping of quantitative trait loci (QTLs) controlling important agronomic traits. An expressed sequence tag (EST) library was constructed using root tissue from two very closely related chickpea genotypes. A total of 106 EST-based

Table 1 Wild *Cicer* species with valuable agronomic characters^(a)

Stress	Species with accessions exhibiting resistance or tolerance	Authors
<i>Fusarium</i> wilt	<i>C. reticulatum</i> ^(b) , <i>C. echinospermum</i> ^(b) , <i>C. pinnatifidum</i> ^(b) , <i>C. juidacum</i> ^(b) , <i>C. bijugum</i> ^(b)	Nene and Haware, 1980; Haware <i>et al.</i> , 1992; Kaiser <i>et al.</i> , 1994; Singh <i>et al.</i> , 1994; Infantino <i>et al.</i> , 1996; Singh <i>et al.</i> , 1998
<i>Ascochyta</i> blight	<i>C. reticulatum</i> ^(b) , <i>C. echinospermum</i> ^(b) , <i>C. pinnatifidum</i> ^(b) , <i>C. juidacum</i> ^(b) , <i>C. cuneatum</i> , <i>C. montbretti</i> , <i>C. anatolicum</i>	Singh <i>et al.</i> , 1981; Singh and Reddy, 1983; Haware <i>et al.</i> , 1992; Singh <i>et al.</i> , 1998; Stagmina <i>et al.</i> , 1998; Collard <i>et al.</i> , 2001
Botrytis gray mold	<i>C. bijugum</i> ^(b)	Haware <i>et al.</i> , 1992
<i>Phytophthora</i>	<i>C. echinospermum</i> ^(b)	Singh <i>et al.</i> , 1994
Cyst nematode	<i>C. reticulatum</i> , <i>C. pinnatifidum</i> ^(b) , <i>C. bijugum</i>	Singh <i>et al.</i> , 1989; Singh and Reddy, 1991; Singh <i>et al.</i> , 1994, 1996, 1998
Leaf miner	<i>C. reticulatum</i> , <i>C. echinospermum</i> ^(b) , <i>C. pinnatifidum</i> ^(b) , <i>C. juidacum</i> ^(b) , <i>C. bijugum</i> ^(b) , <i>C. chorassanicum</i> ^(b) , <i>C. cuneatum</i> ^(b)	Singh <i>et al.</i> , 1994, 1998
Bruchid (seed beetle)	<i>C. reticulatum</i> ^(b) , <i>C. echinospermum</i> ^(b) , <i>C. pinnatifidum</i> , <i>C. bijugum</i> ^(b) , <i>C. Juidacum</i> ^(b) , <i>C. cuneatum</i>	Singh <i>et al.</i> , 1994, 1998
Cold	<i>C. reticulatum</i> , <i>C. echinospermum</i> ,	Chandel, 1984; Van Der Maesen and Pundir,
Drought	<i>C. bijugum</i> ^(b) , <i>C. pinnatifidum</i> , <i>C. juidacum</i> , <i>C. microphyllum</i>	1984; Singh <i>et al.</i> , 1991, 1995, 1998; Kaiser <i>et al.</i> , 1993
Pea streak carlavirus	<i>C. microphyllum</i> , <i>C. anatolicum</i> , <i>C. canariense</i> , <i>C. microphyllum</i> , <i>C. oxyodon</i>	

^(a)Reproduced from Croser *et al.*, 2003^(b)Higher rating species

markers were designed from 477 sequences with functional annotations and these were tested on *C. arietinum*. Forty-four EST markers were polymorphic when screened across nine *Cicer* species. Generated EST markers have detected high levels of polymorphism for both common and rare alleles. This suggests that they would be useful for allele mining of germplasm collections for identification of candidate accessions in the search for new sources of resistance to pests/diseases, and tolerance to abiotic stresses (Buhariwala *et al.*, 2005). Comparative biology and genomics are used to discover or validate the function of key genes. Resistance response to *Ascochyta* blight in four chickpea genotypes was studied using microarray technology and a set of chickpea unigenes, grasspea (*Lathyrus sativus* L.). ESTs and lentil (*Lens culinaris* Med.) resistance gene analogues. The four genotypes included resistant, moderately resistant, susceptible and wild relative of chickpea. The time course expression patterns of 756 microarray features resulted in the differential expression of 97 genes in at least one genotype at one time point (Coram and Pang, 2005, 2006).

Comparisons between genotypes resistant and susceptible to *A. rabiei* revealed potential gene “signatures” predictive of effective *A. rabiei* resistance. These genes included several pathogenesis-related proteins, SNAKIN2 antimicrobial peptide, proline-rich protein, disease resistance response protein DRRG49-C, environmental stress-inducible protein, leucine-zipper protein, polymorphic antigen membrane protein, Ca-binding protein, and several unknown proteins. The information generated enhances the understanding of this plant–pathogen relationship and may aid breeding programs directed toward the production of resistant cultivars (Coram and Pang, 2006). Bhattarai and Fettig (2005) reported that a wild relative of chickpea, *C. pinnatifidum*, is more tolerant than chickpea itself to various abiotic stresses, including drought. A complementary DNA (cDNA) clone encoding a dehydrin gene, *cpdhn1*, was isolated from a cDNA bank prepared from ripening seeds of *C. pinnatifidum*. The polypeptide deduced to correspond to this gene, *cpdhn1*, consists of 195 amino acid residues with a molecular mass of 20.4 kDa. Northern

blot analyses showed that *cpdhn1* expression was induced not only during seed development, but also in leaves in response to drought, chilling, and salinity and also to treatment with abscisic acid (ABA) or methyl jasmonate. The induction of *cpdhn1* expression by methyl jasmonate and ABA indicates that the gene may also be involved in the response to biotic stress. The CpDHN1 protein may thus improve the tolerance of chickpea to a variety of environmental stresses, both abiotic and biotic. In another work, Chen *et al.* (2004) identified three chickpea accessions, PI 559361, PI 559363, and W6 22589, showing a high level of resistance to *Didymella rabiei* (anamorph *Ascochyta*) pathotypes I and II, and can be utilized as resistance sources in chickpea breeding programs for resistance to *Ascochyta* blight. Previous studies on the genetics of chickpea resistance used undefined isolates of *D. rabiei*, and resulted in different genetic hypotheses involving one, two, or more resistance genes or QTLs (Hafiz and Ashraf, 1953; Singh and Reddy, 1983; Santra *et al.*, 2000; Tekeoglu *et al.*, 2000). Recent studies employing pathotype I or pathotype II isolates showed that resistance to pathotype I is conditioned by a single (major) gene, whereas resistance to pathotype II is conditioned by two or more independent loci (Udupa and Baum, 2003; Cho *et al.*, 2004). Another major factor limiting chickpea production is susceptibility to wilt disease (*F. oxysporum* f. sp. *ciceri*) that affects susceptible cultivars within 25 days after sowing and affected seedlings show drooping of leaves followed by complete collapse. Studies at ICRI SAT, Patancheru, India, revealed that at least three genes are involved in conferring resistance to *Fusarium* wilt, and earliness or lateness of wilting depends directly upon the number of genes contributing to the trait (Upadhyaya *et al.*, 1983).

2.2 Designing the Transgene for Effective Expression

Transgenic plant technology has become a flexible platform for cultivar improvement as well as for studying gene function in plants. As the process of transformation protocols was elucidated important information came to light that made the development of efficient plant transformation vectors possible. It is known that

the major determinant of gene expression (level, location, and timing) is the upstream of the coding region, which is termed as “the promoter”, and is of vital importance. Any genes that are to be expressed in the transformed plant have to possess a promoter that will function in plants. This is an important consideration as many of the genes that are to be expressed in plants, particularly reporter genes and selectable marker genes are of bacterial origin. They, therefore, have to be supplied with a promoter that will drive their expression in plants. Transgenes also need to have suitable “terminator” sequences at their 3' terminus to ensure that transcription ceases at the correct position. Failure to stop transcription can lead to the production of aberrant transcripts and can result in a range of deleterious effects, including inactivation of gene products, and increased gene silencing (Slater *et al.*, 2003b). Husnain *et al.* (1997) investigated the effects of different promoters, actin, cauliflower mosaic virus (CaMV), and Win6 on the expression of β -glucuronidase and kanamycin resistance marker genes introduced into zygotic embryos of chickpea. The CaMV promoter exhibited maximum efficiency at 44% followed by actin and Win6 promoters. Seed specific promoters are also useful for expression of foreign genes in the seeds. Shasany and Koundal (2000) isolated a *C. arietinum* legumin promoter, which shows similarity to chickpea 5' part of the legumin structural gene and strong homology with pea promoter and pea *legumin A* gene sequence. This promoter can be utilized for expression of foreign genes in seeds of chickpea. A chimaeric, truncated bacterial *cryIA(c)* gene construct was developed for plant expression with the CaMV 35S promoter, *nos* terminator, an initiatory kozak sequence, and a translational enhancer sequence of tobacco mosaic virus. This *cryIA(c)* gene was co-transferred with a plasmid-containing *nptII* gene as the selection marker. The *cryIA(c)* gene was inhibitory to the development of the feeding larvae of *Heliothis armigera* Hubner, the chickpea pod-borer (Kar *et al.*, 1997).

The efficient production of transgenic plants requires stringent selection procedures supported by a selectable marker gene that confers resistance to agents, such as antibiotics or herbicides. Several such selection systems have recently been described for grain legumes, based on the marker genes neomycin phosphotransferase

II (*nptII*), hygromycin phosphotransferase (*hph*, *aphIV* or *hyg*), phosphinothricin acetyltransferase (*bar* or *pat*), conferring resistance to kanamycin, hygromycin, and the herbicide phosphinotricin (BASTAM), respectively. Transformation of chickpea was done by a seed specific alpha amylase inhibitor (αAII) gene from *P. vulgaris* and the *nptII* gene as selectable marker (Sarmah *et al.*, 2004). At present, only a small number of reporter genes are in wide use in plant transformation vectors, these being β -glucuronidase (*uidA* or *gus*), green fluorescent protein (*gfp*), luciferase genes (*lux* and *luc*) and, to a lesser degree, the chloramphenicol acetyltransferase gene (*cat*) (Slater *et al.*, 2003c). Senthil *et al.* (2004) described the stable integration and the expression of marker genes through three generations of transformed chickpea plants. pGIN1 binary plasmid construct included an intron-containing *uidA* gene (coding for GUS) (Vancanneyt *et al.*, 1990) under the control of a CaMV 35S promoter and a CaMV 35S terminator along with a *bar* gene (bialaphos resistance) driven by a CaMV 35S promoter element with a nopaline synthase (*nos*)pA terminator sequence. Earlier reports (Fontana *et al.*, 1993; Kar *et al.*, 1996) did not provide substantial evidence for the stable integration of transgenes into the progeny of the primary transformants. Krishnamurthy *et al.* (2000) obtained a single sterile T₁ plant containing the *GUS* gene, which had no detectable expression of the marker gene. Tewari-Singh *et al.* (2004) described T₀ transformants using three different selection systems, and one of these transformants gave transgenic progeny. Polowick *et al.* (2004) constructed a plasmid contained a bi-functional fusion gene conferring both *gus* and *nptII* activities, under the control of a 35S35SAMV promoter for chickpea transformation. The plasmid contains a bi-functional fusion gene (*gus:nptII*) conferring both *gus* and *nptII* activities (Datla *et al.*, 1991) with a 35S35SAMV promoter (Datla *et al.*, 1993), a NOS (nopaline synthase) terminator, and an intron (Vancanneyt *et al.*, 1990). Sarmah *et al.* (2004) reported transformation of chickpeas with a seed-specific chimaeric gene encoding bean αAII , using *nptII* as the selectable marker gene. The selectable markers *bar* and *nptII* have both been used for production of transformed chickpeas (Molvig *et al.*, 1995; Kar *et al.*, 1996). But Sarmah *et al.* (2004) used *nptII* that confers resistance to kanamycin, has proved to be a reliable selection system.

Despite the improvements made in vector design and advances in our understanding of both the mechanisms of transgene integration and plant gene expression, plant transformation is still in many ways an imprecise art. If more than one gene are in the vector, different promoters and terminators should be used for each of them. The use of the same promoter and/or terminator can lead to an increase in gene silencing. Multiple genes in one vector should not be immediately adjacent to each other, and should be in the same orientation. This avoids adjacent inverted repeats that cause plasmid instability in bacteria and increased gene silencing in plants (Slater *et al.*, 2003d).

2.3 *In Vitro* Regeneration: A Prerequisite for Genetic Transformation

A reproducible, reliable transformation system, combined with traditional breeding techniques, could aid in improving both the quality and yield of a given crop (Polowick *et al.*, 2004). One of the prerequisites for successful gene transfer in plants is the availability of a suitable protocol for transformation, which is compatible with *in vitro* plant regeneration methods of the targeted species (Kar *et al.*, 1996). Two methods of plant regeneration are widely used in transformation studies, i.e., somatic embryogenesis and organogenesis. In somatic embryogenesis, embryolike structures (embryoids), which can be developed into whole plants in a way analogous to zygotic embryo, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. On the other hand, organogenesis relies on the production of organs, either directly from an explant or from a callus culture by suitable manipulation of growth medium. Key to success in integrating plant tissue culture technology into plant transformation strategies is the realization that a quick and efficient regeneration system must be developed. However, this system must also allow high transformation efficiencies from whichever transformation technique is adopted (Slater *et al.*, 2003a).

In vitro plant regeneration in chickpea has been achieved from shoot meristem through organogenesis (Bajaj and Dhanju, 1979; Sharma *et al.*, 1979), immature cotyledons (Shri and Davis,

1992) and through embryogenesis from immature cotyledons (Sagare *et al.*, 1993). *In vitro* plant regeneration in chickpea has also been reported from leaflet callus (Barna and Wakhlu, 1993; Kumar *et al.*, 1994). Regeneration studies using various explants were reported to produce shoots, either indirectly through a callus phase (Khan and Ghosh, 1984; Barna and Wakhlu, 1994; Altinkut *et al.*, 1997) or directly (Shri and Davis, 1992; Kar *et al.*, 1996; Subhadra *et al.*, 1998; Chakrabarty *et al.*, 2000; Shikha *et al.*, 2001; Sarmah *et al.*, 2004). High numbers of shoots per explant were produced from surface sterilized half embryonic axes attached to a cotyledon (Sarmah *et al.*, 2004). An efficient protocol for the regeneration of whole chickpea plants using embryonic axes after removal of the shoot and root tips as well as the axillary bud has been shown by Jayanand *et al.* (2003). The concentration of growth regulator was first indicated as critical for growth and morphogenesis by Skoog and Miller (1957). They reported that a higher cytokinin to auxin ratio promotes shoot growth in contrast to Bajaj and Dhanju (1979), who reported that only cytokinin has significant effect on the induction of multiple shoot. Rao (1990) and Fontana *et al.* (1993) reported higher regeneration frequency in chickpea in presence of 6-benzylaminopurine (BAP). Kar *et al.* (1996) achieved multiple

shoot induction in chickpea using BAP and α -naphthaleneacetic acid (NAA) in regeneration medium. Thidiazuron in combination with BAP was also reported to produce multiple shoots in chickpea (Murthy *et al.*, 1996). Tissue culture and transformation conditions are strongly dependent on the genotype of explant (Adkins *et al.*, 1995). Genotype dependency of regeneration efficiency has been reported by Altinkut *et al.* (1997) using callus-derived plantlets and seedlings of chickpea. Reports on *in vitro* regeneration process of chickpea are presented in Table 2.

It is very important to identify a set of germplasm of donor gene pools selected from individual crops for gene transfer studies and crop improvement. The contrasting growing regimes of cultivated chickpea and its wild progenitor may have resulted in a different allelic repertoire at different loci in the wild and cultivated gene pools. In recent years, attempts have been made to use wild *C. reticulatum* as a genetic resource for chickpea improvement (Abbo *et al.*, 2002). The world collection of chickpea germplasm contains genotypes that remain uncultivated because of poor agronomic characteristics, but possess a strong capacity for resistance to *A. rabiei*. Wild relatives of *C. arietinum* also possess strong resistance, and may be bred with cultivated varieties to incorporate potential

Table 2 Reports on *in vitro* regeneration process of chickpea (*Cicer arietinum* L.) using various explants and different media compositions

S. no.	Explants	Optimized growth media	Processes of regeneration	References
1	Freeze preserved meristem	MS+11.4 mg l ⁻¹ IAA+2.3 mg l ⁻¹ Kin	Shoot meristem organogenesis	Bajaj and Dhanju, 1979
2	Immature cotyledon	B5+2,4-D+2,4,5-T+NAA+IAA+BA+Kin+Zeatin+ABA	Organogenesis from cotyledon like structure	Shri and Davis, 1992
3	Mature seeds	MS+10 mM TDZ	Direct organogenesis	Malik and Saxena, 1992
4	Immature cotyledon	MS+3 mg l ⁻¹ 2,4,5-T	Direct somatic embryogenesis	Sagare <i>et al.</i> , 1993
5	Leaf	MS+25 μ M 2,4-D	Organogenesis from callus	Barna and Wakhlu, 1993, 1994
6	Meristem tips	DKWC+4.4 μ M BA+0.05 μ M IBA	Direct organogenesis	Bradt and Hess, 1993
7	Embryonic axes without apices	MS+B5 Vit+2.0 mg l ⁻¹ BAP+0.05 mg l ⁻¹ NAA	- do -	Kar <i>et al.</i> , 1996
8	Epicotyl	Basal medium+ BAP+Kin+IAA	Original paper was not found	Vani and Reddy, 1996
9	Cotyledonary nodes	MS+10 μ M TDZ+ 10 μ M L-Proline	Direct somatic embryogenesis	Murthy <i>et al.</i> , 1996
10	Hypocotyl	B5+BA	Direct organogenesis	Islam <i>et al.</i> , 1999
11	Internode	MS+B5 Vit+2,4-D+BAP+NAA+Kin+IAA	Organogenesis from callus and direct organogenesis	Huda <i>et al.</i> , 2000
12	Embryo axes and germinating seeds	MS+2iP+TDZ+Kin+GA3+IBA+NAA	Direct organogenesis	Jayanand <i>et al.</i> , 2003

resistance genes (Collard *et al.*, 2001). Targeted transfer of genes from the wild *Cicer* species into the cultivated species would represent an elegant application of transformation technology. Chickpea transformation, now considered as a routine procedure in chickpea improvement, has brought the application of this technology much closer to reality (Fontana *et al.*, 1993; Hamblin *et al.*, 1998; Chakrabarty *et al.*, 2000; Krishnamurthy *et al.*, 2000; Sharma and Ortiz, 2000; Jaiwal *et al.*, 2001). The applicability of this technology will, however, depend on the identification of key genes, the number of genes controlling a particular character, and public acceptance of cultivars resulting from transformation technology (Croser *et al.*, 2003).

2.4 Genetic Transformation

Two methods, namely *Agrobacterium*-mediated and particle bombardment, have extensively been employed for genetic transformation of crop plants. Regeneration via the callus lends itself easily (compared to explants regenerating directly) to *Agrobacterium*-mediated transformations, while direct regeneration is more amenable for particle bombardment (Chandra and Pental, 2003). *Agrobacterium*-mediated transformation has been used successfully in grain legumes for over a decade (Christou, 1997). Reports on genetic transformation of chickpea are presented in Table 3. Early transformation experiments, which relied on callus cultures, failed due to poor shoot regeneration but demonstrated the potential of *Agrobacterium tumefaciens* as a transformation vector for chickpea (Islam *et al.*, 1994). Fontana *et al.* (1993) first reported successful chickpea transformation. They used embryonic axes as explants, which were co-cultivated with *A. tumefaciens* and produced at least two independent plants, which were confirmed at molecular level. Subsequently, using almost similar experimental protocols, the formation of multiple shoots from different genotypes and the production of primary transgenic plants were reported (Kar *et al.*, 1996; Krishnamurthy *et al.*, 2000).

In the above-mentioned transformation protocols, the transgenic plants were selected via multiple cycle *in vitro* on media containing kanamycin (Fontana *et al.*, 1993; Kar *et al.*, 1996)

or phosphinothricin (Krishnamurthy *et al.*, 2000). Transformation frequencies and reproducibility in these early breakthroughs were low and limited their practical applicability. However, both transformation frequency and reproducibility have been improved recently in four separate studies (Polowick *et al.*, 2004; Sarmah *et al.*, 2004; Senthil *et al.*, 2004; Sanyal *et al.*, 2005), enabling the routine application of transformation technology to chickpea. In these protocols embryonic axes were used as explants. Cotyledonary explants containing half embryonic axes were used by Sarmah *et al.* (2004), while longitudinally sliced embryonic axes were used by Polowick *et al.* (2004) and Senthil *et al.* (2004). Sanyal *et al.* (2005) had precultured the explants (L2 layer) for 24 h on solidified MS-basal medium supplemented 1 mg l^{-1} BAP. The four systems appear equally useful and have some common elements like mature seeds are explant source, embryonic axes contain the target tissue, submersion of explants in *Agrobacterium* suspension followed by co-cultivation, frequent subcultures on selection medium, and transfer of rooted/grafted shoots to soil in the greenhouse. Although different groups have reported successful transformation of chickpea, the overall frequency of transformation is still very low (0.1–1.0%). Thus, to generate a sufficient number of transgenic lines with desired expression level a large number of explants need to be co-cultivated. In crops such as, maize and soybean, frequency of transformation has been dramatically enhanced with the use of Thiol compounds and L-cystine (Olhoft and Somers, 2001; Olhoft *et al.*, 2003). Similar efforts may also be made to improve the chickpea transformation efficiency. Use of super virulent strains of *A. tumefaciens* may be another option.

The biolistic gene (gene gun method) delivery where tungsten or gold particles are coated with the DNA that is to be used to transform the plant tissue has been successful in producing transgenic lines (Slater *et al.*, 2003e). There have been a few reports on production of transgenic chickpea plants using biolistic gene delivery. Kar *et al.* (1997) demonstrated the expression of *cryIA(c)* gene of *Bacillus thuringiensis* in transgenic chickpea plants. Explants and regeneration procedure were the same as their previous *Agrobacterium*-mediated transformation method, but only the gene construct and transformation

Table 3 Reports on genetic transformation of chickpea (*Cicer arietinum* L.) using various ex-plants, cultivars, methods of gene transfer, marker genes, and genes of interest

S. no.	Explant	Cultivar	Method of gene transfer	Genes transferred	Reference	Remark
1	Embryonic axis without apical meristem	Unknown	AT (LBA 4404)	<i>nptII, gus</i>	Fontana <i>et al.</i> , 1993	Low reproducibility, expression and integration of transgene in T ₀ by Western and Southern analysis, respectively
2	- do -	ICCV-1, ICCV-6, Desi	AT (LBA 4404)	<i>nptII, gus</i>	Kar <i>et al.</i> , 1996	Low reproducibility, transgene integration by Southern analysis in T ₀
3	Embryonic axis	ICCV-1, ICCV-6	MPB	<i>nptII, CryIAc</i>	Kar <i>et al.</i> , 1997	Low reproducibility, transgene transmitted to T ₁ (PCR analysis)
4	- do -	PG 1, Chafa, Turkey, PG 12	AT (C58, C1, GV2260)	<i>nptII, pat, gus</i>	Krishnamurthy <i>et al.</i> , 2000	Low reproducibility, <i>gus</i> gene expressed in T ₀ , transmission into T ₁ (PCR)
5	Cotyledons with half embryonic axis	Vijay	AT (AGL-1)	<i>nptII, Bt-CryIAc</i>	Das and Sarmah, 2003	Transgene transmitted to T ₁ (PCR for <i>nptII</i>)
6	Embryonic axis	P 362, P 1042, P 1043	AT (EHA 101)	<i>nptII, gus, bar</i>	Tewari-Singh <i>et al.</i> , 2004	Low reproducibility, <i>gus</i> gene expressed in T ₀ , transmission to T ₁ (PCR)
7	Cotyledons with half embryonic axis	Semsen	AT (AGL 1)	<i>nptII, αAII</i>	Sarmah <i>et al.</i> , 2004	Reproducibility good, gene integration, transmission up to T ₁ and expression in T ₁ shown by Southern and Western analysis, respectively, high <i>αAII</i> activity in T ₁ seeds
8	Sliced embryonic axis	ICCV-5, H 208, ICCL 87322, K 850	AT (AGL 1)	<i>bar, gus, PGIP</i>	Senthil <i>et al.</i> , 2004	Reproducibility good, transmission and expression of <i>gus</i> up to T ₃
9	- do -	CDC Yuma	AT (EHA 105)	<i>gus, nptII</i>	Polowick <i>et al.</i> , 2004	Reproducibility good, transmission and expression of <i>gus</i> up to T ₃
10	L2 layer of cotyledonary nodes	C 235, BG 256, Pusa 362, Pusa 372	AT (LBA 4404)	<i>gus, nptII, CryIAc</i>	Sanyal <i>et al.</i> , 2005	Reproducibility good, transmission and expression of <i>nptII</i> and <i>CryIAc</i> in T ₁ was shown, good <i>CryIAc</i> activity

method were different. Bio-Rad Biolistic 1000/He particle gun was used for delivering gene to the explants. Bacterial *cryIA(c)* gene was modified for plant expression. Transgenic kanamycin resistant chickpea plants were obtained through multiple shoot formation. Molecular analyses of the transformants indicated the presence of the transferred functional *cryIA(c)* gene in plant. The expression level of the *cryIA(c)* gene was

inhibitory to the development of the feeding larvae of *Heliothis armigera* Hubner, the chickpea pod borer.

Transient expression of marker genes in the zygotic embryos of chickpea was demonstrated and conditions for optimum transient expression of *gus* and *nptII* genes were established. When 12 µgm of plasmid DNA per milligram of tungsten particles accelerated with helium discharge at a

60 kg cm⁻² of mercury from a distance of 24 cm resulted in optimal transient expression of the *gus* and *nptII* gene in chickpea embryos (Husnain *et al.*, 1997).

2.5 Selection of Transformed Plants

The efficient production of transgenic plants requires stringent selection procedures supported by a selectable marker gene that confers resistance to agents such as antibiotics or herbicides. Several such selection systems have recently been described for grain legumes, based on the marker genes *nptII*, hygromycin phosphotransferase (*hph*, *aphIV*, or *hyg*), phosphinotricin acetyltransferase (*bar* or *pat*) conferring resistance to kanamycin, hygromycin, and the herbicide phosphinotricin (BASTAM), respectively (Chandra and Pental, 2003). In some cases, only a few transformed plants have been regenerated. Further optimizing the transformation parameters such as inoculation, co-culture condition, and selectable marker could increase transformation frequency. Although kanamycin has been the most favored selectable agent, it has not been proved an efficient selectable marker for grain legumes. The development of efficient uptake of selective agents by the regenerating tissues has increased recovery of transformed shoots, as has been shown by efficient selection in soybean on glufosinate-containing medium (Zhang *et al.*, 1999). The selectable markers *bar* and *nptII* have both been used for production of transformed chickpeas (Molvig *et al.*, 1995; Kar *et al.*, 1996). Sarmah *et al.* (2004) have used *nptII* in conjunction with kanamycin which has proved to be a reliable selection system where all of the plants selected based on kanamycin were proved to be transformed.

A rapid and reliable selection strategy has deterred chickpea improvement programs (Somers *et al.*, 2003). Tewari-Singh *et al.* (2004) developed an efficient and reliable nonantibiotic selection strategy using the *pat* and aspartate kinase (*AK*) genes for the production of transgenic chickpea. Kanamycin has been used for selection in most of the chickpea transformation studies reported (Fontana *et al.*, 1993; Kar *et al.*, 1996, 1997; Krishnamurthy *et al.*, 2000). There is only one report on the use of phosphinotricin (PPT) as a selective agent for chickpea (Krishnamurthy

et al., 2000), although it has been successfully used for selection in other legumes such as pea (Schroeder *et al.*, 1993, 1995; Bean *et al.*, 1997). Tewari-Singh *et al.* (2004) reported the use of the AK/LT selection system for the production of fertile transgenic chickpea. The results also showed that kanamycin and PPT can be used as selective agents for chickpea transformation. PPT was found to be a better selection marker than kanamycin as transgenic plants could be identified more easily and rapidly using the former.

3. FUTURE ROAD MAP FOR TRANSGENIC CHICKPEA

Although there has been an incremental increase in the productivity of chickpea during the past two decades using conventional breeding approaches, the productivity continues to be rather low, and far below the potential. The global chickpea demand in 2101 is estimated at 11.1 million metric tons, an increase of 29% from its level of 8.6 million metric tons during 2003–2004. Thus, a combination of productivity enhancement through genetic improvement might help achieving this target. Genes for transformation can be broadly divided into those that will be used to overcome the agronomic limitations (high yield potential, biotic and abiotic stresses) and the ones that could be used to enhance the value-added traits. Although major emphasis is currently being placed on improving the primary constraints, the manipulation of value-added traits, such as improving quality parameters like nutrition will be of much concern for chickpea improvement using transgenic technology. Transgenic technology could conceivably be used in chickpea for the introduction of disease–pest resistance; drought and salinity stress tolerance as well as value-added traits such as improved vitamins, micronutrients, and protein content thus enhancing the crop product value, quality, and safety. Current efforts include incorporating immunity or very high resistance to several viral and fungal diseases through transformation of chickpea cultivars that have very high demand for which no adapted resistant chickpea genotypes are available. Improved crop protection through the transfer and expression of disease resistance genes will decrease

or eliminate the usage of insecticides, pesticides, which are costly to the grower and may be harmful to the environment.

Finally, there are numerous traits that potentially could be manipulated with single or few gene introductions to produce more disease and pest resistant, drought and salinity tolerant, healthier, higher quality chickpeas.

3.1 Insect Resistance

The extent of crop losses by pod borer (*Helicoverpa armigera* Hubner) has been estimated to over US\$ 1 billion annually where in chickpea it is estimated at over US\$ 400 million in South Asia alone. Thus, improving chickpea resistance to this pest through genetic transformation is likely to contribute to sustainable crop protection and environmental conservation. For intractable pest problems such as *Helicoverpa*, the presumption is that no single tactic will suffice in itself to contain this pest. It has long been recognized that host plant resistance would be one of the most effective management options, but thus far, the levels of resistance in the available chickpea germplasm have been found to be very low. Genes encoding insecticidal proteins can be extensively used in generating chickpea transgenic plants for resistance to pod borer. Advancement in transgenic technology has made it possible to impart resistance to this devastating insect-pest of chickpea using different insecticidal genes from microorganisms, such as *Bt* crystal protein genes. Toxins genes from *Bt* deployed through transgenic plants are environmentally benign and incentive is to have improved resistance to this damaging pest while reducing reliance on synthetic pesticides. Transgenic chickpeas using *cry1Ab*, *cry1Ac*, and *SBTI* genes have already been developed at ICRISAT and are being subjected to insect bioassays and evaluated under contained field trials (K.K. Sharma, personal communication). Besides use of *Bt* genes, genes of plant origin like, lectins, diverse proteases, protease and amylase inhibitors also hold great promise in development of insect resistance in chickpea. Another possibility is to achieve an early and selective control of pod borer by targeting its specific physiology. Hence, insect chitinase-based strategy with different or multiple chitinase genes for the control of *H. armigera* is worth using for

pod borer resistance in chickpea. These can also be used as companion transgenes, which, when engineered into the plant together with a *Bt* gene, multiply the effect of *Bt* δ -endotoxin by weakening the peritrophic membrane surrounding the insect midgut, thus improving resistance to the legume pod borer.

3.2 Disease Resistance

Chickpeas are susceptible to a number of fungal diseases, which affect the growth and productivity of this crop. Diseases of economic importance include *Fusarium* wilt (*F. oxysporum* f. sp. *ciceris*), *Ascochyta* blight (*A. rabiei*), botrytis gray mold (*Botrytis cinerea*), collar rot (*Sclerotium rolfsii*), and dry root rot (*Rhizoctonia bataticola*) among others. In chickpea, varieties need to be developed having multiple resistance against *Fusarium* wilt, collar and root rots, *Ascochyta* blight, and botrytis gray mold to succeed in farmers' fields. The way is now open to the testing of genetic transformation approaches designed to enhance fungal resistance in chickpeas. Hydrolytic enzymes such as chitinases and glucanases, which degrade the fungal cell wall components, also pose as attractive candidates for development of disease resistant chickpeas. The endochitinase derived from the biocontrol fungus *Trichoderma harzianum* appears to be particularly effective in inducing resistance to fungi and this version of endochitinase can also be used for genetic transformation purposes. Besides, another set of antifungal genes encoding for polygalacturonase-inhibiting proteins (PGIP) (that inhibit one or more key enzymes used by *Botrytis* and some other pathogenic fungi during invasion, thereby delaying the disease long enough for other defenses to take over) may be explored for transformation purposes in chickpea. Resistance to fungal diseases in chickpea can be achieved by using another group of plant derived secondary compounds known as phytoalexins, which have a direct inhibitory effect on the growth of fungal pathogens.

3.3 Biofortification

Improvement in nutritional quality traits is important for providing better nutrition to the consumers. In developed countries, there is already

a growing interest in use of chickpea as functional food or nutraceuticals and in developing dietary supplements (P.M. Gaur, personal communication). Chickpea is deficient in the sulfur-containing essential amino acids methionine and cystine, which lower its dietary and nutritional value. The nutritional quality of chickpea can be improved by either raising the level of sulfur-containing amino acids of storage proteins or by changing the proportion of methionine-rich proteins already present in the seeds. High methionine trait cannot be produced by conventional breeding methods because of its failure to detect genotypes containing desirable levels of methionine. There are reports on transferring a gene encoding a methionine-rich seed 2S albumin from sunflower to lupins. The albumin constituted 5% of the total protein in the seeds of the transgenic lupins and led to the doubling of the seed protein methionine. This approach might as well prove to be an alternative approach for developing methionine-rich chickpeas.

Although chickpea already contains higher amounts of carotenoids such as β -carotene, cryptoxanthin, lutein, and zeaxanthin than genetically engineered “golden rice” (Abbo *et al.*, 2005), the recent success in chickpea transformation technology might enable further enhancement according to the RDA (recommended dietary allowances) recommendations. The potential of such approaches is still largely unrealized but should yield seeds with enhanced nutritional quality for the future.

3.4 Enhancing Quality Traits

Chickpea does not contain any specific major antinutritional factors, such as ODAP in grasspea, vicin in faba bean, and trypsin inhibitors in soybean. However, A PA2-homologous protein, isolated from chickpea (*C. arietinum*), has been shown to have lectinlike properties and has been implicated in allergic responses in chickpea-sensitive individuals. This indicates that a reduction or removal of PA2 using genetic transformation approaches or gene silencing could lead to significant improvements in chickpea seed quality for food uses. Another negative factor ascribed to chickpea consumption is more flatulence due to higher concentrations of raffinose

family oligosaccharides (RFOs) than any other dry legumes. Genetically manipulating the level of RFOs has been achieved by inhibiting the enzyme galactinol synthase, which catalyzes the first committed reaction in the pathway involving the synthesis of galactinol from UDP-Gal and myo-inositol. However, a better strategy involving the activation of α -galactosidase isolated from a thermophilic bacterium (*Thermotoga neapolitana*) for degradation of RFOs can be used considering the physiological importance of the RFOs during seed development and storage. Promoting the synthesis of a galactosyl cyclitol “ciceritol” in chickpea that is more slowly hydrolyzed by α -galactosidase than the RFOs could be another alternative for the reduction of RFOs thereby imparting protection during seed development and storage.

3.5 Abiotic Stress Resistance

Chickpea improvement for adaptation to abiotic stresses is crucial for stabilizing the yield in this major food legume crop. Genetic transformation provides prospects to enhance tolerance of chickpea to abiotic stresses including drought, salinity, and low temperature. Enhancing the production of chickpea under water deficits is vital as it is a rainfed crop. However, multigenic and quantitative nature of drought makes it difficult to breed for abiotic stress tolerance using conventional plant breeding. Knowledge of key genes involved in tolerance may allow genetic transformation of chickpea using such genes thus speeding up the breeding process. For example, efforts on enhancing the drought tolerance in chickpea are ongoing in ICRISAT, where transgenics carrying *P5CSF129A* gene encoding Δ^1 -pyrroline-5-carboxylate synthetase driven by CaMV 35S promoter are being developed for overproduction of an osmolyte proline, which is known to have a role in osmotic adjustment and cell protection under water deficits. In another effort, *DREB1A* cDNA from *Arabidopsis thaliana*, capable of transactivating DRE-dependent transcription in plant cells under the control of stress inducible *rd29* promoter was introduced into a popular chickpea cultivar for improving drought and salinity tolerance in this important pulse crop (K.K. Sharma, unpublished results).

3.6 Nutrient Responsive Genotypes

With a shrinking area of quality arable land for agriculture and its increasing allocation to staple cereal or high value crops, it is unlikely that in future chickpea will be grown on lands that are better endowed with nutrients than on which it is grown at present. Mineral nutrient deficiency will, therefore, continue to be a major and increasing constraint to chickpea production. Mineral nutrient deficiencies commonly observed in major chickpea producing areas are: nitrogen (N) (due to suboptimal nitrogen fixation), phosphorus (P), sulfur (S), iron (Fe), zinc (Zn), and boron (B). It seems, therefore, realistic to expect some recovery of losses in yield due to nutrient deficiency through genetic improvement for increased nutrient acquisition. Detailed information about genetic aspects of plant mineral nutrition should be derived to augment research strategy for developing nutrient use efficient genotypes in chickpeas. Outputs of transgenic research in other crops, in due course of time, could be extended for enhancement of nutrient uptake mechanisms and salinity tolerance in chickpea.

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Lupin

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The genus *Lupinus* comprises herbaceous annuals and shrubby perennials in two main geographical distributions. One of these, the largest with around 500 taxa, occurs in the Americas from Alaska to the Andean highlands of Peru, Brazil, Uruguay, and Argentina. As opposed to the New World taxa, the Old World group comprises only 11 species that occur within the Mediterranean basin and extend into East Africa. Among this group are six species with rough seeds and six smooth-seeded species. It is species from this latter group (*Lupinus angustifolius*, *Lupinus albus*, and *Lupinus luteus*) that have received the greatest attention from plant breeders seeking to develop suitable crop types. Of the rough-seeded species only *Lupinus cosentinii* has had any breeding effort although there is renewed interest in this group and preliminary breeding work has been initiated with collected material for a number of species (Buirchell and Cowling, 1998). *Lupinus mutabilis* has been used as a grain crop for human consumption in the Andean region since pre-Columbian times and this is the only species from the New World that has received attention from plant breeders.

1.2 Botanical Description

The taxonomy of the genus in the New World has not been well studied and considerable uncertainty remains as to their origin and evolution. The smooth-seeded Mediterranean species seem most likely to have originated in western Asia (Turkey and Syria) but today comprise three distinct monospecific groups (*L. angustifolius*, *L. albus*, and *Lupinus micranthus*) plus the *L. luteus*/*Lupinus hispanicus* complex (Gladstones, 1998). Lupins in general are not tolerant to heavy shading, occurring in open cleared habitats and especially as pioneer species on disturbed sites. A few selections are able to persist in slightly alkaline soils but most thrive on deep well-drained acid/neutral soils. In the New World taxa, the common chromosome number is $2n = 48$ (e.g., *L. mutabilis*) but variations from 36 to 96 have been noted. In the Old World rough-seeded species chromosome numbers vary from $2n = 32$ in *L. cosentinii* to $2n = 42$ in *Lupinus pilosus*. The smooth-seeded species have higher chromosome numbers, with $2n = 40$ in *L. angustifolius*, 50 in *L. albus*, and $2n = 52$ in the remaining species. A full taxonomic/botanical treatment of the Mediterranean lupin species can be found in Gladstones (1974, 1998). There have been frequent

attempts to generate interspecific hybrids with an aim to secure favorable traits in the improved species, but there appear to be a number of genetic barriers and there has been little success to date (Atkins *et al.*, 1998).

1.3 Economic Importance

Across the whole genus lupins generally contain high levels of quinolizidine alkaloids in both foliage and mature seeds (Wink *et al.*, 1995; Wink, 2003). The seeds particularly are “bitter” and unpalatable, as well as toxic to both monogastric and ruminant animals. As a consequence, lupins that have been used traditionally for human food in Latin America and Egypt, for example are debittered by washing. Breeding in *L. angustifolius*, *L. albus*, and *L. luteus* has led to the development of varieties with much lower levels of alkaloids and as a result greater palatability in stock feed formulations or for human consumption.

The most important crop lupin is *L. angustifolius* (“narrow-leaved lupin” or NLL, and in the United States known as “blue lupine”) and in Australia is the major “pulse” crop where as much as 1 million hectares has been planted with more than 90% of this area in Western Australia. The Western Australian Lupin breeding program was driven by the need for a grain legume crop to sustain cereal production through rotation in cropping systems. Because of the predominance of deep coarse-textured, sandy, acid soils in the wheat belt, narrow-leaved lupin is the best adapted pulse for this purpose, producing grain yields of a ton per hectare or more of high protein grain and contributing a significant rotational benefit to a succeeding wheat/barley crop. The benefit stems from residual N in the soil together with a beneficial disease break and other less well-defined positive impacts (on soil structure, K availability and nutrient cycling). In this environment the soils are low in available N and lupins nodulate profusely, typically deriving more than 90% of their N from the atmosphere. Typically the symbiosis is hydrogenase uptake negative (hup^- ; Layzell *et al.*, 1979) and there is growing evidence that the hydrogen gas produced by root systems nodulated with hup^- rhizobia leads to further rotational benefits as a consequence of effects on the soil biota (Dong *et al.*, 2003).

1.4 Traditional Breeding

The major breeding goal before and following the Second World War was to select “sweet” genotypes that had lower levels of alkaloids and as a consequence greater palatability to grazing animals and increased potential as grain for human consumption (von Sengbusch, 1942; Gladstones, 1970). Equally important was selection for nonshattering pods and permeable seeds, earlier flowering, and shorter periods for grain filling. These traits were selected and used to develop a most successful breeding program for *L. angustifolius* (NLL) in Western Australia beginning in the 1950s and continuing today (Cowling *et al.*, 1998). Both natural and induced mutations were exploited in achieving success. Additional breeding goals included selection for resistance to a range of fungal and viral pathogens, frost tolerance, yield potential especially under increasing stand density, and plant architecture. More recently the NLL breeding program has sought to improve grain quality and specifically protein content. Current commercial NLL cultivars contain in excess of 35% protein on a whole seed basis with a relatively low content of S-containing amino acids (especially methionine), typical of many pulse grains (Pettersson, 1998). *L. luteus* cultivars have significantly higher protein levels and greater concentrations of essential amino acids (cysteine, methionine, and lysine). Lupins are unusual among grains in that mature seeds contain essentially no starch. The major carbohydrate reserves are heavily developed secondary-thickened cell walls in the cotyledons. Together with the thick seed coats, that are typical of all lupins, this material contributes to the relatively high fiber content of the grain. Carbohydrates in the seed coats are structural polysaccharides (cellulose and hemicelluloses) and in the cell walls of the cotyledons nonstructural polysaccharides rich in galactose, arabinose, and uronic acids (Pettersson, 1998).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

A significant limitation to NLL yield potential in cereal-based rotations is inadequate and incomplete weed control particularly by annual ryegrass

(*Lolium* spp.) that displays a high level of resistance to grass-specific herbicides, especially the triazines. Effective weed control is critical in dry land farming where early exploitation of soil moisture by highly competitive weeds at seeding and later in the season during grain filling seriously impacts yield. Consequently using genetic modification to introduce resistance to nonselective herbicides like glyphosate (Roundup®) and glufosinate (Basta®, Liberty®) in NLL could provide an innovative and effective means to address this need.

Lupins, like many other legume species, are characterized by prolific flowering but high rates of floral abortion and abscission (Pigeaire *et al.*, 1992). A range of environmental factors influence flower abortion and as a consequence lupins show low and variable harvest index and unstable yield. While significant improvement to pod set has been achieved from newer breeding lines of narrow-leaved lupin, there is good evidence for a central role of plant hormones, especially cytokinins, in determining abortion and a transgenic approach has been suggested (Atkins and Pigeaire, 1993).

Similarly, levels of S-containing amino acids are relatively low in legume grain proteins and this is also the case for lupins (Pettersson, 1998). While there is some variation in the methionine levels in seeds among different lupin species the slight variation within a species does not encourage selection for this trait from the available germplasm. It has been proposed that transformation with genes encoding plant proteins richer in essential amino acids and particularly methionine would improve the amino acid composition of the seed and sunflower albumin has been introduced into *L. angustifolius* to address this (see below).

Lupins are prone to severe yield restriction as a consequence of both fungal and viral diseases (Sweetingham *et al.*, 1998). Conventional breeding has progressively introduced significant levels of resistance for some of the fungal pests (*Pleiochaeta* leaf spot and root rots, *Phomopsis*, *Sclerotinia*, and to some extent Anthracnose), for one of the major viruses, cucumber mosaic virus (CMV), but not at all for the major viral disease, bean yellow mosaic virus (BYMV). Transgenic approaches to provide resistance for BYMV in narrow-leaved lupin have been outlined (Atkins *et al.*, 1998).

2. DEVELOPMENT OF TRANSGENICS

2.1 Donor Genes

Genetic modification of lupins has used a number of different genes and constructs that are summarized in Table 1.

2.2 Transformation Methods Employed

All methods published for stable transformation of lupins use *Agrobacterium tumefaciens* as a vector. Stable transformation methods have been developed for *L. angustifolius* (Molvig *et al.*, 1997; Pigeaire *et al.*, 1997), *L. luteus* (Li *et al.*, 2000), and *L. mutabilis* (Babaoglu *et al.*, 2000). The explant used for inoculation in the method developed by Molvig *et al.* (1997) was slices of the embryonic axis isolated from immature seeds. Pigeaire *et al.* (1997) used the shoot apex of the embryonic axis of a mature seed as an explant for inoculation (Figure 1). The apex was wounded with a fine needle before introduction of *Agrobacterium* as this increased the infection significantly. Four different strains of *A. tumefaciens* were tested (LBA4404, EHA101, K61, AgL0) for infection and AgL0 was the most effective as judged by β -glucuronidase (GUS) staining in explants after 3 days of co-cultivation. Later experiments showed that AgL0 was more effective than AgL1 for transformation generating more transgenic shoots 6 months after inoculation (P.M.C. Smith, unpublished data). Many cultivars were also screened to determine their susceptibility to infection by *A. tumefaciens*. Of the 19 cultivars tested initially, Unicrop was most efficiently infected based on transient GUS assays on explants after co-cultivation (Pigeaire *et al.*, 1997). Li *et al.* (2000) used the method of Pigeaire *et al.* (1997) for transformation of *L. luteus*.

Babaoglu *et al.* (2000) developed a method for *A. tumefaciens*-mediated transformation of *L. mutabilis* using a wounded shoot apex as the explant. The initial cell layers of the apex were removed before co-cultivation with *A. tumefaciens* strain 1065.

In addition to these methods for stable transformation of lupin species, *Agrobacterium rhizogenes* has been used to develop transformed hairy roots in *L. mutabilis* (Babaoglu *et al.*, 2004) and *L. albus* (Udhe-Stone *et al.*, 2005). For

Table 1 Genes used in transformation of lupin species

Species transformed	Desired trait	Donor gene (promoter, terminator)	Donor gene source	Selectable marker (promoter, terminator)	Other components (promoter, terminator)
<i>L. angustifolius</i>	Herbicide resistance ^(a)	<i>bar</i> (CaMV ^(g) 35S, <i>ocs</i> ^(h))	<i>Streptomyces hygroscopicus</i>	As for donor gene	<i>Uida</i> [GUS] (CaMV 35S, <i>nos</i>)
<i>L. angustifolius</i>	Herbicide resistance	<i>bar</i> (CaMV 35S, <i>ocs</i>)	<i>Streptomyces hygroscopicus</i>	As for donor gene	<i>Uida</i> [GUS] (CaMV 35S, CaMV 35S)
<i>L. angustifolius</i>	High methionine in seeds ^(b)	SSA ⁽ⁱ⁾ (pea vicilin, pea vicilin)	<i>Helianthus annuus</i> (sunflower)	<i>bar</i> (CaMV 35S, <i>ocs</i>)	
<i>L. luteus</i>	BYMV resistance ^(c)	<i>Nta</i> (CaMV 35S, <i>nos</i>)	Bean yellow mosaic virus	<i>bar</i> (CaMV 35S, ?)	
<i>L. mutabilis</i>	Confirm transformation ^(d)	Gus-intron (CaMV 35S, ?)	<i>Escherichia coli</i>	<i>nptII</i> (<i>nos</i> , ?)	
<i>L. angustifolius</i>	Stable yield ^(e)	<i>ipt</i> ^(k) (<i>TP12</i> ^(l) , <i>nos</i> ^(m))	<i>Agrobacterium tumefaciens</i>	<i>bar</i> (CaMV 35S, CaMV 35S)	
<i>L. angustifolius</i>	Stable yield ^(e)	<i>ipt</i> (<i>con γ</i> ⁽ⁿ⁾ , <i>nos</i>)	<i>Agrobacterium tumefaciens</i>	<i>bar</i> (CaMV 35S, CaMV 35S)	
<i>L. angustifolius</i>	Viral resistance ^(f)	BYMV replicase (<i>S4S4</i> ^(o) , <i>MeI</i> ^(p))	Bean yellow mosaic virus	<i>bar</i> (CaMV 35S, <i>ocs</i>)	Twin T-DNA construct
<i>L. angustifolius</i>	Fungal disease resistance ^(f)	<i>AMP1</i> ^(q) (<i>ScI</i> ^(r) , <i>Sc3</i>)	<i>Macadamia integrifolia</i>	<i>bar</i> (<i>S4S4</i> , <i>MeI</i>)	
<i>L. angustifolius</i>	Fungal disease resistance ^(f)	<i>SphX6</i> ^(s) (<i>Sc4</i> , <i>Sc5</i>)	<i>Stylosanthes humilis</i>	<i>bar</i> (CaMV 35S, <i>ocs</i>)	

^(a)Pigeaire *et al.*, 1997^(b)Molvig *et al.*, 1997^(c)Li *et al.*, 2000^(d)Baoglu *et al.*, 2000^(e)P.M.C. Smith and C.A. Atkins, unpublished data^(f)S. Wylie, S. Barker, and P.M.C. Smith, unpublished data^(g)CaMV, cauliflower mosaic virus^(h)*ocs*, octopine synthase⁽ⁱ⁾SS4, sunflower seed albumin^(j)?, element not defined^(k)*ipt*, isopentenyl transferase^(l)*TP12*, TP12 floral specific promoter from tobacco^(m)*nos*, nopaline synthase⁽ⁿ⁾*con γ*, conglutin gamma seed specific promoter from *L. angustifolius*^(o)*S4S4*, double promoter from subterranean clover stunt virus genomic segment 4^(p)*MeI*, MeI terminator from *Flavaria bidentis*^(q)*AMP1*, antifungal protein^(r)Sc, subclover stunt virus^(s)*SphX6*, peroxidase gene

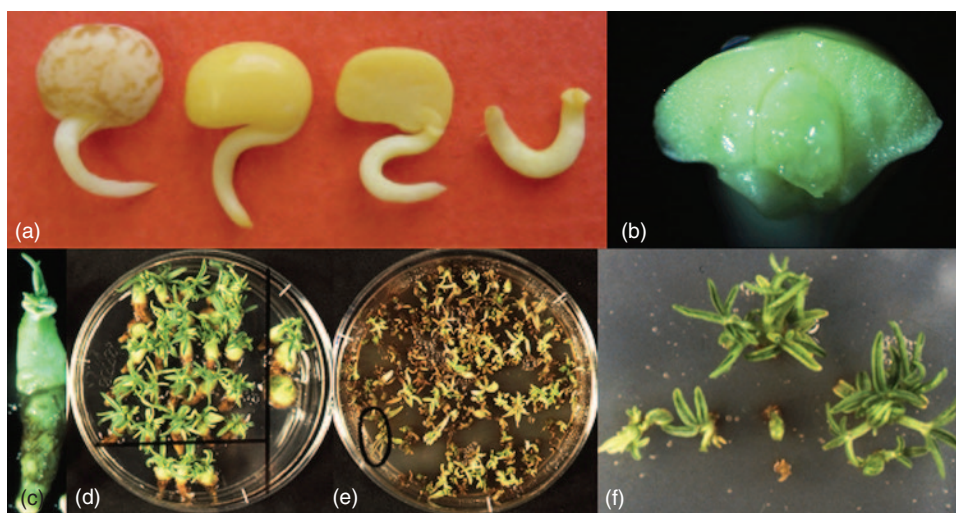


Figure 1 Lupin transformation process using the shoot apex as an explant (Pigeaire *et al.*, 1992). (a) Preparation of explants from germinated seeds. (b) The shoot apex explant that is infected with *Agrobacterium tumefaciens*. (c) An explant after co-cultivation, selection, and regeneration of shoots. (d) A plate of explants with regenerated shoots before they are subcultured. (e) Shoots removed from original explants after the first round of selection. (f) Shoots after three rounds of micropropagation with selection. The shoot in the middle was the original chimeric shoot. Axillary shoots removed from it can be seen around it. Those with more transformed tissue grow well while others that lack transformed cells do not survive on selective media

L. mutabilis hypocotyl, epicotyl, and stem explants were inoculated (Babaoglu *et al.*, 2004) and for *L. albus* the base of the root of a germinating seedling was excised and the wound inoculated (Udhe-Stone *et al.*, 2005). Much higher concentrations of isoflavones were produced in the transformed roots of *L. mutabilis* than in nontransformed roots and these levels could be sustained over a long period of time (Babaoglu *et al.*, 2004). This makes them an ideal potential source for commercial production of isoflavones.

2.3 Selection of Transformed Tissue

The *bar* gene from *Streptomyces hygroscopicus*, which encodes phosphinothricin acetyl transferase, was used as a selectable marker for transformed tissue in combination with the herbicide glufosinate (Basta®) for selection in both *L. angustifolius* (Molvig *et al.*, 1997; Pigeaire *et al.*, 1997) methods and for *L. luteus* (Li *et al.*, 2000). The *nptII* gene has also been tested as a selectable marker gene for *L. angustifolius* but it was not as effective as the *bar* gene with transformation frequencies approximately one third lower (Molvig

et al., 2003). Pigeaire *et al.* (1997) and Li *et al.* (2000) used a drop of Basta® on the top of the explant (shoot apex) for the initial selection after co-cultivation rather than incorporating the herbicide in the media. This method improved the efficiency of selection as the apex was still attached to the embryo and well removed from the media. After regenerating shoots had been removed for subculturing the Basta® was incorporated in the micropropagation medium.

For *L. mutabilis* Babaoglu *et al.* (2000) found use of the *nptII* gene as a selectable marker gene with kanamycin selection to be effective.

2.4 Regeneration of Whole Plants

In the *L. angustifolius* transformation method developed by Molvig *et al.* (1997), regeneration was achieved via organogenesis. Clumps were divided to produce single shoots and these were subcultured until ready for treatment with indolebutyric acid (IBA, 1 mg l⁻¹) to produce roots. This method was cultivar specific with only cv. Warrah producing transgenic plants and the transformation frequency was low (0.01%).

In the method of Pigeaire *et al.* (1997) for transformation of *L. angustifolius*, transformants are regenerated from axillary buds that develop as shoots on the apical dome explant (Figure 1). Since the process does not involve a *de novo* regeneration step (the axillary buds exist at the time of explant preparation) chimeric shoots may be produced. To increase the chance of obtaining transgenic progeny from these chimeras micropropagation on selective media is used (Figure 1). Micropropagation involves removal of axillary buds that develop on the shoots to fresh media. If these buds arose from a transgenic sector they survive and usually have more transgenic tissue than the parent shoot (Figure 1). At least four rounds of micropropagation are routinely done before transfer of the shoots to the glasshouse. Even with this micropropagation step, the germ cells are not always transformed and so the transgene is not transferred to progeny. Transgenic progeny are recovered from approximately 30% of transformed T₀ shoots. While the original shoots had to be grafted onto a nontransgenic root stock, growth of shoots on IBA (3 mg l⁻¹) is now used to promote root growth before shoots are transferred to the glasshouse (Atkins *et al.*, 1998). This method is used routinely for transformation of a range of *L. angustifolius* cultivars. Unicrop is still the most efficiently transformed (6.2% transformation frequency) but many of the more advanced breeding lines have now been trialed with transformation frequencies ranging from 1.1% for cv. Quilnock to 3.3% for cv. Merrit. Recent experiments where L-cysteine was incorporated into co-cultivation media for *L. angustifolius* increased transformation efficiency significantly (170% of control for plants on 200 or 400 µg l⁻¹) (L. Hogson, S. Chapple, and P.M.C. Smith, unpublished data). This modification to the Pigeaire *et al.* (1997) method may make transformation of *L. albus* possible with a number of transgenic shoots generated (N. Fletcher and P.M.C. Smith, unpublished data). There are still difficulties in transferring shoots to the glasshouse as IBA induction of roots does not work and grafting is difficult but recent work to produce hairy roots in *L. albus* (Udhe-Stone *et al.*, 2005) and to successfully graft *L. albus* shoots (Lee, 2005) may solve this problem.

A regeneration process similar to that for *L. angustifolius* (Pigeaire *et al.*, 1997) was used for *L.*

luteus transformation (Li *et al.*, 2000). However, these lupins shoots did not micropropagate in the same manner so the original shoot was used for seed production. Roots developed on IBA media for only 10% of shoots and grafting was used in most cases to obtain seed in the glasshouse (Li *et al.*, 2000). Transformation efficiency was low, ranging from 0.09% for cv. Juno to 0.32% for cv. Wodjil.

For *L. mutabilis* regenerated buds developed at the periphery of the apical meristem explant and transgenic shoots developed from these. The shoots were derived from elongating epicotyls (Babaoglu *et al.*, 2000). All the transgenic plants described by Babaoglu *et al.* (2000) originate from the same explant and are likely to represent a single transformation event so it is not possible to gauge the effectiveness of the protocol.

2.5 Testing for Activity and Stability of Transgenes, Adverse Effects on Growth, Yield, and Quality

For *L. luteus* and *L. mutabilis*, there are no subsequent reports describing transgenic lines after the initial publications. Research involving transgenic *L. angustifolius* has been concentrated in three main areas; herbicide resistance, improved seed quality, and disease resistance (Hamblin *et al.*, 1998). Narrow-leaved lupins resistant to the herbicide Basta® (glufosinate or phosphinothricin) were produced by Pigeaire *et al.* (1997) through introduction of the *bar* gene. These lines were grown for five generations with stable expression of the transgene. They had significant resistance to the herbicide and in field trials their yields were equivalent to the parent cultivar, Merrit (Atkins and Smith, 2003). There was potential for their use to control weeds in the wheat-lupin rotations used in Western Australia, however, commercial development was blocked due to intellectual property restrictions associated with use of the *bar* gene.

The most detailed characterization of a transgenic lupin line has been for the high-methionine *L. angustifolius* line produced by Molvig *et al.* (1997). Lupin seeds are deficient in sulfur containing amino acids and this limits their use as a source of protein in animal feed. Molvig *et al.* (1997) introduced the sunflower seed albumin

(*SSA*) gene, encoding a rumen stable protein rich in methionine and cysteine, into *L. angustifolius* cv. Warrah to address this problem. The gene was expressed from a seed-specific pea vicilin promoter. The lines were grown for a number of generations with stable expression of the transgene. Seeds in the resulting transgenic plants had 94% more methionine but 12% less cysteine than seeds of nontransgenic plants with an overall 19% increase in the total seed sulfur content. It was suggested that the reduction in cysteine in the transgenic seed occurs as a result of reduced accumulation of one of the endogenous storage proteins that are high in cysteine. It appears that the seed's ability to import sulfur may limit the amount of sulfur that can be accumulated even when the sink strength for sulfur has been increased (by expressing *SSA*) (Tabe and Droux, 2002).

Feeding trials have been used to compare the nutritive value of *SSA* expressing transgenic seed to that of the parent cultivar in rats (Molvig *et al.*, 1997), sheep (White *et al.*, 2000), broiler chickens (Ravindran *et al.*, 2002), and fish (Glencross *et al.*, 2003). There were no adverse effects from using transgenic seed as feed for animals. The transgenic seed gave a significant increase in live weight gain when compared with the nontransgenic seed in rat and sheep trials (Molvig *et al.*, 1997; White *et al.*, 2000) and for sheep there was an 8% higher rate of wool growth. Use of transgenic lupins in feeds for broiler chickens reduced the requirement for supplemental methionine in bird diets (Ravindran *et al.*, 2002). There appeared to be little value in the use of high methionine lupins as feed for fish (Glencross *et al.*, 2003). These results highlight the different nutritional requirements of different animals.

Seed meal from high methionine lupins has also been tested as a vaccine to suppress experimental asthma in mice (Smart *et al.*, 2003). This was tested as *SSA* has been suggested as a potential allergen. Mice fed seed meal from the transgenic lupin were less likely to develop the symptoms of experimental asthma when challenged with *SSA* than those fed meal from nontransgenic lupins. These results support the theory that plant-based vaccines may be therapeutic for protection against allergic diseases.

The high methionine transgenic lines have also been used to estimate the potential for gene flow from transgenic *L. angustifolius* crops (Hamblin

et al., 2005). Within 1.5 m from the transgenic crop, outcrossing was estimated at 2.7×10^{-4} . No outcrossing was detected at distances greater than 2.25 m.

A number of different genes have been tested for their efficacy in improving resistance to fungal and viral diseases in lupins (S. Wylie, S. Barker, and P.M.C. Smith, unpublished data) (see Table 1). A peroxidase gene from *Stylosanthes humilis* significantly increased tolerance of the transgenic line to *Pleiocheata* root rot but the extent of the resistance was no greater than resistance found in a conventionally bred line (J. Thomas, S. Chapple, S. Barker, and P.M.C. Smith, unpublished data). The most promising results are for resistance to the BYMV; the most important viral disease of lupins worldwide. A nontranslatable inverted repeat of part of the RNA-dependent RNA polymerase (replicase) gene segment of BYMV has been expressed in narrow-leaved lupin. Three lines carrying this synthetic resistance gene have been identified, which are immune to infection (S. Wylie, unpublished data). These lines are still in the early stages of development and further testing will be required to confirm their resistance.

The isopentenyl pyrophosphate transferase (*ipt*) gene has been expressed in narrow-leaved lupin with a flower specific promoter (TP12) to increase cytokinin levels during pod set in an attempt to reduce pod abortion (C.A. Atkins, P.M.C. Smith, and N.E. Emery, unpublished data). The resulting transgenic plants have patterns of branching quite different from that of the parent cultivar. While in nontransgenic lupins branches develop only from the lowest few axillary buds and from three or four buds directly below the main stem inflorescence, in the *ipt* transgenic lines branches develop from all axillary buds. The implications of the modification on yield are currently being investigated.

3. FUTURE ROAD MAP

3.1 Expected Products

Development of transgenic lupin varieties in Australia has all but stopped in the last 3 years. There are lines available that, with further development, could be released commercially if the legal (intellectual and regulatory) and funding issues (see below) were solved. All these lines are

L. angustifolius and include lupins with Basta® resistance, improved methionine content of seeds, and BYMV resistance.

3.2 Expected Technologies

Freedom to operate issues caused by use of the *bar* gene as a selectable marker and its presence in lines to be commercialized forced some changes to the protocol for transformation of *L. angustifolius*. Groups working on transformation of lupins in Australia now use twin transfer DNA (T-DNA) constructs for transformations where the product may be commercialized. In this method the selectable marker and gene of interest are carried on two separate T-DNAs that may integrate into the plant genome at different positions. This means that after selection of transgenic shoots (T₀ generation) segregation of the marker gene away from the gene of interest can occur to allow marker-free transgenic plants to be recovered in the T₁ generation. In the experiments using this technology to date, approximately 25% of lines showed segregation of the gene of interest away from the *bar* gene (L. Molvig, S. Wylie, S. Barker, T.J. Higgins, and P.M.C. Smith, unpublished data). Use of twin T-DNAs increases the amount of work required to produce a transgenic line as about four times more explants need to be infected and subsequently processed. The screening to identify plants segregating in the T₁ generation is also more arduous. However, production of marker-free transgenic plants carrying only the gene of interest will reduce problems associated with use of third party intellectual property (IP), which may impede release of a commercial product. In addition any regulatory issues associated with release of herbicide-resistant plants will be overcome and the transgenic plants produced are more likely to be accepted by the public.

3.3 IP Rights, Public Perceptions, Industrial Perspectives, and Political and Economic Consequences

IP problems associated with use of the *bar* gene blocked release of Basta® resistant lupins in the 1990s and since that time work done in Australia has sought to ensure that there is freedom to

operate with any transgenic lupin variety used. To achieve this, researchers have attempted to reduce their reliance on third party IP in the constructs used for transformation. For example, regulatory sequences from subclover stunt virus have been tested and found effective in controlling transgene expression and the CaMV 35S promoter is no longer used for lines that may be released commercially.

In Australia where most of the work with genetically modified lupins has been done there is currently a moratorium on growth of genetically modified plants in all but two states and territories. It is not clear when this moratorium will be lifted. Although field trials can be conducted, the public distrust of transgenic plants along with the inability to release new lines has reduced funding for the work on genetically modified lupins and the programs that existed have now been scaled back to almost nothing.

Note added in proof. The moratorium on release of GM crops has now been lifted in two Australian states, Victoria and New South Wales.

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Alfalfa

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The genus *Medicago* comprises over 60 annual and perennial species. Alfalfa (*Medicago sativa* L. and *Medicago falcata* L.) is a perennial species that has a long history of cultivation around the world (Russelle, 2001). The center of origin for alfalfa is likely the Middle East and the steppes of Central Asia. The record of its use by humans dates back to the 10th millennium BC at Abu Hureyra in present day Syria. By the 1st century BC, it had spread to Greece and China and the Roman Columella planted alfalfa in southern Spain by 100 AD. Alfalfa spread to North and South America by the 1700s and to New Zealand and Australia by around 1800. The crop was so revered as an excellent source of nutrition for livestock that people carried alfalfa with them and established the crop on six out of the seven continents by the early 1800s.

1.2 Botanical Description

Alfalfa develops an extensive, well-branched root system that is capable of penetrating deep into the soil. Root growth rates of 1.8 m per year are typical in loose soils (Johnson *et al.*, 1996) and metabolically active alfalfa roots have been found

18 m or more below ground level (Kiesselbach *et al.*, 1929). This deep root system allows alfalfa plants to access water and nutrients that are not available to more shallowly rooted annual plants, enabling established alfalfa plants to produce adequate yields under less than optimal rainfall conditions. Alfalfa has a chromosome complement of $x = 8$ with both diploid ($2n = 2x = 16$) and tetraploid forms ($2n = 4x = 32$) (Quiros and Bauchan, 1988).

1.3 Economic Importance

Alfalfa is widely grown for animal feed throughout the world. Alfalfa is known as the “Queen of the Forages” because it contains a high amount of crude protein, digestible fiber, and is an excellent source of vitamins and minerals for animals. In the United States, alfalfa is the fourth most widely grown crop with over 8.9 million hectares of alfalfa harvested in 2005 (USDA-NASS, 2006). In 2005, the US alfalfa cultivation for dry hay was over 70 million metric tons with a total direct value of over \$7 billion. It is typically harvested for 4 years (an establishment year plus three subsequent years). Depending on location, alfalfa is harvested three or more times each year by cutting the stems near ground level. On average across the United States, alfalfa yields 7.6 Mg of dry matter (DM) per hectare each year although yields can

vary by location from 4.5 (North Dakota) to 15.5 (California) Mg ha⁻¹ (USDA-NASS, 2006). In 2005, the national harvest of alfalfa was over 68 million metric tons (USDA-NASS, 2006). The technology for cultivation, harvesting, and storing alfalfa is well established, machinery for harvesting alfalfa is widely available, and farmers are familiar with alfalfa production. There is a well-developed industry for alfalfa cultivar development, seed production, processing, and distribution.

The high biomass potential of alfalfa is based on underground, typically unobserved traits. Alfalfa roots engage in a symbiotic relationship with the soil bacterium *Sinorhizobium meliloti*. This partnership between the plant and bacterium results in the formation of a unique organ, the root nodule, in which the bacterium is localized. The bacteria in root nodules take up nitrogen gas (N₂) and “fix” it into ammonia. The ammonia is assimilated through the action of plant enzymes to form glutamine and glutamate. The nitrogen-containing amide group is subsequently transferred to aspartate and asparagine for transport throughout the plant. On average, alfalfa fixes approximately 152 kg N₂ ha⁻¹ on an annual basis as a result of biological nitrogen fixation, which eliminates the need for applied nitrogen fertilizers (Russelle and Birr, 2004). Although a significant proportion of the fixed nitrogen is removed by forage harvest, fixed nitrogen is also returned to the soil for use by subsequent crops. This attribute of increasing soil fertility has made alfalfa and other plants in the legume family crucial components of agricultural systems worldwide. Cultivation of alfalfa has also been shown to improve soil quality, increase organic matter, and promote water penetration into soil. In addition, alfalfa sprouts are consumed by humans and alfalfa juice is being used in health food products for its flavonoid compounds.

1.4 Traditional Breeding

Alfalfa, a perennial with perfect flowers is naturally cross-pollinated by bees, tolerates comparatively little inbreeding, and can be vegetatively propagated by stem cuttings. All cultivars are autotetraploids and the inheritance of economic traits is, therefore, quite complex. The introgression of *M. falcata* into *M. sativa* increased the genetic variation and the range of

adaptation of this crop to temperate climates of all continents (Rumbaugh *et al.*, 1988). Nine highly diverse, distinct germplasm sources were introduced into North America from different regions of the world (Barnes *et al.*, 1977). Since the 1920s, geneticists have employed recurrent phenotypic selection taking advantage of additive genetic variance to successfully improve alfalfa for resistance to diseases and insects, and extend geographic adaptability. However, more complex quantitatively inherited traits, such as forage yield, have essentially not changed for the past 60 years (Brummer, 1999; Lamb *et al.*, 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Comprehensive genomic analysis of alfalfa is limited due to the large size of the genome and obligate outcrossing autotetraploid nature of alfalfa. Thus, varieties are synthetic populations, consisting of a heterogeneous mixture of heterozygous genotypes. Although improved varieties of alfalfa have been developed, little is known about the genes controlling desirable traits due to the plant's complex genetic system. The traits needed to improve alfalfa are complex and require a multifaceted approach to first understand the molecular underpinnings of the characteristic and subsequently to select or engineer improved plants. To increase the crop value and utility of alfalfa a number of characteristics need to be enhanced. In areas of severe winters, winter hardiness needs to be improved to extend stand life and retain high yields over multiple years. In areas with low rainfall, water use efficiency and tolerance to salinity need to be increased. In acid soils, tolerance to aluminum is needed as well as increased tolerance to waterlogged soils. Improving forage fiber digestibility would enhance nutritional quality for ruminant animals and decrease processing costs when using alfalfa fiber as a feedstock for bioethanol production.

The basic tools required for modern genome analysis are largely not available in alfalfa. Direct study of alfalfa in isolation is unlikely to yield rapid improvements for these characteristics. The use of an annual relative of alfalfa, *Medicago truncatula*, is an ideal candidate for parallel analysis of agronomically important genes in alfalfa.

2. DEVELOPMENT OF TRANSGENIC ALFALFA

2.1 Promoters for Transgenic Alfalfa

The cauliflower mosaic virus 35S (CaMV 35S) promoter is the most widely used promoter in alfalfa transgenic work. In an attempt to find alternative constitutive promoters for transgenic alfalfa, several authors evaluated many promoters for their efficacy driving the reporter gene β -glucuronidase (*gusA*) or green fluorescent protein (GFP). In one such work, Winicov *et al.* (2004) generated transgenic alfalfa that contained the MsPRP2 (proline rich protein 2) gene promoter from alfalfa driving GFP and NOS 3' transcription terminator. Transformation of alfalfa was accomplished by co-cultivation of alfalfa leaf discs with *Agrobacterium tumefaciens*. Kanamycin resistance conferred by a constitutive neomycin phosphotransferase (*nptII*) gene was used as a selectable marker. The MsPRP2 promoter showed root-specific reporter gene expression in transgenic alfalfa plants (Winicov *et al.*, 2004).

Samac *et al.* (2004b) generated transgenic alfalfa that contained the CaMV 35S promoter, the cassava vein mosaic virus (CsVMV) promoter, or the sugarcane bacilliform badnavirus (ScBV) promoter. Each promoter was fused separately to the reporter *gusA* gene and NOS 3' transcription terminator. Transformation of alfalfa was done by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Kanamycin resistance conferred by the *nptII* gene was used as a selectable marker. The highest enzyme activity as well as *in situ* staining for β -glucuronidase (GUS) was obtained in transgenic alfalfa plants that contained the CsVMV promoter. In transgenic alfalfa leaves, GUS activity was observed in the order of CsVMV promoter > CaMV 35S promoter > ScBV promoter. The CsVMV promoter was expressed in all tissues examined. The 35S promoter was expressed in leaves, roots, and stems at moderate levels, but the promoter was not active in stem pith cells, root cortical cells, or in the symbiotic zones of nodules. The ScBV promoter was active primarily in vascular tissues throughout transgenic alfalfa plants studied.

The potato protease inhibitor II (PinII) promoter fused to the *gusA* gene and NOS 3' transcription terminator was introduced into alfalfa

by *Agrobacterium*-mediated transformation of leaf discs (Samac and Smigocki, 2003). Transformant alfalfa was selected by kanamycin resistance conferred by the *nptII* gene. Constitutive GUS expression was observed in leaf and root vascular tissues of transgenic alfalfa. In some transgenic alfalfa plants, GUS expression was observed in leaf mesophyll cells. Approximately twofold more GUS expression was observed following mechanical wounding of leaves compared to unwounded control alfalfa leaves (Samac and Smigocki, 2003).

2.2 Alfalfa Forage Quality Improvement

2.2.1 Manipulation of lignin and plant cell wall composition for forage quality improvement

Lignin is a polymer of monolignols that is found in secondarily thickened plant cell walls and is critical for structural integrity of the wall and the strength of stems. Alfalfa contains two major monolignols, monomethoxylated guaiacyl (G) and dimethoxylated syringyl (S) units. Increased lignin content and S/G ratio resulting from stem maturity have been linked with reduced forage digestibility by grazing animals and hence represent poor forage quality variables. Over the years, considerable effort has been made toward improving forage quality by suppressing the expression of plant genes that are thought to play a role in lignin content or modifying lignin composition in alfalfa. Improving forage digestibility provides a means to enhance animal production.

A cytochrome P450 gene from *M. truncatula* in antisense orientation, driven by the vascular-specific bean phenylalanine ammonia lyase (PAL2) promoter and NOS 3' transcription terminator was used to generate transgenic alfalfa (Reddy *et al.*, 2005). Transformation of alfalfa leaf pieces was following co-cultivation with *A. tumefaciens*. Kanamycin resistance conferred by the *nptII* gene was used as a selectable marker. Transgenic alfalfa showed a range of differences in lignin content and composition. In cytochrome P450 transgenic alfalfa, lignin content was negatively related to rumen digestibility, but no relationship was observed between lignin composition and rumen digestibility (Reddy *et al.*, 2005).

Transgenic alfalfa plants containing the coumaroyl shikimate 3-hydroxylase (C3H) gene from *M. truncatula* in the antisense orientation were generated (Reddy *et al.*, 2005; Ralph *et al.*, 2006). The antisense C3H clone was driven by the vascular-specific bean PAL2 promoter and NOS 3' transcription terminator. *Agrobacterium*-mediated transformation was used to transform alfalfa leaf pieces. Selection of alfalfa transformants was achieved by kanamycin resistance conferred by the *nptII* gene. Down-regulation of C3H transcript in alfalfa altered lignin composition as the proportion of p-hydroxyphenyl (P) units was increased compared to the usually dominant G and S units of lignin. Transgenic alfalfa plants displayed improved digestibility values (Reddy *et al.*, 2005; Ralph *et al.*, 2006).

In a separate study, transgenic alfalfa contained caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT) genes from alfalfa in antisense orientation (Guo *et al.*, 2001; Marita *et al.*, 2003). The transgenes were driven by the vascular-specific bean PAL2 promoter and NOS 3' transcription terminator. Alfalfa transformation was by co-cultivation of leaf pieces with *A. tumefaciens*. Kanamycin resistance conferred by the *nptII* gene was used as the selectable marker. Transgenic plants down-regulated for COMT showed reduced lignin content and altered lignin composition. Down-regulation of COMT transcript in transgenic alfalfa was accompanied by an increase in cellulose, resulting on average a 30% increase in cellulose:lignin ratio. Digestibility of transgenic alfalfa was also improved. On the other hand, lignin content in transgenic alfalfa down-regulated in the expression of CCoAOMT was reduced, but the structure of lignin was similar to that of nontransformed control plants. Nevertheless, a much greater improvement in digestibility values of alfalfa forage was reported for transgenic alfalfa down-regulated in CCoAOMT (Guo *et al.*, 2001).

Uridine diphosphate (UDP)-glucose dehydrogenase is a key enzyme in the biosynthesis of uronic acids. Uronic acids represent a large proportion of the primary cell wall matrix in plants. Samac *et al.* (2004a) reported the generation of transgenic alfalfa overexpressing a soybean UDP-glucose dehydrogenase gene in the sense direction. The transgene was driven by an Arabidopsis class III chitinase promoter

or an alfalfa phosphoenolpyruvate carboxylase promoter and NOS 3' transcription terminator. Transformation was following co-cultivation of leaf pieces with *A. tumefaciens* LBA 4404. Plant transformation vectors contained the *nptII* gene for selection of transformants by resistance to kanamycin. UDP-glucose dehydrogenase enzyme activity in transgenic lines grown in the greenhouse was up to sevenfold greater than in nontransformed control plants. However, enzyme activity of field-grown transgenic plants showed a maximum of 1.9-fold more enzyme activity than the nontransformed control plants. No significant increase in pectin or uronic acids was seen in the polysaccharide fraction of field-grown transgenic plants. However, lower cell wall polysaccharide content and mannose concentrations were accompanied by higher Klason lignin content in transgenic plants. Ectopic expression of UDP-glucose dehydrogenase showed a 15% increase in xylose content in stem tissues of most transgenic alfalfa lines.

2.2.2 Reducing protein loss for forage quality improvement

Ensiling is a popular method of preserving forage crops. Wounding of forage crop leaves during crop harvest and ensiling releases cellular proteases resulting in degradation of plant protein to small peptides and amino acids. Red clover (*Trifolium pratense* L.) experiences less protein loss than alfalfa when ensiled. Proteolytic inhibition of ensiled forage seen in red clover was hypothesized to occur through the activity of polyphenol oxidase (PPO) on endogenous *o*-diphenols. To test this idea, the *PPO* gene from red clover was placed under the control of a constitutive CsVMV promoter and NOS 3' transcription terminator to produce transgenic alfalfa (Sullivan *et al.*, 2004). Transformation of alfalfa leaf pieces was mediated by co-cultivation with *Agrobacterium* strain LBA 4404. Kanamycin resistance conferred by the *nptII* gene was used to select transformed alfalfa. The red clover PPO was expressed in transgenic alfalfa and the protein was shown to be active in alfalfa extracts, as evidenced by *o*-diphenol-dependant extract browning and quantitative assays of PPO activity (Sullivan *et al.*, 2004; Sullivan and Hatfield, 2006).

Leaf extracts of transgenic alfalfa showed a nearly fivefold *o*-diphenol-dependent decrease in proteolysis compared to those of nontransformed control alfalfa (Sullivan and Hatfield, 2006). In small-scale ensiling experiments, proteolysis was reduced for PPO-expressing transgenic alfalfa (Sullivan and Hatfield, 2006).

2.2.3 Improving dietary proteins in alfalfa forage

Most dietary proteins ingested by ruminants are rapidly degraded by rumen bacteria. Rumen microbial proteins in turn become sources of protein for the animals. However, microbial proteins are poor in sulfur amino acids. A small number of proteins appear to be relatively resistant to degradation in the rumen and presumably are assimilated by ruminants in the lower gastrointestinal tract. Ovalbumin, one such protein that is found to be resistant to degradation by rumen bacteria, is also regarded as nutritionally valuable. Schroeder *et al.* (1991) placed a chicken ovalbumin gene under the control of the CaMV35S promoter and NOS 3' transcription terminator to generate transgenic alfalfa plants. Transformation of alfalfa leaf pieces was done by co-cultivation with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. Transgenic alfalfa plants expressed the recombinant protein in leaf tissues and ovalbumin expression was estimated from 0.001% to 0.01% of total soluble protein. Recombinant ovalbumin expression was stable and was detected in leaf tissues of all ages (Schroeder *et al.*, 1991).

2.3 Abiotic Stress Tolerance

2.3.1 Overexpression of transcription factor to improve salinity tolerance

Transcription factors modulate gene expression patterns through sequence-specific DNA binding and/or protein-protein interactions. They are capable of acting as switches of gene expression regulatory cascades by activating or repressing transcription of target genes. One such gene, *Alfin1*, encodes a putative transcription factor

in alfalfa, and the recombinant protein binds DNA in a sequence-specific manner, including the promoter region of the salt (NaCl) inducible gene *MsPRP2* (Winicov and Bastola, 1999). *Alfin1*-binding sites were shown to occur in promoters of genes expressed in roots of a wide variety of plant species (Winicov, 2000). Transgenic alfalfa plants containing the alfalfa *Alfin1* cDNA (complementary DNA), in the sense or antisense direction, placed under the control of the CaMV 35S promoter and NOS 3' transcription terminator were generated (Winicov and Bastola, 1999). Transformation of alfalfa leaf pieces was *Agrobacterium*-mediated. Selection of transformant alfalfa was by kanamycin resistance conferred by the *nptII* gene. Transgenic calli and plant roots overexpressing *Alfin1* showed enhanced levels of endogenous *MsPRP2* mRNA (messenger RNA) accumulation. Calli overexpressing *Alfin1* were more resistant to growth inhibition by 171 mM NaCl than vector-transformed controls, whereas calli expressing *Alfin1* in the antisense orientation were more sensitive to NaCl inhibition. Similarly, transgenic plants overexpressing *Alfin1* showed salinity tolerance comparable to a NaCl-tolerant control plant (Winicov and Bastola, 1999). Further more, *Alfin1*-overexpressing transgenic alfalfa plants showed increased root growth under normal and salt stress conditions (Winicov, 2000). In contrast, the antisense transgenic plants grew poorly in soil, demonstrating that *Alfin1* expression is also essential for normal plant root development.

2.3.2 Overexpression of transcription factor to improve drought tolerance

The *WXP1* gene, an AP2 domain-containing putative transcription factor from *M. truncatula*, placed under the control of the CaMV 35S promoter and NOS 3' transcription terminator was used to transform alfalfa (Zhang *et al.*, 2005). Transformation of alfalfa leaf pieces was following co-cultivation with *A. tumefaciens* strain C58C1. Transformed lines were selected for resistance to the herbicide Basta conferred by the *bar* gene. Cuticular wax loading on leaves of transgenic alfalfa was significantly increased. The total leaf wax accumulation per surface area increased approximately 30–38% in the transgenic alfalfa

lines. Transgenic alfalfa leaves showed reduced water loss and chlorophyll leaching, as well as delayed wilting after watering was ceased and quicker and better recovery when the dehydrated plants were rewatered (Zhang *et al.*, 2005).

2.3.3 Improvement of tolerance to aluminum phytotoxicity in acid soils

Development of acid soils that limit crop production is an increasing problem worldwide. Many factors contribute to phytotoxicity of these soils, however, in acid soils with a high mineral content, aluminum (Al) is the major cause of toxicity. Toxicity can be reduced through lime application to raise soil pH, however, this amendment does not remedy subsoil acidity, and liming may not always be practical or cost effective. Addition of organic acids to plant nutrient solutions alleviates phytotoxic Al effects, presumably by chelating Al and rendering it less toxic.

In an effort to increase organic acid secretion and thereby enhance Al tolerance in transgenic plants, Tesfaye *et al.* (2001) produced transgenic alfalfa using the nodule-enhanced form of malate dehydrogenase (*neMDH*) gene from alfalfa. The *neMDH* gene in the sense direction was placed under the control of the CaMV 35S promoter and NOS 3' transcription terminator. Transformation of alfalfa leaf discs was *Agrobacterium*-mediated. Kanamycin resistance conferred by the *nptII* gene was used as the selectable marker. Malate dehydrogenase enzyme specific activity in root tips of selected transgenic alfalfa was 1.6-fold higher than in nontransformed control plants. A 4.2-fold increase in root concentration as well as a 7.1-fold increase in root exudation of citrate, oxalate, malate, succinate, and acetate was measured in transgenic alfalfa lines compared with nontransformed control alfalfa plants. In acid soils and hydroponic culture, transgenic alfalfa plants showed increased biomass accumulation compared to nontransformed control alfalfa.

2.3.4 Winter freezing tolerance

Transgenic alfalfa plants that overexpress manganese superoxide dismutase (Mn-SOD) cDNA

from alfalfa were produced (McKersie *et al.*, 1999). The transgene was driven by the CaMV 35S promoter and NOS 3' transcription terminator in the sense direction. Alfalfa petioles were transformed by co-cultivation with *A. tumefaciens*. Kanamycin resistance conferred by the *nptII* gene was used as the selection agent. In two field experiments, transgenic alfalfa plants showed over 25% better winter survival than the nontransgenic alfalfa controls (McKersie *et al.*, 1999).

Similarly, the Arabidopsis iron-superoxide dismutase (Fe-SOD) was expressed in transgenic alfalfa (McKersie *et al.*, 2000). The Fe-SOD cDNA was under the control of the CaMV 35S promoter and NOS 3' transcription terminator. Alfalfa petioles were transformed by co-cultivation with *A. tumefaciens*. Kanamycin resistance conferred by the *nptII* gene was used as the selection agent. Transgenic alfalfa plants overexpressing the Arabidopsis Fe-SOD transcripts showed greater survival after two winter seasons than the nontransformed control alfalfa plants (McKersie *et al.*, 2000).

2.4 Herbicide Resistance and Weed Control

In fall 2005, the first biotechnology-enhanced alfalfa, Roundup Ready[®] alfalfa, was commercialized in the United States. The plant transformation vector for this transgenic alfalfa contained an *Agrobacterium* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) fused to a transit peptide (Samac and Temple, 2004). The transgene was driven by a strong constitutive promoter and NOS 3' terminator (Samac and Temple, 2004). Transformation of alfalfa leaf discs was achieved by co-cultivation with *A. tumefaciens*. The transgenic alfalfa is resistant to glyphosate (active ingredient of Roundup herbicide). This trait is expected to provide growers with improved weed control and increased flexibility in timing of herbicide application.

2.5 Disease and Pest Resistance

As a potential strategy to improve disease resistance in plants, Masoud *et al.* (1996) generated transgenic alfalfa utilizing constitutive expression of chitinase and glucanase genes separately or

in tandem for coexpression. The acidic glucanase (*AgluI*) gene from alfalfa and the rice basic chitinase (*RCH10*) gene were placed under the control of CaMV 35S and NOS 3' transcription terminator. Plant transformation vectors contained each transgene separately, or in tandem for coexpression in a single transgenic plant. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. Both transgenes were expressed in transgenic alfalfa plants. However, the level of expression of each transgene by transgenic alfalfa plants with both transgenes in tandem was much less than transgenic alfalfa plants carrying a single transgene. Only transgenic plants expressing *AgluI* displayed reduced disease symptoms from *Phytophthora megasperma* f. sp. *medicaginis* inoculations. Transgenic alfalfa plants did not tolerate infections from other fungi infections including *Stemphylium alfalfae*, *Colletotrichum trifolii*, or *Phoma medicaginis* (Masoud *et al.*, 1996).

Alfalfa mosaic virus (AMV) can cause significant yield losses to forage legumes. An AMV RNA 4 gene encoding the coat protein of AMV was placed under the control of the CaMV 35S promoter and T-DNA ORF 25 RNA terminator (Hill *et al.*, 1991). Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. Production of the coat protein by transgenic alfalfa plants ranged from <100 ng to nearly 500 ng coat protein per milligram soluble leaf protein. While nontransformed alfalfa plants showed systemic infections of the virus following inoculations with 10 $\mu\text{g ml}^{-1}$ AMV, transgenic alfalfa plants develop resistance to AMV with inoculations of up to 50 $\mu\text{g m l}^{-1}$ AMV (Hill *et al.*, 1991).

Phytocystatins, inhibitors of cysteine proteinases, are found in a number of plants where they may play a role in defense against pathogens and pests. The cDNAs of the phytocystatins of rice, oryzacystatin I (*OC-I*) and oryzacystatin II (*OC-II*), were expressed in transgenic alfalfa plants under the control of the *PinII* promoter (Samac and Smigocki, 2003). Transformation was by co-cultivation of leaf discs with *A. tumefaciens*. Transformants were selected by

kanamycin resistance conferred by the *nptII* gene. Populations of the root-lesion nematode (*Pratylenchus penetrans*) in alfalfa roots from one transgenic alfalfa line containing the *PinII::OC-I* transgene and one transgenic line containing the *PinII::OC-II* transgene were reduced by 29% and 32%, respectively, compared with a vector only transgenic control line (Samac and Smigocki, 2003).

2.6 Transgenic Alfalfa for Industrial Applications

Plants are capable of carrying out acetylation, phosphorylation, and glycosylation as well as other post-translational protein modifications required for the biological activity of many recombinant proteins. Indeed, numerous recombinant proteins have been produced in transgenic plants. Alfalfa has primarily been used as a source of forage and animal feed. Improving the economic value of forage legumes could be achieved by developing new uses and value-added products of these crops. The ease of genetic transformation and large-scale cultivation makes alfalfa suitable for production of recombinant proteins.

2.6.1 Production of biodegradable plastic

A biodegradable polymer, polyhydroxybutyrate (PHB), is made naturally by many bacteria under nutrient-limiting conditions, and can be produced commercially by fermentation (Saruul *et al.*, 2002). Three genes from *Ralstonia eutropha* required for PHB synthesis, *pha*, *phb*, and *phc*, were each placed between the CaMV 35S promoter and NOS 3' transcription terminator, in the same plant transformation vector. Alfalfa transformation was by co-cultivation of leaf discs with *A. tumefaciens*. Kanamycin resistance conferred by the *nptII* gene was used as the selectable marker. Polymer granules similar to bacterial PHB indicating the production of the bioplastic were observed in chloroplasts of transgenic alfalfa. The amount of PHB accumulation in transgenic alfalfa was estimated at approximately 0.025–1.8 g kg^{-1} dry weight. Transgenic alfalfa exhibited growth similar to nontransformed plants, and F_1 hybrid progeny, obtained from crosses of PHB transgenic plants

with elite alfalfa germplasm exhibited leaf PHB levels similar to the transgenic parental line.

2.6.2 Phytoimmunogen production in transgenic alfalfa

Vaccines produced in transgenic plants offer a system for oral delivery of recombinant immunogens. This approach is especially relevant when used for enteric pathogens since oral immunization may be capable of eliciting appropriate immune mechanisms for the induction of protective responses.

In an attempt to use alfalfa plants for vaccine production, a 96bp coding sequence for the bovine rotavirus VP4, eBRV4 peptide, fused with the *gusA* gene was driven by the CaMV 35S promoter and NOS 3' transcription terminator (Wigdorovitz *et al.*, 2004). Transformation of alfalfa petioles was *Agrobacterium*-mediated. Kanamycin conferred by the expression of the *nptII* gene was used as a marker gene for selection of transformants. The recombinant protein was shown to induce protective lactogenic immunity against a virulent rotavirus in suckling mice born from immunized adult female mice (Wigdorovitz *et al.*, 2004).

2.6.3 Production of industrially important enzymes and phytopharmaceuticals

In an effort to utilize transgenic alfalfa for the production of industrially useful enzymes, Austin *et al.* (1995) used an α -amylase gene from the bacterium *Bacillus licheniformis* to transform transgenic alfalfa. α -amylase is involved in starch degradation and the production of a recombinant protein using plants has potential industrial-scale applications in starch processing. The transgene was driven by the MAC promoter and mannopine synthase transcription terminator from *Agrobacterium*. The MAC promoter is a hybrid fragment that contains some elements from the CaMV 35S promoter and the nopaline synthase promoter from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. Transgenic alfalfa

plants looked normal throughout the growth period, and recombinant protein expression was estimated in the range of 0.001–0.01% of total soluble protein extracts. Field-grown transgenic alfalfa plants also expressed the recombinant α -amylase enzyme with no effect on biomass production (Austin *et al.*, 1995).

Another recombinant enzyme that was produced using transgenic alfalfa was manganese-dependent lignin peroxidase (Mn-P) from the fungus *Phanerochaete chrysosporium* (Austin *et al.*, 1995). The objective was to produce a recombinant enzyme using plants for large-scale application as a bleaching agent in the biopulping process (Austin *et al.*, 1995). The transgenic alfalfa is also thought to improve digestibility of crude fiber as ruminant feed. The transgene was driven by the MAC promoter and mannopine synthase transcription terminator from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. Expression of the recombinant protein was estimated in the range of 0.01–0.5% of total soluble protein extracts. Field-grown transgenic alfalfa plants also expressed the recombinant Mn-P protein with an estimated yield of up to 0.15% (high expressing plants) of total soluble protein (Austin *et al.*, 1995). Transgenic alfalfa plants expressing Mn-P looked normal for the first 2–3 weeks of growth, but high expressing plants showed foliar yellowing accompanied by stunted and slow growth rate.

Most phosphorus (P) in plants is stored as phytic acid, and phytic acid is not readily digested by monogastric animals, such as poultry and swine. To help overcome this problem, a phytase enzyme, secreted by *Aspergillus niger*, has been widely incorporated in animal feeds to help improve P availability by degradation of phytate. Ullah *et al.* (2002) reported the production of transgenic alfalfa carrying the *phyA* gene from *Aspergillus ficuum*. The transgene was driven by the MAC promoter and mannopine synthase transcription terminator from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. The recombinant enzyme was recovered from alfalfa leaf tissues

and was found to be active and stable, with an approximate 17-fold increase in phytase enzyme activity values in leaves of transgenic alfalfa plants (Ullah *et al.*, 2002).

Production of transgenic plants that express cellulase enzymes are thought to have great potential for use in the conversion of lignocellulosic biomass to ethanol. Ziegelhoffer *et al.* (1999) produced transgenic alfalfa plants carrying the E2 and E3 cellulase genes of *Thermomonospora fusca* under the control of the MAC promoter and manopine synthase transcription terminator from *Agrobacterium*. Transformation of alfalfa was by co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Selection of transformed alfalfa was by kanamycin resistance conferred by the *nptII* gene. Recombinant cellulase expression by transgenic alfalfa plants ranged from 0.04% of total soluble protein for E3 cellulase to 0.1% of total soluble protein for E2 cellulase (Ziegelhoffer *et al.*, 1999).

The extraction and purification of proteins from biochemically complex plant tissues is a laborious and expensive process that presents a major limitation to large-scale protein production using transgenic plants. Exudation of recombinant proteins from roots may offer an alternative for use of alfalfa in the production of value-added phytopharmaceuticals. Transgenic alfalfa plants were produced following transformation with a fungal cDNA from *Trichoderma atroviride* encoding an endochitinase gene (*ech42*) fused in frame to the signal peptide of a white lupin acid phosphatase and under the control of the CsVMV promoter and NOS 3' terminator (Tesfaye *et al.*, 2005). Plant transformation vectors were introduced into alfalfa by *Agrobacterium*-mediated transformation. Kanamycin resistance was used as the selectable marker for transformed alfalfa. Chitinase mRNA and protein synthesis in transformed plants was accompanied by chitinase enzyme activity. The recombinant ech42 enzyme was secreted into the rhizosphere. Chitinase activity in root exudates of transgenic alfalfa was 7.5–25.7 times higher than in the nontransformed control alfalfa plants. The secreted recombinant endochitinase retained its lytic activity against glycol chitin substrate and also showed antifungal activity by inhibition of spore germination of two fungal pathogens.

2.6.4 Phytoremediation of herbicide contaminated soils

Atrazine is one of the most widely used herbicides in the United States. In specific Gram-negative soil bacteria, atrazine chlorohydrolase (*atzA*) is the first enzyme in a pathway that catalyses the hydrolytic dechlorination and detoxification of atrazine to hydroxyatrazine. The potential of transgenic alfalfa expressing an *atzA* gene to take up, dechlorinate, and detoxify atrazine was investigated (Wang *et al.*, 2005). Transgenic alfalfa plants were generated containing an *atzA* gene modified for plant expression, *p-atzA*, under the control of the CsVMV promoter and NOS 3' transcription terminator. Plant transformation vectors were introduced into alfalfa following co-cultivation of leaf discs with *A. tumefaciens* strain LBA 4404. Kanamycin resistance conferred by the *nptII* gene was used as the selectable marker for transformed alfalfa. Transgenic alfalfa plants expressing *p-atzA* grew over a wide range of atrazine concentrations. Thin layer chromatography analyses indicated that *in planta* expression of *p-atzA* resulted in the production of hydroxyatrazine. Hydroponically grown transgenic alfalfa dechlorinated atrazine to hydroxyatrazine in leaves, stems, and roots (Wang *et al.*, 2005).

3. FUTURE ROAD MAP

3.1 Expected Products

The development and commercialization of Roundup Ready® alfalfa has paved the way for development of additional biotechnology-derived traits in alfalfa. However, the traits developed must be of high economic value in order to recover the considerable costs of developing and releasing transgenic crop varieties. Because alfalfa is used primarily for livestock feed, the direct value of a biotechnology-derived trait would be targeted to the producer of alfalfa or livestock, rather than to the general public. Traits of interest to livestock producers include increasing forage digestibility and decreasing the potential for bloat. Recently, significant advances have been made to reduce the lignin content and/or composition of alfalfa to increase digestibility (Guo *et al.*, 2001; Marita

et al., 2003; Reddy *et al.*, 2005; Ralph *et al.*, 2006). Additionally, a greater understanding of the genes for proanthocyanidin (condensed tannin) production has been achieved (Ray *et al.*, 2003; Xie *et al.*, 2003), which will accelerate the development of bloat-safe alfalfa. Forage yield is of primary importance to alfalfa producers. A significant decrease in leaf senescence, with a concomitant delay in plant flowering, has been achieved by controlled expression of isopentenyl transferase, an enzyme involved in cytokinin biosynthesis (Gan and Amasino, 1995; Sandman *et al.*, 2003). In addition to potentially increasing yields, a delay in flowering will provide producers with greater flexibility in crop harvest. Enhancing crop adaptation, such as tolerance to salt, cold, or acid soils using transgenic approaches may not have the economic return for commercializing these traits.

3.2 Risks and Concerns

Alfalfa is an insect-pollinated crop; therefore, safeguards are needed to ensure trait purity of the biotechnology-derived trait and to mitigate the dissemination of a regulated trait. Field studies were conducted in the seed-producing areas of Canyon County, Idaho using leaf cutter bees (Fitzpatrick *et al.*, 2003) and Kings County, California using honeybees (Teuber *et al.*, 2004) to measure pollen flow. Based on these studies, 1.6 km minimum isolation for production of Roundup Ready® alfalfa seed was established.

3.3 Expected Technologies

Transformation of alfalfa chloroplasts, via optimization of biolistic transformation, would facilitate development of specific biotechnology-derived traits. Chloroplast transformation in other crops has been shown to increase gene copy number and protein production. Enhanced product accumulation would be useful in production of vaccines and other high value proteins. In addition, retaining some protein products, such as cell wall hydrolases, within the chloroplast may protect the cell from deleterious effects of production. Finally, the chloroplast is an ideal location for synthesis of some biopolymers, such as polyhydroxyalkanoates. Because chloroplasts are

found in alfalfa pollen, chloroplast transformation would not be a means of managing trait dissemination.

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Clovers

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The true clovers (*Trifolium* spp.), may have been cultivated in Europe as early as the 4th Century AD (Zohary and Heller, 1984), but largely remained wild until around 1000 AD when they were domesticated in Spain. By the 17th century, clovers, accompanied by rhizobial bacteria and honeybees as pollinators, had spread throughout Europe where they were to change the world by providing the nitrogen (N) that drove the agricultural revolution of the 18th and 19th centuries (Kjaergaard, 2003). This co-evolved plant-bacterium-insect species association provided the basis of a human population explosion and literally transformed 18th century landscapes into scenes flowing with milk and honey. Interestingly, after the 1940s, and the high-energy mass production of nitrate fertilizers, clovers progressively declined, first in the northern hemisphere, and later in the southern hemisphere. In the 21st century, they are poised to return for widespread use again as the age of cheap oil and abundant energy come to an end (Kjaergaard, 2003).

A recent DNA sequence phylogenetic analysis (Ellison *et al.*, 2006) revealed that the genus *Trifolium* is monophyletic with three major centers of diversity—the Mediterranean basin, western North America, and the East African

highlands. Of these, the Mediterranean center has the most species and is the likely location of origin of the genus. The American species apparently arose from a single origin while the African species may have arisen from three separate dispersals. The genus has approximately 250 species, of which about 15 have been widely commercialized for agriculture (Zohary and Heller, 1984). The most important perennial pastoral species are red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.). There are several important annual clovers, including berseem (*Trifolium alexandrinum* L.), Persian clover (*Trifolium resupinatum* L.), and subterranean clover (*Trifolium subterraneum* L.) that are used as cool season annuals in many countries and a number of species with minor agricultural use in various countries (Taylor, 1985). To date, red, white, and subterranean clovers have been the main targets of forage biotechnology.

Red clover appears to have originated in Southeast Europe and Asia Minor (Smith *et al.*, 1985). The species became widespread throughout Eurasia and is morphologically highly variable as the result of the development of locally adapted ecotypes. It has become one of the most widely used species in temperate agriculture. *T. repens* is even more widespread than *T. pratense*. It is naturally distributed throughout Europe, northern and Central Asia, all Mediterranean countries, and northern Africa. It has been introduced into

many other countries where it is cultivated and often provides excellent grazed feed because of its prostrate habit. It is one of the best honey plants known.

1.2 Botanical Descriptions

Over 80% of *Trifolium* species evaluated by DNA phylogeny have $2n = 2x = 16$ chromosomes and $x = 8$ is the confirmed basic number for *Trifolium* (Ellison *et al.*, 2006). However, *T. pratense* ($2n = 2x = 14$, Section *Trifolium*) has the reduced basic number $x = 7$. White clover is one of very few allotetraploids in *Trifolium* ($2n = 4x = 32$), and is classified in section *Trifoliastrum* with 16 other taxa that are predominantly $x = 8$. A recent DNA sequence phylogeny (Ellison *et al.*, 2006) revealed that the alpine species *Trifolium pallescens* may be a progenitor, perhaps in combination with *Trifolium occidentale*. However, this is yet to be verified.

Red clover grows from a thick root and a short crown that produces branched stems that do not usually root from the nodes, although some “stoloniferous” forms with nodal rooting have been described (Smith and Bishop, 1993; Rumball *et al.*, 2003). The rootstock may last for many years in some environments, but in many situations, especially under frequent grazing, it can become damaged or diseased after 2–5 years. Consequently, it is a short-lived perennial. Red clover is adapted to slightly acid to neutral, free-draining soils with good water-holding capacity and it will not tolerate waterlogging. *T. pratense* is seen growing in meadows, on roadsides, forest clearings, and near water in moist regions worldwide. It requires adequate P and potassium (K) to maintain production. Populations are highly polymorphic for flowering requirements, varying in need for cold treatment and critical photoperiod. Zohary and Heller (1984) provided a tentative and incomplete subdivision of the species into six varieties.

White clover is tap rooted in its first year or 18 months of seedling growth, and produces indeterminate stolons that root at the nodes and branch in the leaf axils (Thomas, 1987a, b). White clover is found in pastures throughout the temperate world with annual rainfall >500 mm. During flowering, inflorescences replace vegetative

branches in some leaf axils. After the death of the tap root, survival and growth become dependent on stolon fragments with nodal roots (Beinhart, 1963). These roots are usually shallow and less robust than the seedling tap root and the plant fragments can be vulnerable to soil surface drying and to pests and diseases. As a consequence, a white clover plant has two growth phases and it can appear to be relatively hardy as a seedling but much less so in its adult fragmented form. As a creeping plant, white clover is a colonizer for which no part survives for more than 12–15 months. Consequently, although it is perennial, it has been described as a “rolling annual” (Hollowell, 1966). It is intolerant of drought, excess water, salinity, and highly alkaline and acid soils (Gibson and Cope, 1985; Williams, 1987a). Flowering requirements vary among populations and greatly among individuals within populations (Thomas, 1987c). Inflorescence initiation is generally promoted by long days but is also affected by low temperatures. Many populations appear to behave as short-long day plants. Much of the seed is hard and can remain in soil seed banks for many years (Harris, 1987). The dual mechanisms of asexual reproduction by stolon proliferation and sexual reproduction through seeds give white clover adaptation to a wide range of habitats.

Both red and white clovers have relatively small genome sizes, red clover having a 1C haploid nuclear genome value of 0.7–0.8 pg (picogram) and white clover 1.0–1.5 pg (Bennett and Smith, 1991). Red clover has regular diploid cytological behavior. White clover is a diploidized allotetraploid and behaves very regularly at meiosis to produce 16 bivalents almost without exception. Inheritance is as a typical amphidiploid. Thus, homeoloci that are present in both subgenomes, for example, shikimate dehydrogenase (SDH), are inherited totally independently, as if the other locus was not present (Williams *et al.*, 1998). Several important traits are conditioned by loci that are present in only one of the subgenomes and are inherited in diploid fashion, for example, the S-incompatibility system, cyanogenesis, V leaf marks (Williams, 1987c). Despite being allotetraploid, the white clover karyotype has only two nucleolar organizer regions (NORs). Cytogenetic fluorescent *in situ* hybridization (FISH) mapping of multigene DNA sequences coding for 18S-5.8S-26S rDNA

(ribosomal DNA) and 5S rDNA in white clover showed that at least one pair of ancestral NORs had been eliminated (Ansari *et al.*, 1999). The 18S-26S rDNA (NOR) was located proximally on the shorter arm of a submetacentric chromosome pair, which also carried a 5S rDNA locus on the longer arm. A second 5S rDNA locus occurred proximally on the longer arm of another submetacentric chromosome pair. White clover and its closest relatives share a unique centromeric repetitive DNA sequence, TrR350 (Ansari *et al.*, 2004).

1.3 Economic Importance

Clovers continue to have major economic importance internationally despite a downturn in use since about 1950 coinciding with the availability of abundant N fertilizers. White clover seed production is currently around 15 000 MT annually, with approximately 4000 MT of this from New Zealand, 1600 MT from Europe, and the bulk from North America. White clover is also the most important legume of grazed pastures, not only in moist temperate (rainfall >750 mm) areas, where it is best adapted, but also on acidic soils in semi-arid regions where it serves as the best legume for grazing. White clover is found on 7.8 million hectares of pasture in Australia (Australian Government, 2004) and 9 million hectares in New Zealand grasslands (Williams, 1983). Its value to New Zealand alone has been estimated to be in excess of US\$2 billion annually (Caradus *et al.*, 1996), and the dairy industry of the state of Victoria, Australia, is estimated to benefit annually by A\$267 million (Mason, 1993).

In general, red clover is better adapted to forage production under cutting for hay or silage while white clover has adaptations that best suit it for grazing. However, both species are highly polymorphic and there are prostrate red clover types suitable for grazing and tall, upright white clovers (e.g., ladino clover) suitable for cutting. Clovers have very high nutritional value for livestock compared to pasture grasses (Essig, 1985; Abberton *et al.*, 2002). Animal performance on white clover is substantially greater than that on temperate grasses as a consequence of high voluntary intake and high quality (Ulyatt, 1970).

Honey production is a very important secondary use of white clover.

Red clover uniquely contains a polyphenol oxidase (PPO) enzyme, which reduces protein breakdown and leads to approximately 40% less proteolysis during ensiling and rumen digestion than other legume forages (Albrecht and Muck, 1991; Jones *et al.*, 1995a, b, c). This leads to larger amounts of intact forage protein passing from the rumen to the intestine and thus red clover is more efficiently utilized by grazing animals (Buxton, 1996). However, red clover contains phyto-oestrogenic isoflavones, which can cause severe reproductive problems in sheep. These isoflavones include biochanin, diadzein, formononetin, and genistein, the most important of which is formononetin (Morley *et al.*, 1966, 1968). Selection for reduced formononetin has been successful (Rumball *et al.*, 1997) and has reduced ewe fertility problems (McDonald *et al.*, 1994). Products such as Promensil, based on red clover extracts, contain isoflavones, which are strong antioxidants and oestrogens. These are being produced as dietary supplements to combat PMS, osteoporosis, and high cholesterol in humans.

1.4 Traditional Breeding

1.4.1 Red clover breeding

Red clover has a gametophytic incompatibility system based on *S*-alleles (Townsend and Taylor, 1985). Self-fertile mutants occur and some selfing also occurs when the incompatibility system fails. This ensures that cross-pollination predominates and that populations consist of highly heterozygous plants. Breeding, therefore, aims to select plants that produce high frequencies of desirable plants in progenies from interpollination with other plants. Selection of red clover thus involves progeny testing of elite parent plants, and varieties are synthetic populations developed by intercrossing in all possible combinations (polycrossing). Inbred lines can be maintained either vegetatively or sexually and higher yielding single or double cross hybrids are theoretically possible (Cornelius *et al.*, 1977), but high yielding hybrids have proved difficult to achieve because of difficulties in obtaining superior inbred lines.

Mutation breeding has been used little in red clover, partly because of the vast amount of genetic diversity already present and partly because the self-incompatibility system that limits the expression of recessive mutations. Chromosome doubling to produce tetraploid varieties with superior yields has been successfully used in several countries but the polyploids can also show low seed yields because of pollination problems (Cope and Taylor, 1985).

Ex situ genetic resources of red clover are extensive. Large collections exist in the United States Department of Agriculture (USDA)-GRIN system and the European Union with smaller collections elsewhere. Further collections are needed, especially in regions of diversity such as eastern Europe, Turkey, Iran, and Iraq, where populations are coming under threat of extinction due to pressures of human population growth. There is considerable genetic diversity for plant type in red clover, from prostrate and creeping, to very tall upright plants and availability of appropriate types for particular end uses (single cut, double cut, grazed pastures, etc.) is a problem mainly where long-lived perennials are desired. Red clover grows strongly in the spring through to late summer and generally grows slowly in the cool seasons. Some Mediterranean-type germplasm from Morocco was found to have cool season activity and these were hybridized with the New Zealand material (of European origin) to produce new varieties for use in maritime climates (Claydon *et al.*, 1993). Persistence continues to be a major breeding objective in most breeding programs. Plants with the crown below the soil surface instead of above may have more longevity (Smith, 2000), as would plants with disease-resistant tap roots that also have large secondary root systems. To date, plants with strong secondary roots or rhizomes have not been found and use of the secondary gene pool (i.e., related species) may be fruitful in this regard. Use of alternative species through interspecific hybridization is likely to contribute to heat, drought, and cold tolerance. The major pests and diseases of red clover vary with region and include the usual spectrum of foliar and root diseases, viruses, insects, and nematodes (e.g., Smith *et al.*, 1985; Smith, 2000). Those that shorten the life of the crown and root are particularly important.

The feed value of red clover has been the subject of some breeding effort. Attempts have been made

to find plants with proteins more resistant to degradation (Broderick *et al.*, 1991), while another approach would be to increase the content of soluble carbohydrates that would provide energy for rumen microorganisms to break down proteins without wasteful ammonia production. In this context, genetic variation for cell wall pectins in red clover has been detected (Hatfield and Smith, 1995). Selection for low formononetin has proved to be important for sheep grazing (McDonald *et al.*, 1994; Rumball *et al.*, 1997). On the other hand, now that red clover has become a source of industrial pharmaceuticals (isoflavones), selection for increased contents of such constituents is equally likely to be important.

Seed yield and high persistence tends to be negatively correlated in red clover (Steiner *et al.*, 1997; Herrmann *et al.*, 2006). This has proved to be a barrier to the commercialization of creeping types, in particular, and the development of persistent varieties with high seed yields remains as an important objective for future breeding.

Framework linkage maps of red clover have been developed by Isobe *et al.* (2003), Sato *et al.* (2005), and Herrmann *et al.* (2006). The latter authors identified 38 quantitative trait loci (QTLs) for eight seed yield components as a step toward the application of marker-assisted breeding for improved seed yields. To date, continuous progress in red clover performances through conventional breeding has occurred. Woodfield (1999) has estimated that genetic gains from red clover breeding in New Zealand have been approximately 1% per year since breeding began.

1.4.2 White clover breeding

White clover is a tetraploid but has a diploid system of gametophytic self-incompatibility involving oppositional *S*-alleles at a single locus. Consequently it is highly heterozygous and, having two independently assorting subgenomes, has intergenomic heterozygosity as well. It is severely affected by inbreeding (Cousins and Woodfield, 2006). Genetically, white clover behaves as a cross-pollinated diploid and varieties are generally structured similarly to those of red clover. As with red clover, single and double cross hybrids theoretically have high potential, although obtaining large numbers of suitable inbred lines for efficient screening is a

difficulty. Self-fertility alleles (*Sf*) have proved to be useful in this regard (Cousins and Woodfield, 2006). Because it is predominantly used as a grazing plant in conjunction with grasses, breeders in New Zealand (Caradus *et al.*, 1991) and, more recently elsewhere (Annicchiarico, 2003; Bouton *et al.*, 2005), have very successfully screened breeding populations under grass competition.

Mutation breeding is not a viable option for most objectives because of the tetraploid and outcrossing nature of the species. The diploid self-pollinating relative, *T. occidentale* has been developed as a possible genetic model for white clover (Jones *et al.*, 2006; Williams *et al.*, 2006a). This species has been successfully mutagenized and some stable condensed tannin-lacking mutants have been produced (Williams *et al.*, 2006a). Polyploidy has been explored in white clover and, although some hexaploid and octoploid families have been tested, cytological problems have occurred and the performance of the polyploid materials has not been adequate to make this approach attractive (Williams, 1987c). White clover is very widespread geographically and ecologically and is easy to collect because the seed heads can remain in place for long periods without completely shattering. Consequently, there are large numbers of accessions in seed banks worldwide. Some of the closely related species (Ellison *et al.*, 2006) carry traits that may benefit white clover and use of this secondary gene pool is a promising approach. A major weakness is the adult plant root system and strongly rooted related species like *Trifolium uniflorum* (Pandey *et al.*, 1987) and *Trifolium ambiguum* (Williams and Verry, 1981), which can hybridize with white clover, offer potential solutions. Intolerance to dry conditions is another weakness that might be corrected by hybridization with *T. ambiguum* (IGER) or *T. uniflorum* (Williams *et al.*, 2006b). *T. occidentale* could be a source of drought and salt tolerance and *Trifolium nigrescens* a source for heat and pest resistance (Hussain *et al.*, 1997). Other close species, which have as yet unknown attributes, include *Trifolium thalii* and *T. palleescens* (Williams *et al.*, 2006b).

White clover populations have traditionally been classified according to leaflet size as this trait is highly correlated with other organ sizes (e.g., stolon thickness) and with ecological fitness. Small-leaved plants are best adapted to closely

defoliated pastures, whereas larger-leaved types can be intolerant of hard grazing (Williams, 1987b). One of the most significant recent advances has been the development of large-leaved varieties that can withstand grazing pressures. This is exemplified by the breeding of Kopu II (Woodfield *et al.*, 2001) by selection under sheep grazing. The key methods used were to combine the genomes of small-, medium-, and large-leaved populations to develop parent plants with high stolon densities that survived under grazing pressures in combination with companion grasses. As a result, the old perceptions about a negative relationship between leaf size and grazing tolerance have been changed, although large-leaved varieties are still unlikely to persist under heavy sheep grazing. Use of diverse germplasm has also led to gains in yield and disease resistances in medium-leaved and medium-large-leaved varieties, for example, Crusader (Woodfield *et al.*, 2001).

Root thickness in white clover is correlated with leaf size so that plants with large leaves tend to have thicker roots (Caradus, 1977). Attempts to break this relationship by looking for thicker roots in smaller-leaved varieties have shown only limited success (Woodfield and Caradus, 1987). It is possible that, if a less fibrous, more thickened root system could be developed for adult white clover, the zone of adaptation of the species could potentially be extended to regions with hot, semi-arid climates. This would best be associated with improved drought tolerance of the shoot tissues. White clover generally grows most rapidly in spring and early summer and its cool season activity is low. The use in the 1960–1970s of Mediterranean germplasm to improve cool season activity especially in the United Kingdom, Australia, and New Zealand is well documented (Williams, 1987c).

White clover is affected by a wide range of pests and diseases (Gaynor and Skipp, 1987; Latch and Skipp, 1987; Skipp and Gaynor, 1987). The most intractable of these are the viruses and insects. The succulent nature of white clover tissue makes it a soft target for chewing insects. To date, no selection for resistance to chewing insects has been successful. Although some excellent advances in breeding for virus resistance have occurred by traditional breeding (Taylor *et al.*, 1995), viruses remain one of the main sources of yield losses in pastures (Barnett and Diachun, 1985; Garrett, 1991).

White clover requires high soil concentrations of phosphate to grow and fix N. Most soils require phosphate fertilizers, which are manufactured from nonrenewable resources that are progressively becoming harder to obtain and more expensive. Considerable effort has been allocated to identification and selection of plants able to grow efficiently at low soil phosphate concentrations but, despite promise, selections did not hold up in the field (Caradus, 1990; Caradus *et al.*, 1992). For both red and white clovers, advances in N-fixation potential have paralleled advances in yield. To date, no method has been developed that successfully disconnects N fixation from yield in the clovers. However, there is significant variation in white clover-*Rhizobium* associations for response to high soil N (Crush and Caradus, 2001) to suggest that the amount of N fixation at high soil N concentrations could be raised or lowered by selection. A more rapid "turning off" of N fixation at moderate soil N concentrations might provide a better grassclover balance by preventing clover elimination from mixed sward due to competition from grasses fed by fixed N.

Framework linkage maps for white clover and its very close diploid model species, *T. occidentale*, are available and marker-assisted approaches to clover improvement are in progress internationally. A set of simple sequence repeat (SSR) markers was characterized by Kolliker *et al.* (2001) and these were used by Jones *et al.* (2003) along with amplified fragment length polymorphism (AFLP) markers to develop a map of 18 linkage groups. Barrett *et al.* (2004), Griffiths *et al.* (2006), and Zhang *et al.* (2006) have reported SSR-based maps with 16 linkage groups with high levels of colinearity with *Medicago truncatula*, while Griffiths *et al.* (2006) have shown that the *T. occidentale* map is highly syntenic with *T. repens*. Using these maps, QTL analyses for agronomic traits have been carried out by Barrett *et al.* (2005) and Cogan *et al.* (2006).

Future breeding objectives for white clover include attention to forage quality. As indicated above, white clover is too low in soluble carbohydrate to enable efficient ruminant digestion of the relatively high protein it carries. Potentially large differences have been found among white clover varieties for soluble carbohydrate content, larger-leaved varieties tending to be higher than smaller-leaved varieties (Woodfield *et al.*, 2001).

Thus, it may be possible to select new varieties with higher soluble sugars that will be more efficiently utilized by grazing animals. White clover does not have high foliar concentrations of phyto-oestrogens but many populations are polymorphic for cyanogenesis. The basic genetics of cyanogenesis is well understood (Corkill, 1942; Williams, 1987c), but some aspects of its inheritance still need clarification. Highly cyanogenic varieties, for example, Grasslands Huia, have been used internationally for decades with few negative effects on grazing animals. Cyanogenic white clover may, however, be unsuitable for rapidly growing young animals during pregnancy (Gutzwiller, 1993).

Condensed tannins (CTs) have been targeted as a mechanism for solving the dual problems of bloat and inefficient protein degradation in the rumen since Jones and Lyttleton (1971) noted their potential. White clover has floral CTs but lacks them in leaf tissues. To date, efforts to obtain foliar CTs in red and white clover have been unsuccessful, and this remains a very desirable future breeding target.

Very significant advances in white clover performance through conventional breeding continue to be made. Genetic gains from New Zealand white clover breeding from 1985 to 1998 have been more than 4% annually for sheep grazing and 2.5% for cattle grazing. These gains come at the end of a 60-year breeding program in which mean annual gains have exceeded 1%, in line with many crop species (Woodfield, 1999).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Relative to most field crop species, the clovers are comparatively undomesticated and not far removed from the wild genetic state. Therefore, they are still responding well to conventional breeding methods. In addition, white clover breeders, in particular, have hardly begun to use the secondary gene pools available in closely related species. Conventional breeding of clovers is thus a long way from reaching its limits. Nevertheless, there are some targets that are already looking to be intractable using conventional sources of genetic diversity and transgenics may provide faster solutions. Both insect and virus resistances are important targets that may be successfully

addressed through transgenic breeding. The addition of foliar CTs to clovers and PPO activity to white clover are potentially important nutritional objectives that may best be approached using genetic modification. Similarly, there have been difficulties to date using conventional breeding to improve the efficiency of the N-fixation system and the efficiency of use of phosphorus by clovers. Clover has lower water-soluble carbohydrate concentrations. These low soluble carbohydrate contents, in combination with high protein, can lead to wastage of protein N in ruminant animals where energy is required to prevent the conversion of ammonia to urea and excretion in urine (Buxton, 1996). Perhaps transgenics might eventually prove to be a useful approach for clover development.

2. DEVELOPMENT OF TRANSGENIC CLOVERS

2.1 Promoters for Transgenic Clovers?

There have been several attempts to evaluate potential promoters for transgenic clover transgenic research. The promoter of the *MtHP* gene, belonging to the *PR10* gene family, from *M. truncatula* was fused to the reporter *gusA* gene and nopaline synthase (NOS) transcription terminator to characterize its expression in transgenic white clover plants (Xiao *et al.*, 2005). Transformation was achieved by co-cultivation of cotyledons of imbibed seed with *Agrobacterium tumefaciens*. Selection of transformants was done via the herbicide phosphinothricin resistance conferred by the *bar* gene sequence encoding the enzyme phosphinothricin acetyltransferase. Strong constitutive β -glucuronidase (GUS) expression was observed in transgenic white clover plants containing the *MtHP* promoter. The level of GUS activity in transgenic white clover containing the *MtHP* promoter was higher than the level of GUS activity in transgenic clovers transformed with the cauliflower mosaic virus (CaMV) 35S promoter (Xiao *et al.*, 2005).

The efficacy of four promoter sequences from *Arabidopsis thaliana* (*atmyb32*, *adh*, *xero2*, and *SAG12*) was assessed in transgenic white clover (*T. repens*) plants generated by *Agrobacterium*-mediated transformation (Lin *et al.*, 2003). The promoters were fused to the β -glucuronidase

gene and NOS 3' transcription terminator (Lin *et al.*, 2003). The *atmyb32* promoter directed GUS expression in leaf and root vascular tissue including lateral roots and nodules with low levels of GUS expression in reproductive organs. Wound response of the *atmyb32* promoter in white clover leaves and stolons was also shown. The *adh* promoter showed anaerobic stress and dehydration stress response. The *xero2* promoter directed strong expression in roots, leaf vascular tissue, inflorescences, anther filaments, and pollen grains. The *SAG12* promoter resulted in senescence-associated GUS expression in transgenic white clover leaves (Lin *et al.*, 2003).

Genomic sequences comprising the 5'-UTR and promoter regions of the 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene (TR-ACO1p, TR-ACO2p, TR-ACO3p, and TR-ACO4p) from white clover were fused to the β -glucuronidase gene and NOS 3' transcription terminator (Chen and McManus, 2006). The constructs were used to transform white clover cv. Huia by co-cultivation with *A. tumefaciens* strain LBA 4404 (Chen and McManus, 2006). Selection of transformants was done via kanamycin resistance conferred by the *nptII* gene sequence. The TR-ACO1 promoter directed the highest GUS expression in the apical tissues, axillary buds, and leaf petiolules in younger tissues and then declined in the ageing tissues. The TR-ACO2 promoter directed GUS expression in both younger, mature green, and in ontologically ageing tissue. The TR-ACO3 and TR-ACO4 promoters directed expression in the ontological older tissues, including the axillary buds and leaf petiolules. All promoters directed GUS expression in the ground meristem tissue of axillary buds, vascular tissue, pith and cortex of the internode and node, and the cortex and vascular tissue of the leaf petiolule (Chen and McManus, 2006).

Transgenic *T. repens* cv. Haifa was produced following transformation with a 1764 bp tobacco basic chitinase promoter driving the β -glucuronidase gene and NOS 3' transcription terminator (Pittock *et al.*, 1997). Transformation was achieved by co-cultivation of cotyledons of imbibed seed with *A. tumefaciens*. The *bar* gene and phosphinothricin selection was used to screen transformants. In transgenic clover plants, strong GUS expression was observed in tap and lateral root meristems. No GUS expression was detected in the pericycle or in the dividing cells of the developing lateral root. Also, mechanical

wounding and aphid (Family Aphididae) feeding resulted in rapid and localized GUS staining (Pittock *et al.*, 1997).

2.2 Improving Clover Forage Quality

Transgenic white clover plants generated by Jenkins *et al.* (2002) contained the gene encoding fructosyltransferase (Ftf) enzyme from the bacterium *Streptococcus salivarius*. The transgene was cloned between the CaMV 35S promoter and NOS 3' transcription terminator. Clover cotyledons were transformed by co-cultivation with *A. tumefaciens* strain AGL1. Selection of clover transformants was by kanamycin resistance conferred by the *nptII* gene. White clover does not normally accumulate fructan as a storage carbohydrate. However, transgenic clover plants accumulated fructan in leaves, petioles, stolons, flowers, and root tissues. Levels of fructan up to approximately 2% dry weight were measured in leaves. Ftf enzyme activity up to 120 nmol min⁻¹ g⁻¹ fresh weight was determined in leaf extracts of the transformed plants, and appeared to be stable throughout leaf development. Most transgenic white clover lines flowered and produced seeds, but the growth rate of some transformed lines decreased. In a transgenic line with relatively low fructan, levels of endogenous carbohydrates such as hexoses, sucrose, and starch were not substantially changed. However, growth and endogenous carbohydrate contents were reduced in transgenic white clover with relatively high fructan accumulation (Jenkins *et al.*, 2002).

Most dietary proteins ingested by ruminants are rapidly degraded by rumen bacteria. Rumen microbial proteins in turn become sources of protein for the animals. However, microbial proteins are poor in sulfur amino acids. A small number of proteins appear to be relatively resistant to degradation in the rumen and presumably are assimilated by ruminants in the lower gastrointestinal tract. In an attempt to improve the rumen-protected level of the sulfur amino acids cysteine and methionine in transgenic clovers, Christiansen *et al.* (2000) introduced the coding sequence of the sunflower seed albumin (SSA) into *T. repens* cv. Haifa by *A. tumefaciens*-mediated transformation. The SSA gene was driven by three different promoters in separate plant transformation vectors. The promoters used in this work

were: (1) the promoter region of the small subunit of ribulose biphosphate carboxylase (Rubisco) gene from *A. thaliana* (*A(ssu)*), (2) the promoter region of the small subunit of Rubisco gene of *Medicago sativa* (*L-ssu*), or (3) the commonly used CaMV 35S promoter. Transformant clovers were selected on the basis of their resistance to the herbicide phosphinothricin conferred by the *bar* gene sequence. The highest observed level of SSA accumulation in transgenic clovers was approximately 0.1% of total extractable leaf protein. Promoter activity as measured by the effect on the level of SSA accumulation was in the order of *A(ssu)* > CaMV 35S > *L-ssu* (Christiansen *et al.*, 2000).

In a separate attempt, a gene encoding a sulfur-rich maize seed storage protein, δ -zein, was introduced into white clover plants by *Agrobacterium*-mediated transformation of cotyledons with or without the apical shoot (Sharma *et al.*, 1998). The transgene construct was under the control of the double 35S promoter of CaMV and the NOS 3' transcription terminator. Selection of clover transformants was by kanamycin resistance conferred by the *nptII* gene. Transgenic clover plants expressed the maize δ -zein mRNA (messenger RNA) and protein in their leaves. The δ -zein protein was also detected in petioles, nodes, internodes, roots, and seeds of transgenic clover plants. In the highest expressing transgenic clover plants, δ -zein accumulation increased with increasing leaf age from 0.3% (young leaves) to 1.3% (old leaves) of total water-soluble protein (Sharma *et al.*, 1998).

Similarly, a transgene encoding the pea albumin 1 (PA1) protein, rich in sulfur amino acids, transcribed from the CaMV 35S promoter and NOS 3' transcription terminator was introduced into the white clover genotype WR8 via *Agrobacterium*-mediated transformation (Ealing *et al.*, 1994). By introducing genes encoding rumen-protected proteins rich in essential amino acids, the objective was to improve the quality of white clover proteins. Transgenic clover plants expressed and accumulated PA1 gene products (Ealing *et al.*, 1994).

2.3 Herbicide Resistance and Weed Control

In Australia, the herbicide bromoxynil (3,5-dibromo 4-hydroxy benzonitrile) is widely applied

to annual legume-based pastures, especially in subterranean clover (*T. subterraneum*) based pastures to control broadleaf weeds (Dear *et al.*, 2003). Although subterranean clover shows a moderate level of tolerance to the herbicide, it still suffers substantial forage yield depression from herbicide application. The *bxn* gene, isolated from the soil bacterium *Klebsiella ozaenae*, codes for a nitrilase enzyme, which converts bromoxynil to a relatively nontoxic product (3,5-dibromo-4-hydroxybenzoic acid) and is then further degraded by plant enzymes. As a result, the bacterial *bxn* gene was placed between the CaMV 35S promoter and NOS 3' transcription terminator. Transformation of subterranean clover tissue pieces (lower hypocotyl together with the radicle from imbibed seeds) was done by co-cultivation with *A. tumefaciens*. Selection of transformants was via spectinomycin resistance conferred by a gene from *Shigella flexneri* cloned between the CaMV 35S promoter and octopine synthase gene 3' transcription terminator from *Agrobacterium*. In field experiments and greenhouse studies, the application of bromoxynil herbicide resulted in a 50% reduction in the herbage yield of the nontransgenic control but no yield reduction was observed in transgenic subterranean clover lines (Dear *et al.*, 2003).

2.4 Disease and Pest Resistance

Serine proteinase inhibitors have been shown to retard the development of feeding insect pests. Consequently, transgenic white clover plants were generated by placing an 850 bp coding region (plus 200 bp of 3' untranslated region) of the soybean (Kunitz) trypsin inhibitor (SBTI) gene between the CaMV 35S promoter and NOS 3' transcription terminator (McManus *et al.*, 2005). Transformation was done by co-cultivation of cotyledons with *A. tumefaciens* strain LBA 4404. Selection of transformant clovers was by kanamycin resistance conferred by the *nptII* gene. The protein was expressed in transgenic clover plants studied. The recombinant inhibitor protein was effective against trypsin-like activity of black field cricket (*Teleogryllus commodus*) and grass grub (*Costelytra zealandica*), but ineffective against trypsinlike activity from light brown apple

moth (*Epiphyas postvittana*) and porina (*Wiseana copularis*) (McManus *et al.*, 2005).

The white clover mosaic virus (WCIMV), a member of the Potexviurs group, is the most widespread pathogen that causes significant yield losses to white clover production worldwide. The genomic organization of WCIMV spanning an approximately 6 kb sense RNA, encodes five genes required for replication (147 kDa), cell-to-cell and systemic movement (26 kDa, 13 kDa, and 7 kDa), and the coat protein (CP). As a potential strategy to produce virus-resistant plants, transgenic approaches were employed using three of the WCIMV genes: the CP gene (Beck *et al.*, 1993), a mutated form of the 13K movement protein gene (Beck *et al.*, 1994), and the 147 kDa replicase gene (Voisey *et al.*, 2001). The WCIMV gene constructs were placed under the control of the CaMV 35S promoter and the NOS 3' or octopine synthase transcription terminators. In all cases, transformations of white clover were done by co-cultivation with *A. tumefaciens* strain LBA 4404. Kanamycin resistance conferred by the *nptII* gene was used as a selectable marker. In one of the experiments, the protein content of the WCIMV CP transgene in four white clover plants ranged from 0.0005% to 0.005% of total soluble leaf protein. Only two transgenic white clover plants expressing relatively higher levels of WCIMV CP showed some resistance to virus inoculation (Voisey *et al.*, 2001). In general, the effect of the CP transgene was associated with a time delay in the systemic spread of the virus on inoculated transgenic plants. In the second transgenic approach, 11 transgenic white clover plants expressing a mutant WCIMV 13 kDa movement protein gene were evaluated for resistance to WCIMV inoculation (Voisey *et al.*, 2001). Of these, three transgenic clover plants were found to be resistant against high levels of WCIMV, although approximately 20% of the transgenic white clover plants were found to be infected when tested for the presence of the virus 40 days post inoculation. The WCIMV resistance displayed in the three transgenic white clovers expressing the mutant 13 kDa WCIMV was stable and was inherited in F₁ progeny obtained from pair crossing of primary clover transformants with commercial clover varieties. In a similar approach, 22 transgenic white clover

plants expressing the WCIMV replicase gene were evaluated for WCIMV resistance (Voisey *et al.*, 2001). Approximately 87% of the transgenic clover plants showed resistance to WCIMV inoculation, with five transgenic white clover plants categorized as having immunity to systemic viral infection.

Alfalfa mosaic virus (AMV) can cause significant yield losses to forage legumes including white clovers. In a study from Australia, transgenic white clover plants were generated using the *CP* gene of an alfalfa mosaic alfamovirus (AMV) (Kalla *et al.*, 2001). The AMV *CP* gene was fused between the CaMV 35S promoter or *Arabidopsis* small subunit ribulose biphosphate carboxylase/oxygenase promoter and the NOS 3' transcription terminators (Kalla *et al.*, 2001). Transformation of white clover plants was by co-cultivation with *A. tumefaciens*. Five AMV-immune transgenic white clover lines and a single resistant transgenic line were identified in controlled experiments (Kalla *et al.*, 2001). After one growing season in field plots, 60% of the nontransgenic control plants were infected with the virus; whereas, all the immune transgenic white clover plants expressing AMV *CP* transgene were found to be resistant to AMV inoculation (Kalla *et al.*, 2001).

2.5 Transgenic Clovers for Industrial Applications

Clovers have primarily been used as a source of animal feed. Plants are capable of carrying out acetylation, phosphorylation, and glycosylation as well as other post-translational protein modifications required for the biological activity of many recombinant proteins. The ease of genetic transformation and large-scale cultivation make clovers attractive targets for the production of recombinant proteins and enzymes for large-scale industrial applications and to deliver oral vaccines. Improving the economic value of clovers could be achieved by developing new uses and value-added products for clovers.

Mannheimia haemolytica is the principal microorganism responsible for bovine pneumonic pasteurellosis, or shipping fever. Leukotoxin (Lkt) has been identified as an important protective antigen of *M. haemolytica*, and a fragment, Lkt50, was shown to produce toxin-neutralizing

antibodies in rabbits. A gene encoding Lkt50, fused to modified GFP5 was placed under the control of the CaMV 35S promoter and NOS 3' transcription terminator. White clover petioles were transformed by co-cultivation with *A. tumefaciens* strains, LBA 4404 and C58. Transgenic white clover plants produced detectable levels of the fusion protein, Lkt50-GFP. A leaf extract containing Lkt50-GFP from white clover was immunogenic and elicited toxin-neutralizing antibodies in experimental rabbits. The recombinant antigen was stable after drying of the transgenic clover tissue at room temperature for 4 days (Lee *et al.*, 2001), or even after oven drying of transgenic tissue at 50 °C (Lee *et al.*, 2003). The recombinant antigen was present in the dried clover after 1 year of storage at ambient temperature. Field- and greenhouse-grown transgenic clovers maintained similar levels of Lkt50-GFP expression (Lee *et al.*, 2003).

3. FUTURE ROAD MAP

3.1 Expected Products

Genetic transformation is routine for a number of clover species and traits of interest to clover producers have been demonstrated to be effective. Published reports demonstrated the stable expression and inheritance of transgenes in transgenic white clover plants (Scott *et al.*, 1996, 1998). Transgenic white clover plants had been outcrossed to cultivar material as a first step toward the breeding and field release of virus-resistant white clover cultivar (Woodfield and White, 1996; Voisey *et al.*, 2001). Breeding strategies for developing transgenic white clover cultivars have been suggested (Woodfield and White, 1996). However, the current highly segmented clover seed production industry and high cost of commercialization of biotechnology traits does not favor the commercialization of transgenic clover in the near future.

3.2 Risks and Concerns

White clover has many of the characteristics of a successful weed and transgenic forms may be considered to be environmental threats in some jurisdictions. A risk assessment carried

out in Australia (Australian Government, 2004) showed that there was a potential risk of weediness, especially in disturbed sites. However, the chances of white clover transferring genes to other organisms were found to be negligible as it does not naturally hybridize with any other species.

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Apple

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Apple (*Malus × domestica* Borkh.) is the most important temperate tree fruit crop grown in North America and around the world, and belongs to the family Rosaceae and subfamily Pomoideae. It has been reported that the domestic apple arose some 8–12 million years ago in refuge fruit forests in Central Asia, primarily in the Tien Shan region, an area extending from the current region of Kazakhstan, through the Xinjiang Province in China, and all the way west to Uzbekistan (Juniper *et al.*, 1999). Through natural distribution, selection, random hybridization, as well as adaptation over millions of years, the new apple of the Tien Shan, *Malus sieversii* Lebed., having fruit ranging in size from small (2 cm) to large (6 cm), has played a major role in the origin of the cultivated apple. It is likely, that to the east of this region, *M. sieversii* could have hybridized with *Malus prunifolia* (Willd.) Borkh., *Malus baccata* (L.) Borkh., and *Malus sieboldii* (Reg.) Rehd. and to the west with *Malus turkmenorum* Juz., *Malus orientalis* L., and *Malus sylvestris* (L.) Mill. (Juniper *et al.*, 1999). Recent studies utilizing molecular

markers, random amplified polymorphic DNAs (RAPDs), along with phylogenetic tree analysis have confirmed that *M. sieversii* is the primary progenitor of the cultivated apple, *M. × domestica* (Lamboy *et al.*, 1996; Zhou and Li, 2000).

Cultivation of the apple has taken place since the age of the ancient Greeks and the Romans, who following their travels and invasions, spread these practices throughout Europe and Asia (Janick *et al.*, 1996; Juniper *et al.*, 1999). Cultivars were selected and propagated ever since, particularly as vegetative propagation, via grafting, was practiced over 2000 years ago. As cultivars were brought into Europe, particularly those making it to the British Isles, they hybridized among themselves, and underwent spontaneous mutations resulting in new cultivars. Later, apples were brought over to North America, and cultivation practices of the colonial Europeans were further adapted and modified. As pioneers of distribution of apple seed throughout North America, such as John “Johnny Appleseed” Chapman (1774–1825), random hybridizations took place that eventually led to the discovery of chance seedlings that became significant apple cultivars, such as “Delicious”, “Golden Delicious”, and “Jonathan” that were adapted for worldwide growing conditions (Juniper *et al.*, 1999).

1.2 Botanical Description

The genus *Malus* has from 25 to 33 species depending on taxonomic treatment, along with several subspecies, arranged into three geographical groups (Chinese, European, and American) and five botanical sections (Janick *et al.*, 1996; Juniper *et al.*, 1999). The genus *Malus* is characterized by fruits consisting of two to five carpels enclosed by a fleshy tissue.

Malus species as well as clones within the same species have varying ploidy levels as these are more than likely collected from different trees and/or different locations (Schuster and Büttner, 1995; Tatum *et al.*, 2005). Botanical varieties and/or genotypes of few other *Malus* species, such as *M. halliana* and *M. hupehensis*, also show varying ploidy levels as well (Schuster and Büttner, 1995). It has been suggested that differences in ploidy levels in the same species is likely attributed to frequent hybridizations within the genus *Malus*. The cultivated apple has a complex genome consisting of 34 chromosomes per diploid genome. The origin of the basic haploid number of $n = 17$ in *Malus* and other *Pomoideae* has been attributed to hybridization between two remote ancestral types, *Prunoideae* ($n = 8$) and *Spiroideae* ($n = 9$) (Derman, 1949; Challice and Westwood, 1973). On the other hand, Darlington and Moffett (1930) have suggested that the basic number $n = 7$ (very common in other genera of *Rosaceae*) was doubled with three of the chromosomes repeated a third time leading to the haploid number of $n = 17$. DNA flow cytometric analysis has indicated that the mean of diploid genotypes investigated has a DNA content of 1.582 pg/2C, and therefore a single chromosome accounts for 0.045 pg (picogram) (Tatum *et al.*, 2005). Given a 0.23 pg variation among diploids, this suggests that nuclear DNA content variation among diploid apple cultivars and species may equal to the DNA amount of almost six chromosomes. Given the complexity of the apple genome and its suggested allopolyploid origin (Schuster and Büttner, 1995; Evans and Campbell, 2002; Tatum *et al.*, 2005), the apple can easily withstand the loss of a certain amount of DNA, and there is a slight tendency toward diminished haploid nuclear DNA content with increased polyploidy (Tatum *et al.*, 2005).

Apple has gametophytic self-incompatibility, and it is controlled by a single multiallelic *S* locus.

The *S* locus has *S2-* (= *Sa-*), *S3-*, *S5-*, *S7-* (= *Sd-*), *S9-* (= *Sc-*), *Sf-*, *Sg-*, *S24-* (= *Sh*), *S26-*, and *S 27-* alleles (Matsumoto and Kitahara, 2000), and these encode RNase linked to *S*-locus (*S*-RNases), many of which have been cloned (Broothaerts *et al.*, 1995). Therefore, when the pollen *S*-allele matches one of the pistil's *S*-alleles, fertilization is inhibited. Several of these alleles of the *S* locus in apple have been identified and their nucleotide sequences have been determined (Broothaerts, 2003). Based on the diversity of nucleotide sequences encoding the *S*-RNase family members, allele-specific polymerase chain reaction (PCR) primers have been developed to selectively amplify and identify particular *S*-alleles, and later this approach has been modified in order to identify additional alleles (Janssens *et al.*, 1995; Broothaerts, 2003). The new numbering introduced *S*₁₆, *S*₁₉, *S*₂₂, and *S*₂₃. Recently, Broothaerts *et al.* (2004b, b) have been able to identify 18 different *S*-alleles, and these have been used to determine *S*-genotypes of 150 diploid and triploid European, American, and Japanese apple cultivars. This has indicated that three *S*-alleles (*S*₂, *S*₃, and *S*₉) are very common in these cultivars, probably attributed to the widespread use of the same breeding parents, and seven alleles are very rare (*S*₄, *S*₆, *S*₈, *S*₁₆, *S*₂₂, *S*₂₃, and *S*₂₆).

1.3 Economic Importance

World apple production is around 42 million metric tons (based on 2004/2005 data), and this is valued at US\$1.5 billion (United States Department of Agriculture (USDA) Agricultural Attache Reports and the US National Agricultural Statistics Service.). China is the leading producer of apples, followed by United States, Turkey, Poland, and Italy. The US apple crop is at 4.6 million metric tons (2004/2005 data). The southern hemisphere has increased production of apples to around 4.4 million metric tons, with Argentina and Chile leading the way. World production of apple juice is around 1.26 million metric tons (USDA-ARS), of which 1.19 million tons are exported. China exports almost 50% of this world total. The United States is one of the world's largest importers of apple juice concentrate. Although global apple consumption has been either stagnant or on the decline, consumption of fresh apples in developed

countries has been on the rise with improved choices and availability of new cultivars. China is the largest consumer of apples, followed by the United States, Turkey, Germany, and Italy.

There are over 7500 cultivars of apples grown in the world. In the United States, over 100 cultivars of apples are grown commercially. Although apples are grown in all the 50 US states, they are commercially grown in only 36 states. In 2002, US consumers ate an average of 42.2 pounds of fresh apples and processed apple products, of which 62% was consumed as fresh fruit.

Apple fruits are characterized as healthful fruits as they do not contribute any fat, sodium, or cholesterol to the human diet. A medium apple contains about 80 calories. Apple fruits are great sources of fiber and pectin, with an average fruit containing five grams of fiber. Apple fruits are rich in sugar and starch. Unripe apples contain as much as 15% starch, while ripe apples contain 8–14% sugar (Reed, 1975). As the size of young fruitlets reach 20 mm in diameter, starch begins to rapidly accumulate (Magein and Leurquin, 2000). As fruit begins to ripen, starch stored in the flesh gradually converts into sugar. Levels of starch and sugar accumulation in apple fruits are genotype dependent. For example, the “Fuji” apple cultivar is a low-starch apple; while “Granny Smith” is a high-starch apple. Next to “Granny Smith”, “Pink Lady” is the second highest starch-accumulating apple grown commercially. Fruit starch content has a strong influence on both eating and processing qualities of apple, as starch together with organic acids are known to greatly contribute to flavor composition. Apples with low starch content combined with low levels of organic acids taste sweet, while those rich in both starch and acid are rather tart. Meanwhile, starch is generally considered to be a problem for processing of apple juice (Carrin *et al.*, 2004). High starch content will contribute to prolonged filtration and result in post processing cloudiness of the apple juice. In order to degrade and eliminate insoluble starch, amylases are widely used during the processing of apple juice production.

1.4 Traditional Breeding

Apple breeding for both fruiting cultivars and rootstocks has evolved over hundreds of years

from accidental and random observations of chance seedlings and spontaneous mutations with desirable fruit and tree characters to deliberate and careful evaluations of populations derived from either open-pollinated seedlings, controlled crosses, or induced mutations. Those individuals exhibiting outstanding or unique fruit characters such as fruit color, pigmentation patterns, acidity and sugar content, juiciness, firmness, as well as various alkaloids and flavor components were the first to be selected. Interest in manipulating and controlling tree growth and stature has led to the identification and selection of desirable rootstocks with dwarfing ability that influences the overall size of the grafted tree, anchorage, as well as resistance to various diseases and pests (Korban and Chen, 1992; Janick *et al.*, 1996).

Over time, breeding objectives continued to incorporate all of the above-mentioned desirable traits, as well as expand to other traits, for both scion cultivars and rootstocks. However, as controlled sexual hybridizations among selected parents became more widely practiced, and breeding programs began to expand and grow, emphasis was placed on selecting parents with desirable fruit characters. Large populations of seedlings were then planted with the anticipated hope of identifying outstanding selections. As the generation cycle of apple breeding can be anywhere from 5 to 7 years, progress was slow. As the genetic control mechanisms of various reproductive and vegetative traits were investigated and became known, breeding efforts made more progress when traits were known to be controlled by qualitative rather than quantitative genes. Approximately, 30 traits or so are known to be controlled by either one or two major genes, and the majority of these traits are primarily associated with either disease and/or insect resistance (Janick *et al.*, 1996). However, most traits, particularly those associated with fruiting and fruit quality, are polygenically controlled.

Over the years, apple breeding programs from all over the world, especially those in New Zealand, Japan, and the United States, have been successful in developing new apple cultivars that have made significant impact on the commercial market. Such cultivars as “Gala”, “Empire”, “Fuji”, “Pink Lady”, “Jonagold”, and “Braeburn”, among others, have become important commercial cultivars worldwide. These cultivars are selections from

F₁ populations derived from controlled crosses between outstanding cultivars used as parents. These cultivars were selected mainly for fruit quality traits, and all these traits are controlled by polygenes. Although most of these apple cultivars are diploids, there are triploid cultivars as well, such as “Jonagold” and “Crispin” (also known as “Mutsu”).

Successful breeding efforts for traits controlled by polygenes can be best exemplified by those cultivars and rootstocks with enhanced tolerance to cold temperatures. Cultivars such as “Honeycrisp” from the Minnesota breeding program and rootstocks such as the Budagovsky series from Russia and the P-series from Poland are also making an impact on the commercial market.

Breeding programs have also been successful in enhancing disease resistance in apple. The best such example is the development of new cultivars with resistance to the fungal disease, apple scab, caused by *Venturia inaequalis* (Cke.) Wint. Having identified single major genes for resistance to apple scab in small-fruited crabapple *Malus* species, the Purdue-Rutgers-Illinois (PRI) cooperative breeding program and later the Cornell breeding program have been able to develop more than 20 apple cultivars with resistance to apple scab. These cultivars resulted from selections following 4–6 generations of controlled crosses using the whole pool of scab-resistant cultivars as “recurrent” parents in crosses (30–40 years). These successful efforts were also replicated in various international breeding programs such as those found in Canada, France, Brazil, England, Australia, Czech Republic, Germany, Romania, and Hungary, among others, in developing many other well-adapted new cultivars with resistance to apple scab. However, these cultivars are yet to have a significant impact on the commercial market, although there is hope that newly released cultivars, such as “GoldRush”, “Juliet”, and “Ariane” may do so in the future.

An example of successful breeding for pest resistance is the development of rootstocks resistant to woolly apple aphid, *Eriosoma lanigerum* Hausm., by incorporating the *Er* gene for resistance, derived from “Northern Spy”, into the Malling rootstock breeding programs in the United Kingdom. The resulting woolly apple aphid-resistant F₁ selections, such as MM106 and MM111, have become commercially important

rootstocks that are grown worldwide. They are now being superseded by rootstocks from the Geneva, NY breeding program that incorporate resistance to fire blight as well as to woody apple aphid.

So far, all apple breeding efforts involve *in vivo* screening of either whole seedlings or detached plant tissues for disease resistance, insect resistance, cold hardiness, dwarf compact habit, or any combinations of these traits. Moreover, this is often followed by *in vivo* screening of whole trees for flowering, fruiting, fruit quality, and tree characters. In recent years, molecular mapping studies of the apple have been underway with over 1000 markers, including isozymes, RAPDs, restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and expressed sequenced tag-SSRs (EST-SSRs) mapped to 17 linkage groups (Xu and Korban, 2000; Liebhard *et al.*, 2002, 2003; Gardiner *et al.*, 2003, 2007; Naik *et al.*, 2006; Silfverberg-Dilworth *et al.*, 2006). These molecular markers have not yet been used in marker-assisted selection although some promising efforts are underway (Bus *et al.*, 2000; Huaracha *et al.*, 2004; Gardiner *et al.*, 2007), and these will aid in expediting early screening of seedlings for various traits, particularly for flowering and fruiting characters.

1.5 Limitations of Conventional Breeding and Rationale for Transformation Breeding

As mentioned above, conventional breeding efforts for improvement of the apple have yielded substantial success in the development of several of cultivars that have been successfully introduced to the commercial industry. However, these breeding efforts have incurred major commitments of time, money, and land space, as well as personnel. As the apple is highly heterozygous, self-incompatible, and has a generation cycle of 5–7 years, the progress made thus far via traditional breeding has been rather modest when compared to other fruit crops, such as peach and table grape, and less so, when compared to herbaceous horticultural crops, such as tomato and beans. As a result of the slow progress made so far, the number

of apple breeding programs worldwide decreased for several years, and has now recovered. In the meantime, research efforts have moved toward identifying molecular markers linked to traits of interests, as mentioned above, developing genetic linkage maps, as well as isolating and cloning genes controlling various desirable traits, including those controlling juvenility, flowering, fruit quality, and disease resistance.

For example, Kotoda *et al.* (2006) isolated and cloned *MdTFL1* (*M. × domestica TFL1*), a gene involved in the maintenance of the juvenile and vegetative phase in apple. However, to confirm the functionality of *MdTFL1*, this gene was introduced into apple, in an antisense orientation, via *Agrobacterium*-mediated transformation, and among recovered transgenic lines, one line was identified that flowered after 8 months following transfer to the greenhouse, while nontransformed control plants had flowered after nearly 6 years. However, flowering after 8 months in the greenhouse has been shown with normal apple seedling (Aldwinckle, 1975). It is important to add that flower organs of trees of this transgenic line were normal in appearance and capable of setting and producing fruit. Interestingly, some flowers of these transgenic apples developed without undergoing dormancy. Therefore, this study suggested that expression of *MdTFL1* gene in apple might influence flower development as well as flower induction. Apple orthologs of several genes controlling juvenility and flowering time, such as *AFL1*, *AFL2*, and *MdAPI* (*MdMADS5*), have also been isolated and characterized (Kotoda *et al.*, 2000, 2002; Kotoda and Wada, 2005). In another example, the *Vf* gene(s) of apple scab resistance was cloned and characterized (Vinatzer *et al.*, 2001; Xu and Korban, 2002), and when two of the four *Vf* paralogs, found clustered within the *Vf* locus, were introduced into scab-susceptible apple cultivars via *Agrobacterium*-mediated transformation, enhanced resistance to apple scab was observed in some transgenic apple lines (Malnoy *et al.*, 2007e).

Various other genes from apple have been cloned and characterized in recent years as well. For example, Gao *et al.* (2005) isolated and characterized two sorbitol transporter genes, *MdSOT1* and *MdSOT2*. Comparisons of expression of transporter genes, especially in various sink tissues including watercored and nonwatercored

fruit tissues indicated that sorbitol transporter expression was evident in all sink tissues tested with the exception of watercore-affected fruit tissues. Therefore, expression of these genes in apple fruit would likely overcome problems of watercore in apple fruit.

In recent years, genomics resources for apple have become available, such as the construction of bacterial artificial chromosome (BAC) libraries (Paton *et al.*, 1999; Xu *et al.*, 2001, 2002) and generation of over 300 000 ESTs (Korban *et al.*, 2005; Newcomb *et al.*, 2006), our capabilities for isolating and cloning of genes controlling various economically important vegetative and reproductive traits have significantly increased. Therefore, efforts to improve the apple via the tools of genetic engineering, and thereby bypassing the lengthy strategies used in traditional breeding have become more critical, and much needed.

2. DEVELOPMENT OF TRANSGENIC APPLES

2.1 Apple Transformation Procedure

Development of an effective system for gene transfer in apple depends largely on the availability of tissue culture techniques that permit efficient DNA delivery, regeneration of shoots, selection of transformants, and propagation of transgenic plants. Increasing leaf regeneration efficiency is critical for the development of a transformation system in apple using an *Agrobacterium tumefaciens* vector (Klee and Rogers, 1989) or by a biolistic process. In many instances, the lack of efficient regeneration systems is the major limiting factor preventing the development of gene transfer technologies for perennial crops (Dandekar, 1992).

The first report of *in vitro* regeneration of adventitious shoots from apple was made by Liu *et al.* (1983a, b) using apple seedlings. Several later reports (Barbieri and Morini, 1987; Welandar, 1988; Fasolo *et al.*, 1989; Sriskandarajah *et al.*, 1990; Swartz *et al.*, 1990; Korban *et al.*, 1992; James *et al.*, 1994) revealed critical factors affecting the frequency of leaf regeneration in apple scion cultivars, rootstocks, and seed explants. They include nitrogen source and concentration, growth regulators, incubation conditions, leaf origin, leaf

maturity and position on the stem, mode of excision, and explant orientation.

Greensleeves was the first apple cultivar to be transformed (James *et al.*, 1989) with an efficiency of transformation between 0.1% and 0.5% on a per explant basis. The method used was based on a leaf disk transformation method using disarmed strains of *A. tumefaciens*. The *GUS* gene in the transformed Greensleeves plant produced in this way displayed stable patterns of expression in fruit and Mendelian segregation in the progeny (James *et al.*, 1995, 1996). To date, variations of this method have been used to transform different *M. × domestica* cultivars (30) and rootstocks (7), *M. prunifolia* (2), and one *M. robusta* variety (Table 1).

The techniques of transformation used for these different apple genotypes are quite similar (Table 1). Due to the fact that all genotypes do not respond similarly to the same procedure of transformation some modifications have been reported.

2.1.1 Nature of the explants

In a large majority of the procedures published, young expanded apple leaves were used as explants. The regeneration of transgenic lines from leaves has been enhanced by placing the leaf segment abaxial side up on the medium, possibly due to increased oxygen exchange since stomata are located abaxially (Yepes and Aldwinckle, 1994). Sriskandarajah and Goodwin (1998) reported that conditioning of apple shoots for several days in an appropriate liquid medium enhances the regenerative capacity of leaf explants by reducing the need for an extended callus phase. However, this conditioning process for explants has not been subsequently reported in transformation protocols. Other procedures reported use of internodal (Liu *et al.*, 1998, 2001), shoot (Pawlicki-Jullian *et al.*, 2002), and stem explants (Yamashita *et al.*, 2004), and protoplast (Ratnasiri *et al.*, 2002; Hyung *et al.*, 1995). The regeneration of plants from protoplasts is a lengthy process compared with standard tissue transformation. Therefore, the transformation of protoplasts is unlikely to be a source of new transgenic plant material, but the system would be useful as a high-throughput approach for transient assay of gene function or protein targeting.

2.1.2 Inoculation procedures

Two inoculation procedures have been used. The first consists of wounding the leaves with a scalpel soaked in the inoculum (Wilson and James, 2003). The second is to bathe prewounded leaves in the inoculum for a few minutes (De Bondt *et al.*, 1994; Norelli *et al.*, 1994) to several hours (Mooney and Goodwin, 1989). Other modes of inoculation have been tested but did not significantly increase the rate of transformation. For example, sonication of the leaf explants in presence of the inoculum has been reported to increase transformation of the apple cultivar Holsteiner but not of Elstar (Szankowski *et al.*, 2001). Vacuum infiltration of Royal Gala leaves was reported to have no effect on transformation (Norelli *et al.*, 1996).

2.1.3 Strains of agrobacterium used

About 95% of apple transformation procedures were based on use of *A. tumefaciens*, with the remaining 5% using *Agrobacterium rhizogenes* for the transfer DNA (T-DNA) integration (Pawlicki-Jullian *et al.*, 2002; Yamashita *et al.*, 2004). The efficiency of transformation depended on the apple cultivars and the strain of *A. tumefaciens* used. For example, the Jork 9 rootstock was transformed at 8.3% efficiency with the strain EHA105 and 1.5–7.2% efficiency with the strain C58C1 (Zhu *et al.*, 2001b).

2.1.4 The selectable marker used

In most cases (90%), kanamycin has been used as a selectable marker. Concentration of kanamycin used for the regeneration of transgenic apple varied depending on the cultivar. Two other antibiotics have been used as selectable agents: hygromycin (Dolgov *et al.*, 2000, 2004a) and phosphonothricin (Szankowski *et al.*, 2003).

2.1.5 Other modifications

Other modifications have been made to these procedures to increase their transformation efficiency. These modifications included the use of plant phenolic compounds (acetosyringone,

Table 1 Genetically transformed apple cultivars

<i>Malus X domestica</i>								
Cultivars	Explants used	Inoculation procedure	<i>Agrobacterium</i> strains	Hormones used for regeneration, nutrients, and sugar	Genes	Selectable marker	Efficiency of transformation	References
1-Ariane	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculums	EHA105	TDZ, IBA MS with sucrose	<i>Chitinase</i> <i>Puroindoline-b</i>	Kanamycin 100 mg l ⁻¹	2%	Faize <i>et al.</i> , 2003, 2004
2-Braeburn	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, 2iP, Ga ₃ MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	De Bondt <i>et al.</i> , 1994, 1996
3-Delicious Red Delicious	Protoplast	Protoplast transformation was via a PEG-mediated protocol	None		<i>GFP</i>	None	0–7.4% GFP protoplast transformant	Ratnasiri <i>et al.</i> , 2002
	Leaf	Leaf cut transversally into strips (2–3 mm) and transferred to inoculum for 36 h	C58C1/pGV3850	BAP MS with sucrose	<i>Nopaline dehydrogenase</i>	Kanamycin 100 mg l ⁻¹	1–2%	Mooney and Goodwin, 1989
		Leaf explants shaken for 20 min 75 rpm in the inoculum at 35 °C	Nd	NAA, TDZ MS with sucrose	<i>Neo Gus</i>	Kanamycin 100 mg l ⁻¹	1–2%	Sriskandarajah <i>et al.</i> , 1994; Sriskandarajah and Goodwin, 1998
		Leaf cut three times perpendicularly to the midrib and shaken in inoculum	EHA101		<i>GFP</i>	Kanamycin 50 mg l ⁻¹	Cell transformed No plants regenerated	Maximova <i>et al.</i> , 1998
(Continued)								

(Continued)

Table 1 Genetically transformed apple cultivars (*Continued*)

	Explants used	Inoculation procedure	<i>Agrobacterium</i> strains	Hormones used for regeneration, nutrients, and sugar	Genes	Selectable marker	Efficiency of transformation	References
4-Elstar	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, 2iP, Ga ₃ MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	DeBondt <i>et al.</i> , 1994, 1996
		Leaf immersed in inoculum for 4–6 min	AGLO	NAA, TDZ MS with sorbitol	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	0.4–0.8%	Schaart <i>et al.</i> , 1995; Puite and Schaart, 1996
		Leaf gently shaken in inoculum for 10 min	EHA105	TDZ, NAA MS with sorbitol	<i>Stilbene synthase PGIP</i>	Phosphonothricin 1–10 mg l ⁻¹	0.17%	Szankowski <i>et al.</i> , 2003
5-Falstaff	Leaf	Leaf discs immersed 20–30 min in inoculum	LBA4044	IBA, TDZ, BAP 2iP, Ga ₃ MS with sorbitol	<i>Gus</i>	Kanamycin	7.5% 3.5%	Wilson and James, 1998
6-Florina	Leaf	Leaf explants dipped in inoculum for 5 min	C58C1	BA, NAA MS with sucrose	<i>RollB</i>	Kanamycin 100 mg l ⁻¹	7.9%	Radchuck and Korkhovoy, 2005
7-Fuji	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, 2iP, Ga ₃ MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	DeBondt <i>et al.</i> , 1994, 1996
		Leaf wounded	LBA4404	TDZ, NAA MS	<i>Polyphenol oxidase</i>	Kanamycin 50 mg l ⁻¹	1.1%	Murata <i>et al.</i> , 2001
		Nd	LBA4404	TDZ, IBA MS with sorbitol	<i>MdMADS2</i> <i>MdMADS4</i> <i>B-peru</i>	Kanamycin 100 mg l ⁻¹	1.4–6.5%	Seong <i>et al.</i> , 2005
	Protoplast	Protoplast electroporation	None		<i>Gus</i>	None	Nd	Hyung <i>et al.</i> , 1995

8-Gala Galaxy Royal Gala	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, BAP 2iP, GA ₃ MS with sorbitol	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	De Bondt <i>et al.</i> , 1994, 1996
		Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Gus Attacin E</i>	Kanamycin 50 mg l ⁻¹	5–20%	Norelli <i>et al.</i> , 1999; Ko <i>et al.</i> , 2000
		Leaf immersed in inoculum for 4–6 min	AGLO	NAA, TDZ MS with sorbitol	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	0.7–0.9% 0.7–8.7%	Schaart <i>et al.</i> , 1995; Puite and Schaart, 1996
		Leaf cut transversally into strips (2–3 mm) and transferred to inoculum for 5 min	LBA4404	BAP, NAA, IBA, TDZ MS with sucrose	<i>Gus Acetolactate synthase</i>	Kanamycin 50 mg l ⁻¹	0.4–4.6%	Yao <i>et al.</i> , 1995
Internodal explants	Internodal explant from etiolated in vitro shoot was dipped in inoculum	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	TDZ, IBA MS with sucrose	<i>Chitinase Puroindoline-b</i>	Kanamycin 100 mg l ⁻¹	1–1.4%	Faize <i>et al.</i> , 2003, 2004
		Internodal explant from etiolated in vitro shoot was dipped in inoculum	EHA105	BAP, IBA, GA ₃ MS with sucrose	<i>Gus</i>	Kanamycin 100 mg l ⁻¹	1.7%	Liu <i>et al.</i> , 1998, 2001

(Continued)

Table 1 Genetically transformed apple cultivars (*Continued*)

	Explants used	Inoculation procedure	<i>Agrobacterium</i> strains	Hormones used for regeneration, nutrients, and sugar	Genes	Selectable marker	Efficiency of transformation	References
9-Golden Delicious	Leaf	Leaf immersed in inoculum for 4–6 min Leaf cut and floating in inoculum for 15–30 min Leaf cut three times perpendicularly to the midrib and shaken in inoculum	AGLO	NAA, TDZ MS with sorbitol	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	0.2–6% 1.6%	Schaart <i>et al.</i> , 1995; Puite and Schaart, 1996
10-Greensleeves	Leaf	Leaf cut three times perpendicularly to the midrib and shaken in inoculum	EHA101		<i>GFP</i>	Kanamycin 50 mg l ⁻¹	Cell transformed No plants regenerated	Maximova <i>et al.</i> , 1998
		Leaf discs immersed 20–30 min in inoculum	LBA4404 C58C1/pGV385	BA, NAA MS with sorbitol and sucrose	<i>Gus</i>	Kanamycin 25 mg l ⁻¹	0.1–0.5%	James <i>et al.</i> , 1989, 1993
		Leaf cut three times perpendicularly to the midrib and shaken in inoculum	EHA101		<i>GFP</i>	Kanamycin 50 mg l ⁻¹	Cell transformed No plants regenerated	Maximova <i>et al.</i> , 1998
		Leaf disc immersed 20–30 min in inoculum	EH101	BAP, NAA, TDZ MS with sorbitol and sucrose	<i>Gus</i> with different promoters	Kanamycin 25 mg l ⁻¹	Nd	Gitins <i>et al.</i> , 2000, 2001, 2003
11-Holsteiner cox	Leaf	Leaf gently shaken in inoculum for 10 min	EHA105	TDZ, IBA MS with sorbitol	<i>Stilbene synthase PGIP</i>	Phosphonothricin 1–10 mg l ⁻¹	0.5–2.68%	Szankowski <i>et al.</i> , 2003
12-Jonagold	Leaf	Leaf gently shaken in inoculum for 10 min	EHA105	TDZ, IBA MS with sorbitol	<i>Pmi</i>	Sorbitol and mannose	1–24%	Degenhardt <i>et al.</i> , 2006
	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, 2IP, Ga ₃ MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	De Bondt <i>et al.</i> , 1994
		Leaf strips of 3–4 mm were incubated for 10–15 min in inoculum	EH101		<i>iaaM-ipt</i>	Kanamycin 50 mg l ⁻¹	Nd	Viss <i>et al.</i> , 2003

13-Jonagold king 14-Jonagored	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, 2iP, Ga ₃ MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	De Bondt <i>et al.</i> , 1994
15-Liberty	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Attaclin E T4</i> <i>lysozyme</i> <i>Endochitinase</i> <i>Exochitinase</i>	Kanamycin 100 mg l ⁻¹	0.03–0.4%	Hanke <i>et al.</i> , 2000
16-McIntosh Wijcik	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	5–20%	Norelli <i>et al.</i> , 1996
17-Melba		Leaf discs immersed 20–30 min in inoculum	LBA4044	IBA, TDZ, BAP 2iP, Ga ₃ MS with sorbitol	<i>Gus</i>	Kanamycin	3.5%	Wilson and James, 1998
		Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	NAA, TDZ N6 with sucrose	<i>Gus</i>	Kanamycin 2.5 mg l ⁻¹	2.6–3.1%	Bolar <i>et al.</i> , 1999
17-Melba		Leaf pieces 1 cm long immersed in inoculum	CBE21	NAA, TIBA MS	<i>Gus</i>	Kanamycin 35–50 mg l ⁻¹	13.3%	Dolgov <i>et al.</i> , 2000
18-Merlijn	Leaf	Leaf cut three times perpendicularly to the midrib and shaken 1 min in inoculum	EH101	IBA, TDZ, 2iP, Ga ₃ MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	Nd	De Bondt <i>et al.</i> , 1994

(Continued)

Table 1 Genetically transformed apple cultivars (Continued)

	Explants used	Inoculation procedure	<i>Agrobacterium</i> strains	Hormones used for regeneration, nutrients, and sugar	Genes	Selectable marker	Efficiency of transformation	References
19-Orin	Leaf	Leaf wounded	LBA4044	TDZ, NAA MS	<i>Polyphenol oxidase</i>	Kanamycin 25–50 mg l ⁻¹	0.1%	Murata <i>et al.</i> , 2000
		Leaf wounded	LBA4004	TDZ, NAA MS sucrose	<i>S6PDH</i>	Kanamycin 50 mg l ⁻¹	0.5%	Kanamaru <i>et al.</i> , 2004
		Leaf immersed in inoculum for 30 min	EHA101	TDZ, NAA MS	<i>MDTFL1</i>	Kanamycin 25 mg l ⁻¹	0.25%	Kotoda <i>et al.</i> , 2006
20-Pink Lady	Leaf	Leaf explants shaken for 20 min 75 rpm in inoculum at 35 °C	Nd	NAA, TDZ MS with sucrose and glucose	<i>Neo Gus</i>	Kanamycin 100 mg l ⁻¹	1–2%	Sriskandarajah and Goodwin, 1998
21-Pinova	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Attacin E T4 lysozyme</i> <i>Endochitinase</i> <i>Exochitinase</i>	Kanamycin 100 mg l ⁻¹	0.03–0.4%	Hanke <i>et al.</i> , 2000
22-Pilot								
23-Pirol								
24-Pingo								
25-Pi-AU								
56-83								
26-Queen Cox	Leaf	Leaf scored transversely 3–8 times with scapel dipped in inoculum	EHA101	NAA, TDZ DKW with sorbitol	<i>Gus</i>	Kanamycin 50–75 mg l ⁻¹	0.5–2.2%	Wilson and James, 2003
27-Remo	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Attacin E T4 lysozyme</i> <i>Endochitinase</i> <i>Exochitinase</i>	Kanamycin 100 mg l ⁻¹	0.03–0.4%	Hanke <i>et al.</i> , 2000
30-Reka	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Attacin E T4 lysozyme</i> <i>Endochitinase</i> <i>Exochitinase</i>	Kanamycin 100 mg l ⁻¹	0.03–0.4%	Hanke <i>et al.</i> , 2000
Rootstocks								
1-A2	Leaf	Leaf gently crushed by forceps and incubated in the inoculum for 20 min	GV31101	NAA, TDZ MS with sucrose	<i>rolA</i>	Kanamycin 50 mg l ⁻¹	0.33%	Zhu <i>et al.</i> , 2001a
2-JET-H	Leaf	Leaf immersed in inoculum for 15 min	LBA4404	TDZ, IAA N6 with sucrose	<i>dpo</i>	Kanamycin 50 mg l ⁻¹	Nd	Sule <i>et al.</i> , 2002

3-Jork 9	Leaf	Leaf wounded with a scalpel and gently shaken in inoculum for 20 min	EHA101 C58C1	NAA, TDZ MS with sorbitol	<i>rolB</i>	Kanamycin 50 mg l ⁻¹	6.5% 3.3%	Sedira <i>et al.</i> , 2001, 2005
4-M.7	Shoot	Base of 5-week-old <i>in vitro</i> shoots was dipped in inoculum	<i>A. rhizogenes</i> C58C1	TDZ, NAA MS with sorbitol	<i>rolB</i>	Kanamycin 50 mg l ⁻¹	Nd	Pawlicki-Julian <i>et al.</i> , 2002
	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	EHA105	BAP, NAA M6 + MS with sucrose	<i>Gus</i>	Kanamycin 50 mg l ⁻¹	5–20%	Norelli <i>et al.</i> , 1996
5-M.26	Leaf	Leaf wounded by crushing with nontraumatic forceps and immersed 5 min in inoculum	LBA4404	BAP, NAA N6 + MS with sucrose	<i>attacinE</i>	Kanamycin 50 mg l ⁻¹	15–50%	Norelli <i>et al.</i> , 1994
	Leaf	Leaf wounded with a scalpel and gently shaken in inoculum for 5 min	C58C1	MS with sucrose	<i>rolB</i>	Kanamycin 25–50 mg l ⁻¹	2.5–7.1%	Welander <i>et al.</i> , 1998
	Leaf	Leaf wounded and infected with inoculum	GV3101	TDZ, NAA MS with sucrose	<i>rolA, phyB</i>	Kanamycin 50 mg l ⁻¹	0.9–3.9%	Holefors <i>et al.</i> , 1998, 2000
6-M9/29	Leaf	Leaf wounded and infected with inoculum	C58C1 EHA 101	NAA, TDZ MS with sorbitol	<i>rolB</i>	Kanamycin 50 mg l ⁻¹	0.02% 0.06%	Zhu <i>et al.</i> , 2001b

(Continued)

Table 1 Genetically transformed apple cultivars (*Continued*)

	Explants used	Inoculation procedure	<i>Agrobacterium</i> strains	Hormones used for regeneration, nutrients, and sugar		Genes	Selectable marker	Efficiency of transformation	References
7-N545	Leaf	Leaf pieces of 1 cm immersed in inoculum	CBE21 EHA105	NAA, TIBA	MS	<i>Gus</i>	Kanamycin 35–50 mg l ⁻¹ Hygromycin 5 mg l ⁻¹	1.5–7.2% 8.3%	Dolgov <i>et al.</i> , 2000, 2004a
<i>Malus prunifolia</i> Marubakaidou	Leaf	Leaf soaked in inoculum	EHA101	TDZ, ABA, IAA	MS with sucrose	<i>rolC</i>	Hygromycin 10 mg l ⁻¹	Nd	Igarashi <i>et al.</i> , 2002
Ringo Asami	Stem	Stem infected with a needle dipped in inoculum	<i>A. rhizogenes</i> MAFF-02-10265	BAP, IBA, GA ₃	MS	Nd		2%	Yamashita <i>et al.</i> , 2004
<i>Malus robusta</i> Rehd. Balenghaitang	Leaf	Leaf cut in three pieces and soaked in inoculum for 5 min	LBA4404	BA, NAA	MS with sucrose	<i>LeIRT2</i>	Kanamycin 50 mg l ⁻¹	1.2%	Qu <i>et al.</i> , 2005

betain phosphate) to increase the expression of several virulence genes in *A. tumefaciens*, and the gelling agent, growth regulator (Table 1), source of carbon (sucrose or sorbitol), nitrogen source, and concentration of AgNO₃ (Seong *et al.*, 2005), binary vector, and antibiotics (cefotaxin, cefoxitin, carboxillin) used. Antibiotics prevent and eliminate contamination by surviving *Agrobacterium* that could occur after the transformation procedure. Hammerschlag *et al.* (2000) reported the generation of *Agrobacterium*-free transgenic apple by vacuum infiltration of explants inoculated with *Agrobacterium* with an acidified medium with a high concentration of antibiotics (500 mg l⁻¹ carbenicillin, cefotaxime, and cefoxitin). Another way to avoid contamination with *Agrobacterium* is to bathe prewounded leaves in the inoculum for a short time.

All these apple transformation procedures allowed regeneration of transgenic apple lines with an efficiency of 0.02–9% with the exception of the procedure described by Norelli *et al.* (1994, 1996), which had an efficiency of 5–50% for the cultivars M.26, McIntosh, and Galaxy. After improvements in their procedures, the rate of transformation for Galaxy and M.26 can be as great as 50% and 80%, respectively (H.C. Aldwinckle, personal communication).

2.2 Traits Improved

2.2.1 Control of transgene expression

The genetic construct integrated into the apple genome to control the expression of the gene of interest must contain a DNA sequence encoding the active protein and several regulatory sequences. These sequences control when, how, and where the protein of interest will be expressed in the plant. Among these regulatory sequences, the promoter identifies the coding region and allows messenger RNA (mRNA) transcription, and also determines when, and in some cases, under what conditions transcription should be initiated. The CaMV 35S promoter from cauliflower mosaic virus (CaMV) has been used widely in transgenic plants. In apple it controls 60% of transgenes integrated in the apple genome because it provides continuous expression regardless of developmental stage in most plant tissues (constitutive expression)

(Table 2). Duplication of the upstream (5') DNA sequences of the 35S promoter results in elevated levels of transcription (Kay *et al.*, 1987). With such a promoter, a gene is expressed in the majority of tissues during most phases of plant growth and development. This limited temporal and spatial regulation may be suitable for proof of concept experiments, but presents a number of potential drawbacks for use in genetically improved crops. For example, the presence of transgenes driven by constitutive promoters may result in homology-dependent gene silencing, particularly when the promoter is also highly active (Vaucheret *et al.*, 1998). Thus, gene expression under the control of inducible promoters (tissue, developmental stage, and pathogen specific) is preferred in any strategy to produce transgenic plants with transgene-mediated improvements in resistance to pathogens. This targeted gene expression could be advantageous in providing resistance where and when needed, limiting the amount of transgenic protein in fruit, reducing the plant energy cost for transgenic protein synthesis, and reducing unnecessary exposure of nontarget organisms to the protein (Norelli and Aldwinckle, 2000).

Knowledge of genes involved in plant pathogen interactions, flowering time, and metabolic pathways are still limited in apple and no homologous apple promoters are able to drive high levels of gene expression, specifically in response to pathogens or the function considered. Only two functional apple promoters have been reported and studied, the scab-resistance promoters (Silfverberg-Dilworth *et al.*, 2005a) and the PR10 promoter (Puhlinger *et al.*, 2000). Therefore, different inducible promoters isolated from tobacco, potato, *Brassica napus*, *Vitis vinifera*, soybean, *A. rhizogenes*, and virus have been used in apple to control the expression of transgenes (Table 2). The responses of some of these promoters to different stresses (pathogen or chemical) have been studied. Their activity differs in different plant organs (Table 2).

The potato promoter *gstI* that encodes a glutathione S transferase was studied for its ability to respond to *Erwinia amylovora*. This promoter mediated rapid and local transcriptional activation in potato in response to fungal and viral pathogens. Transcription from this promoter is not induced by environmental abiotic stimuli, such as wounding or heat shock (Martini *et al.*, 1993; Strittmatter *et al.*, 1996). The ability of the *gstI* promoter

Table 2 Traits expressed in transformed apple cultivars

Trait	Apple cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
Bacterial resistance <i>Agrobacterium tumefaciens</i> (crown gall) <i>Erwinia amylovora</i> (fire blight)	Jonagold	<i>iaaM</i> and <i>ipt</i> (silencing)	<i>A. tumefaciens</i>	CaMV 35S FMV	Silencing of <i>iaaM</i> gene expression was observed. Reduction and abolition of Crown Gall formation was observed.	Viss <i>et al.</i> , 2003
	Gala	SB37 (+/-sp) Shiva 1 (-sp)	Synthetic peptide	Pin2 CaMV 35S	Some SB-37 transgenic lines with partial resistance in the orchard	Norelli <i>et al.</i> , 1999; Aldwinckle <i>et al.</i> , 2003a
	Royal Gala	MB39 (+sp)	Modified cecropin	Osmotin (wound inducible)	Three of seven transgenic lines showed an increase in resistance in greenhouse (2.5–3.3 fold more resistance than the control)	Liu <i>et al.</i> , 2001
	Royal Gala	MB39 (+sp)	Modified cecropin	Osmotin (wound inducible)	Three of seven transgenic lines showed an increase in resistance in greenhouse (2.5–3.3 fold more resistance than the control)	Liu <i>et al.</i> , 2001
	M.26	<i>Attacin E</i>	<i>Hyalophora cecropia</i> (giant silk moth)	Pin2 CaMV 35S	Some transgenic lines showed partial resistance in greenhouse and in the orchard. Increased resistance when signal peptide and translation enhancer (AMV) were used	Norelli <i>et al.</i> , 1994; Ko <i>et al.</i> , 2000; Aldwinckle <i>et al.</i> , 1998, 2003a
	Pinova, Pilot, Pirol, Pingo, Elstar, Remo, Liberty, Reka, Pi-AU 56-83 Gala	Attacin E (-sp) T4 Lysozyme	<i>Hyalophora cecropia</i> (giant silk moth) <i>T4 Bacteriophage</i>	Pin2 CaMV 35S	Some transgenic lines showed partial resistance in greenhouse	Hanke <i>et al.</i> , 2000
		T4 Lysozyme alone or associated with attacin E	<i>T4 Bacteriophage Hyalophora cecropia</i>	Pin2 (attacin E) CaMV 35S (T4ly)	Some transgenic lines showed partial resistance in greenhouse. No increase of resistance when this gene was combined with attacin gene	Ko <i>et al.</i> , 1998, 2002; Aldwinckle <i>et al.</i> , 1998, 2003a

Pinova	<i>Dpo</i>	Phage ϕ Ea1h	CaMV 35S	Sixty-one out of 83 transgenic lines appeared significantly less susceptible than parental parent Pinova after inoculation of detached leaves <i>in vitro</i> . Three lines appeared less susceptible after inoculation in greenhouse.	Hanke <i>et al.</i> , 2003
M.26			CaMV 35S Gst1	Increased of resistance to fire blight. Higher resistance observed when the <i>Dpo</i> gene was driven by CaMV 35S promoter.	Borejsza-Wysocka <i>et al.</i> , 2007
M.26	<i>HrpN</i>	<i>Erwinia amylovora</i>	Gst1 (wound inducible)	Most of the transgenic lines had partial resistance to <i>E. amylovora</i> in greenhouse and in the field. Two of these lines showed a level of resistance similar to the resistant rootstock M.7.	Abdul-Kader <i>et al.</i> , 1999; Aldwinckle <i>et al.</i> , 2003a; Malnoy <i>et al.</i> (submitted)
Galaxy M.26	<i>MpNPR1</i>	Apple	Pin2 CaMV 35S	Significant reduction in susceptibility to <i>E. amylovora</i> of 33–86% for Galaxy; M.26 showed a less substantial reduction in susceptibility compared to Galaxy (0–70%).	Malnoy <i>et al.</i> , 2004, 2007e
Galaxy	<i>DIPM</i> (silencing) 4 different genes	Apple	CaMV 35S	Some transgenic lines showed silencing of the DIPM genes and an increase in resistance to <i>Erwinia amylovora</i> .	Borejsza-Wysocka <i>et al.</i> , 2006
Royal Gala	<i>Avidin Streptavidin</i>	Nd	CaMV 35S	High level of resistance to larvae of lightbrown apple moth (<i>Epiphyas postvittana</i>). Mortality of the larvae was between 80% and 90% in the transgenic lines compare to 14% in the untransformed apple.	Markwick <i>et al.</i> , 2003

(Continued)

Table 2 Traits expressed in transformed apple cultivars (*Continued*)

Trait	Apple cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
Fungal resistance						
Fungal resistance	Melba	<i>Thaumatin</i>	<i>Thaumatooccus danielli</i> (Plant)	CaMV 35S	Nd	Dolgov <i>et al.</i> , 2004
<i>Alternaria mali</i>	McIntosh	<i>Rab</i>	Plant nd	Wound-inducible promoter	Smaller disease lesion and spot size in transgenic lines compared to untransformed	Kim <i>et al.</i> , 2007
<i>Venturia inaequalis</i> (apple scab)	McIntosh	<i>Endochitinase</i> (<i>ech42</i>) <i>Exochitinase</i> (<i>nag70</i>)	Trichoderma atroviride (fungus)	2CaMV 35S	Negative correlation between growth of transgenic lines and endochitinase activity was observed Six of eight transgenic lines expressing endochitinase were more resistant than control. Disease severity was reduced by 0–99.7% (number lesion) and 0–90% (% of leaf area infected) Exochitinase was less effective than endochitinase Some plants expressing both genes were more resistant than plants expressing either single gene	Bolar <i>et al.</i> , 2000, 2001
	Ariane Galaxy	<i>Endochitinase</i> (<i>ech42</i>) <i>Exochitinase</i> (<i>nag70</i>)	Trichoderma atroviride	2CaMV 35S	Negative correlation between growth of transgenic lines and endochitinase activity was observed Reduced of growth appeared to be associated with high lignin content, peroxidase, and glucanase activity All the lines with high endochitinase activity exhibited significant reduction of scab symptoms	Faize <i>et al.</i> , 2003
	Jonagold	<i>Ace-AMPI</i> <i>Rs-AFP2</i>	Onion Radish	CaMV 35S	<i>Rs-AFP2</i> expressing shoot showed eightfold to 32-fold antifungal activity compared to the control <i>Ace-AMPI</i> expressing shoot showed fourfold increased antifungal activity relative to control plants	De Bondt <i>et al.</i> , 1999

Gala Elstar Ariane Galaxy	<i>Ai-AMP</i> <i>Hordothionin</i>	Nd Barley	CaMV 35S CaMV 35S	Nd Decrease in scab symptom development	Broothaerts <i>et al.</i> , 2000 Janse <i>et al.</i> , 2002
	<i>Puroindoline-b</i>	Wheat	CaMV 35S	Reduction of symptoms in transgenic Galaxy (55% for best lines) and in Ariane (64%) after inoculation with the apple scab race 6 No increase in resistance was observed in the transgenic Galaxy lines after inoculation with the apple scab race 1 Hervf2 confers scab resistance to the susceptible apple cultivar Gala Acquired resistance is race specific	Faize <i>et al.</i> , 2004
Gala	<i>HcrVf2</i>	Apple	CaMV 35S	Transgenic lines expressing either <i>Vf1</i> or <i>Vf2</i> showed a significant increase in resistance to <i>Venturia inaequalis</i> Transgenic lines expressing <i>Vf4</i> gene were as, or more susceptible than control	Barbieri <i>et al.</i> , 2003; Belfanti <i>et al.</i> , 2004; Silverberg-Dilworth <i>et al.</i> , 2005a Malnoy <i>et al.</i> , 2007d
Galaxy McIntosh	<i>Vf1</i> , <i>Vf2</i> and <i>Vf4</i>	Apple	Their own native promoter		
Environmental stress resistance					
High and low temperature stress	Royal Gala	Pea	Nd	Transgenic lines showed different degrees of increased resistance to high and low temperature stress Resistance to freezing injury was 1–3° greater and up to 7° increase to acute heat stress Two of 9 transgenic lines were tested for their iron deficiency tolerance One of these two lines showed a higher resistance to iron deficiency (21–34% greater than control)	Artlip <i>et al.</i> , 2006
Iron deficiency tolerance	Balenghaitang Malus robusta	Tomato	CaMV 35S		Qu <i>et al.</i> , 2005

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Table 2 Traits expressed in transformed apple cultivars (Continued)

Trait	Apple cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
Rooting ability/ Dwarfism to rootstock	A2	<i>rolA</i>	<i>A. rhizogenes</i>	<i>rolA</i> promoter	The two transformed lines showed a reduction in height and shortened internode length	Zhu <i>et al.</i> , 2001a
	Florina	<i>rolB</i>	<i>A. rhizogenes</i>	CaMV 35S	All transgenic lines produced roots <i>in vitro</i> in hormone-free medium Transgenic plant does not show any phenotypical difference with the exception of root parts after 2 months of growth <i>in vitro</i> or in greenhouse compared to the control	Radchuck and Korkhovoy, 2005
	Jork 9	<i>rolB</i>	<i>A. rhizogenes</i>	Nd	Increase in adventitious root production Some clones showed reduced apical dominance, slow initial growth, and shorter internodes	Pawlicki-Julian <i>et al.</i> , 2002
	M.26	<i>rol A</i>	<i>A. rhizogenes</i>	<i>rolB</i> promoter <i>rolA</i> promoter	Increase in root production All transformants had reduced stem growth Some showed reduced length, leaf area, and dry weight (shoot, root, and plant)	Sedira <i>et al.</i> , 2001, 2005 Holefors <i>et al.</i> , 1998
		<i>Phytochrome B</i>	<i>Arabidopsis thaliana</i>	CaMV 35S	No decrease in rooting ability of the transformed lines Shoot, root, and plant dry weight were reduced in all transformed lines	Holefors <i>et al.</i> , 2000
					Nine of 13 transgenic lines showed a stem length reduction	
		<i>rolB</i>	<i>A. rhizogenes</i>	<i>rolB</i> promoter	Transgenic lines had greater rooting ability was higher than the control	Welander <i>et al.</i> , 1998; Zhu and Welander, 2000
	M.9/29	<i>rolB</i>	<i>A. rhizogenes</i>	<i>rolB</i> promoter	All transgenic lines produced roots <i>in vitro</i> in hormone-free medium 15% of the transgenic lines showed reduction in node number and stem length	Zhu <i>et al.</i> , 2001b

Marubakaidou	<i>rolC</i>	<i>A. rhizogenes</i>	rolC promoter	Introduction of <i>rolC</i> results in short plants with shortened internodes, smaller leaves, and reduced apical dominance compared to the nontransformed plants	Igarashi <i>et al.</i> , 2002
Herbicide resistance	N545	<i>bar</i>	CaMV 35S	Greenhouse plants treated with 1% solution of BASTA showed no damage	Dolgov and Skryabin, 2004b
Flowering time	Orin	<i>MdTFLL1</i>	CaMV 35S	Transgenic apple expressing MdTFLL1 antisense RNA first flowered 8–22 months after transfer to the greenhouse, whereas nontransgenic plants flowered 69 months after transfer to the greenhouse	Kotoda <i>et al.</i> , 2006
	Pinova	<i>BpMADS4</i>	CaMV 35S	13 weeks after transformation first flower was observed <i>in vitro</i> Flower appeared in greenhouse after a few weeks but abnormal growth was observed	Hanke <i>et al.</i> , 2007b
Self-fertility	Elstar	S3 gene silencing	CaMV 35S	Production of transgenic apple tree with true self-fertility This self-fertility was stable for several years without any obvious adverse effects on tree growth or fruit appearance	Broothaerts <i>et al.</i> , 2004a

(Continued)

Table 2 Traits expressed in transformed apple cultivars (*Continued*)

Trait	Apple cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
Modified metabolism Production of the phytoalexin resveratrol	Elstar Holsteimer cox	<i>Stilbene synthase</i>	<i>Vitis vinifera</i>	Stilbene promoter (UV, wound and pathogen inducible)	Transgenic lines produced a resveratrol glucoside (piceid) that is accumulated in the plant, skin, and flesh of the fruit. Accumulation of piceid after induction by UV treatment does not affect the accumulation of other compounds of the flavonoid and phenylpropanoid pathway Expression of the stilbene synthase gene does not affect the leaf shape, flower morphology or color, or fruit shape and size compared to control plants and fruit	Szankowski <i>et al.</i> , 2003; Ruhmann <i>et al.</i> , 2006
Decreased flesh browning	Orin Fuji	<i>Polyphenol oxidase antisense</i>	Apple	CaMV 35S	Transgenic lines, in which PPO expression is reduced, had less flesh browning	Murata <i>et al.</i> , 2000, 2001
Sugar accumulation	Orin	<i>Sorbitol 6 phosphate dehydrogenase (S6PDH)</i>	Apple	CaMV 35S	Transgenic lines showed different expression level of S6PDH Lines with less activity contained only a low level of sorbitol but showed sixfold to sevenfold increase in sucrose. The growth of this plant stopped early during the summer due a deficiency of sugar	Kanamaru <i>et al.</i> , 2004
	Greensleeves	<i>Aldose 6 phosphate (A6PR) antisense</i> also called <i>S6PDH</i>	Apple	CaMV 35S	Lines with increased amount of S6PDH activity had increased sorbitol and sucrose content Antisense inhibition of A6PR expression significantly decreased A6PR activity and sorbitol synthesis, but increased concentration of sucrose and starch at both dusk and predawn Silencing leaf sorbitol synthesis alters long-distance partitioning and apple fruit quality	Cheng <i>et al.</i> , 2005; Zhou <i>et al.</i> , 2006; Teo <i>et al.</i> , 2006

Down-regulation of ethylene production	Royal Gala	<i>Aminocyclopropane 1 carboxylic (ACC) synthase 2 antisense</i>	Apple	CaMV 35S	Production of apple tree with down regulation of ethylene production. Some lines had fruits with delayed softening.	Hrazdina <i>et al.</i> , 2003
	Greensleeves	<i>ACC synthase antisense ACC oxidase antisense</i>	Apple	CaMV 35S	Some transgenics were significantly suppressed in ethylene production The fruit of these apple trees was firmer and displayed increased shelf life No difference was observed in sugar or acid accumulation in these fruit compared to the control. However, a significant and dramatic suppression of the synthesis of volatile esters was observed	Dandekar <i>et al.</i> , 2004; Defilippi <i>et al.</i> , 2004, 2005a, 2005b
Cell adhesion	Royal Gala	<i>Polygalacturonase</i>	Apple	CaMV 35S	Phenotypic modification of apple tree (silvery colored leaves and leaf shedding) Mature leaves with malfunctioning and malformed stomata	Atkinson <i>et al.</i> , 2002
Apple allergen	Elstar	<i>Mal dl Rnai</i>	Apple	CaMV 35S	Reduction of <i>Mal dl</i> expression in the transgenic silenced apple. This translated into significantly reduced <i>in vivo</i> allergenicity	Glissen <i>et al.</i> , 2005

(Continued)

Table 2 Traits expressed in transformed apple cultivars (*Continued*)

Trait	Apple cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
<i>Promoter</i> Gene expressed in restricted area	Greensleeves		<i>Tomato soybean</i>	Rubisco small subunit (SSU) promoter CaMV 35S	SSU promoters are active in the photosynthetically active leaf mesophyll and palisade cells. The mean activity of the two SSU promoters in apple leaves appeared to be approximately half that of the constitutive promoter CaMV 35S SSU soybean promoter was strictly light dependent	Gitins <i>et al.</i> , 2000
Gene expressed in young stem tissue and load bearing regions	Greensleeves		<i>Brassica napus</i>	940 extA promoter	(-)940 extA promoter has an activity in apple stem comparable to that obtained with CaMV 35S. Its activity was lower in petioles (50%), roots (20%), and leaves compared to the activity of CaMV 35S	Gitins <i>et al.</i> , 2001
Gene expressed in vegetative tissues	Greensleeves		<i>A. rhizogenes</i> <i>Commelina</i> yellow mottle virus	RoIC promoter CoYMV promoter	The two promoters showed an activity lower than the CaMV 35S. RoIC promoter was less active than CoYMV promoter in apple These promoters are active in the vascular system, particularly the phloem	Gitins <i>et al.</i> , 2003
Gene expressed in response to pathogen attack	M.26 Royal Gala		<i>Potato</i>	GstI promoter	In the two apple genotypes, GstI exhibited a low level of expression after bacterial and fungal inoculation compared to CaMV 35S (15% and 8%, respectively). It is activated by salicylic acid but not by wounding	Malnoy <i>et al.</i> , 2007b
Chemical inducible and light-inducible promoter	M.26		Nd	XVE promoter	Promoter currently being evaluated	Norelli <i>et al.</i> , 2007

Selectable marker <i>Nonantibiotic- based selection</i>	M.26	Phosphomannose isomerase (<i>pmi</i>)	Nd	CaMV 35S	Mannose (2 g l ⁻¹) can be used as selection agent but the selection efficiency seems to be lower than that of kanamycin	Zhu <i>et al.</i> , 2004
	Pinova Pilot Elstar Regine	<i>pmi</i> <i>GFP</i>		CaMV 35S	Some putative transgenic lines were regenerated with the GFP gene as reporter gene Any transgenic lines were regenerated with the <i>pmi</i> gene as selectable marker	Flachowsky <i>et al.</i> , 2004
		<i>pmi UDP- glucose:galactose- 1-phosphate uridylyltrans- ferase (galT)</i> <i>Vt-ERE</i>	Nd		First regenerants were obtained, with the <i>pmi</i> gene as selectable marker, and tested gus positive No transgenic lines yet regenerated with the <i>galT</i> gene	Szankowski and Degenhardt, 2006; Degenhardt <i>et al.</i> , 2006
	Galaxy		<i>Vigna radiata</i>	CaMV 35S	Benzaldehyde (BA) is able to inhibit adventitious bud regeneration Ba can be used instead of kanamycin but the efficiency of the selection pressure is not yet proven	Chevreau <i>et al.</i> , 2007
Excision by recombination	Elstar	<i>Cre-lox</i> system			Antibiotic-resistance marker gene free transgenic Elstar apple lines have been produced	Schaart <i>et al.</i> , 2004
No selectable marker	M.26 Galaxy	None			Transgenic lines of M.26 and Galaxy were regenerated without any selectable marker with an efficiency of 22% and 12%, respectively	Malnoy <i>et al.</i> , 2007c

to drive expression of the *GUS* reporter gene was determined in two genotypes of apple: the fruit cultivar Royal Gala and the M.26 rootstock (Malnoy *et al.*, 2007d). In both apple genotypes, the *gst1* promoter exhibited a low level of expression after bacterial and fungal inoculation compared to the level obtained with the *CaMV* 35S promoter (15% and 8%, respectively). The *gst1* promoter was systematically activated in apple from the infection site with a fungal pathogen. It was also activated after treatment with salicylic acid but not after wounding. In apple, the *gst1* promoter is less active than the *CaMV* 35S promoter, but its pathogen responsiveness could be useful in driving the expression of transgenes to promote bacterial and fungal disease resistance.

Norelli *et al.* (2007) reported the use of a chemically induced promoter *XVE* in apple. The ability of this promoter to drive the *gus* gene in apple is under investigation.

Constitutive and nonspecific promoters such as *CaMV* 35S and *A. tumefaciens* nopaline synthase (*nos*) have been used as experimental tools to assess the effects of transgene expression. With such promoters, a gene is expressed in the majority of tissues throughout plant development. This lack of temporal and spatial regulation may be suitable for proof of concept experiments but has a number of potential drawbacks for use in genetically improved crops (Gittins *et al.*, 2000). Moreover there has been some discussion in the scientific press (Ho *et al.*, 1999) and the media as to the safety of using the *CaMV* 35S promoter in any transgenic plant strategy due to concerns regarding its unpredictable recombinogenic potential (Kohili *et al.*, 1999). To address the problems associated with constitutive promoters, Gittins *et al.* (2000, 2001, 2003) undertook a study on efficacy of various tissue-specific promoters in apple. They studied the level and location of expression in apple of the *GUS* reporter using promoters from *Rubisco* small subunit (*SSU*) genes of tomato and soybean (Gittins *et al.*, 2000), from a form of the *B. napus* extension A gene (*extA*) (Gittins *et al.*, 2001), and from *rolC* and *CoYMV* genes (Gittins *et al.*, 2003).

The *Rubisco* gene catalyzes the competing reactions of photosynthetic carbon fixation and photorespiration in higher plants and green algae. It is a multimeric enzyme composed of eight large and eight small subunits. The large subunit is

encoded by the chloroplast genome, whereas the *SSU* polypeptides are encoded by a nuclear gene family. Both of the *Rubisco* *SSU* promoters of tomato and soybean exhibited expression with half of the activity of the *CaMV* 35S promoter in apple tissues. This activity appeared to be localized in the leaf mesophyll and palisade cells of the photosynthetically active leaf (Gittins *et al.*, 2000). Both promoters would be appropriate for confining the expression of beneficial transgenes to green photosynthetic tissues. Effective leaf-specific promoters would also be useful in directing the expression of pest and disease resistance factors.

The *extA* gene of *B. napus* encodes one member of an extensin gene family. The *extA* transcript is highly expressed in roots, with lower levels in stems and petioles, and very little, if any, expression in leaves (Shirsat *et al.*, 1996). Extensin genes from a number of plant species are induced by wounding treatments (Elliott and Shirsat, 1998). Deletion analysis of the *extA* promoter localized the wound responsive elements to within the -940 to -3500 bp region (-940 *extA* promoter) (Elliott and Shirsat, 1998). The ability of the -940 *extA* promoter to drive expression of the *gus* reporter gene was determined in the apple genotype Greensleeves (Gittins *et al.*, 2001). In this genotype, the -940 *extA* promoter did not express very highly in roots compared to the *CaMV* 35S promoter. It is likely that those control elements responsible for root specificity are located outside the *extA* promoter fragment used in this study. However, the -940 *extA* promoter has strong activity in apple stems comparable to that obtained with *CaMV* 35S. This activity was lower in petioles (50%), and leaves compared to the activity of *CaMV* 35S. On the basis of the results of Gittins *et al.* (2001), there is the potential to use this promoter to drive transgenes whose gene products are active in stems to bring about dwarfing, or to alter lignin composition, both major goals of tree biotechnology.

The *rolC* gene and *Commelina* yellow mottle virus (CoYMV) are active in the phloem, and vascular parenchyma. The ability of the promoter to drive the expression of the reporter gene *gus* in the vascular system of apple was determined in the apple genotype Greensleeves (Gittins *et al.*, 2003). In Greensleeves both promoters showed activity in the vascular system, particularly in the phloem, but with lower activity than with *CaMV* 35S.

The entire group of heterologous promoters tested in apple showed similar patterns of expression as in their host plants.

2.2.2 Engineering disease resistance

The most important diseases of apple are fire blight, caused by the bacterium *E. amylovora* and scab, caused by the fungus *V. inaequalis*. Both diseases are favored by humid climates and cause significant losses in many apple-growing regions. Different strategies have been used to increase resistance to these two pathogens.

2.2.2.1 Fire blight

Fire blight is a disease of American origin and was first reported in the Hudson Valley of New York in 1784. It has been known as the major destructive bacterial disease of the *Maloideae* (pear, apple, and quince) and other members of the *Rosaceae* for over 200 years, and is in fact, the first plant disease proved to be of bacterial origin (Norelli *et al.*, 2003). This disease is caused by the necrogenic endobacterium *E. amylovora*, which is capable of infecting blossoms, fruits, vegetative shoots, woody tissues, and rootstock crowns. Upon entering plants through natural openings or wounds, the bacteria cause necrosis that develops quickly along the shoots to the main branches, producing the characteristic symptom of this disease: a necrotic shoot blight that can kill a tree in one season (Thomson, 2000). In nonhost plants, such as *Arabidopsis thaliana* or tobacco, *E. amylovora* elicits a hypersensitive response (HR) characterized by a rapid and localized collapse of tissues.

E. amylovora, *Enterobacteriaceae*, is a gram negative bacterium and its anatomy, physiology, and serology have been well described (Paulin, 2000). Twenty years ago, factors involved in its pathogenicity and virulence were identified. Transposon mutagenesis led to the identification of three key groups of genes responsible for pathogenicity: (1) The cluster of *dsp* genes (disease specific gene, Boucher *et al.*, 1987) is strictly required for pathogenicity (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998b). Two *dsp* genes were isolated, *DspA* or *E*, which encodes a pathogenicity factor homologous to the *Avr E* gene

of *Pseudomonas syringae*. The *DspB* or *F* gene encodes a protein similar to a chaperone protein of pathogenic bacteria such as *Yersinia* and *Shigella* (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998a, b). (2) The *ams* gene (amylovoran synthesis gene) is required for synthesis of extracellular polysaccharides (EPSs) (Belleman and Geider, 1992). (3) The *hrp* (hypersensitive reaction and pathogenicity gene) cluster is necessary for pathogenicity on host plants and HR elicitation on nonhost plants (Barny *et al.*, 1990; Kim and Beer, 2000). The *hrp* gene cluster has been characterized in several other pathogenic bacteria such as *Xanthomonas* and *Ralstonia* (Lindgren, 1997). This cluster encodes components of a type III secretion pathway (Hueck, 1998), and regulatory and secreted proteins. Today, at least seven proteins of *E. amylovora* are known to be secreted via the Hrp pathway: *DspA/E*, *OrfB*, *HrpW*, *HrpA*, *HrcQ*, *HrpJ*, and *HrpN_{Ea}* (Kim and Beer, 2000). *Harpin N_{Ea}* was the first secreted protein characterized by molecular analysis (Wei *et al.*, 1992). This protein is a 44 kDa glycine-rich protein, which lacks cysteine, is heat stable, and is able to induce an HR when purified and infiltrated into tobacco leaves. Besides three key pathogenicity factors, *E. amylovora* produces a siderophore belonging to the desferrioxamine family, which acts as virulence factor (Dellagi *et al.*, 1998).

The economic impact of fire blight is difficult to determine, as losses are rarely recorded when they are low (a few flower clusters on a few trees killed within a season) and a single outbreak can disrupt orchard production for several years. However, in recent years, fire blight has caused serious losses around the world (Norelli *et al.*, 2003). While outbreaks are sporadic in occurrence, they often result in significant losses when they do occur, in terms of trees damaged and killed, or loss of crops. In 2000, southwest Michigan experienced a severe fire blight epidemic and reported a \$42 million economic loss for the region that resulted in the removal of 300 000 apple trees covering approximately 2000 acres. Losses and costs in many other states and countries, such as New Zealand, Italy, and Washington State in 1998 were also very substantial.

The strategies of ribosomal DNA (rDNA) technology to improve the resistance to pathogens are aimed at producing incompatible interactions between the plant and the pathogen, by means

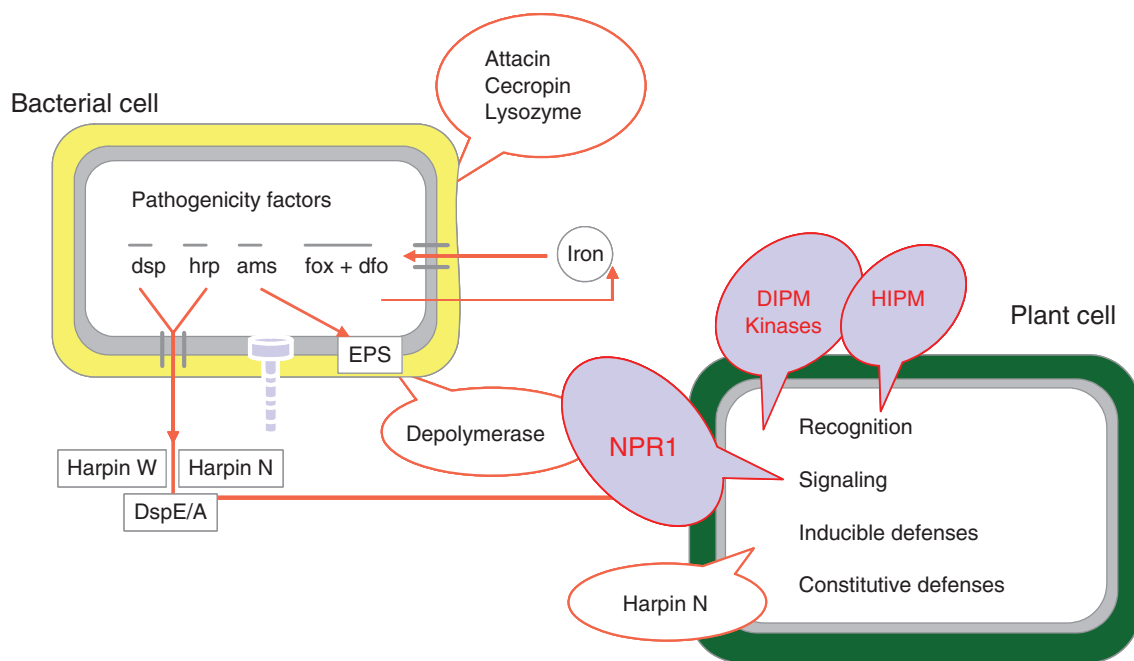


Figure 1 Drawing representing the different strategies used in apple for increased resistance to *Erwinia amylovora*

of restricting the multiplication of the pathogen in the plant after infection. These outcomes can be obtained in different ways: (1) production of antimicrobial proteins, (2) inhibition of bacterial pathogenicity factors, and (3) enhancement of natural plant defense. These three different ways have been used to increase resistance to fire blight (Figure 1; Table 2).

Production of antimicrobial proteins Antibacterial proteins are important components of the overall antimicrobial defense mechanisms of many groups of animals, including arthropods, amphibians, and mammals (Mourgues *et al.*, 1998). Probably acting in synergy, they have a bactericidal action on a large range of gram negative and gram positive bacteria. Genes encoding antimicrobial proteins that have been cloned and expressed in apple and pear in an attempt to confer resistance to fire blight disease include: attacin E, cecropins, and lysozymes (Table 2).

Attacins and cecropins are antimicrobial peptides found in the hemolymph of *Hyalophora cecropia* in response to bacterial infection (Hultmark *et al.*, 1980). These two peptides have no known enzymatic activity. They are active against the

membrane of gram negative bacteria. Attacin causes an increase in the permeability of the outer membrane (Carlsson *et al.*, 1991) and cecropin induced pore formation in the bacterial membrane (Flink *et al.*, 1989), which results in death of the bacterial cells. Attacin E and synthetic analogs of cecropin (SB-37, Shiva 1, and MB39) under the control of inducible (Pin2) and constitutive (CaMV 35S) promoters have been introduced into various apple genotypes to enhance resistance to *E. amylovora* (Table 2). These genes were reported to cause partial resistance to fire blight.

Attacin expression in apple trees in the greenhouse and in the field has significantly increased the level resistance to fire blight (Aldwinckle *et al.*, 1998, 2003a; Ko *et al.*, 2000). This increased fire blight resistance was reported to be correlated with the expression level of attacin (Ko *et al.*, 2000). The presence of a peptide signal, which allowed the secretion of attacin into the intracellular space, increased the resistance of the transgenic apple in spite of a lower attacin content (Ko *et al.*, 2000). Attacin E expression is increased when this gene is under the control of the alfalfa mosaic virus (AMV) translational enhancer (Ko *et al.*, 2000). Hanke *et al.* (2000), showed

similar increased resistance to fire blight when the attacin gene was expressed in different apple cultivars.

In 1997, approximately 120 field grown plants of 13 Royal Gala transgenic lines containing the cecropin SB-37 transgene were evaluated for their resistance following inoculation of vigorously growing shoot-tips with *E. amylovora*. In this test, several of the transgenic lines developed less disease than Royal Gala, but only transgenic line T245 (12% shoot length infected) was significantly more resistant than Royal Gala (67%). In addition, the cecropin B analog MB39 under the control of the wound-induced osmotin promoter from tobacco has been transferred to Royal Gala and few lines showed partial fire blight resistance (Liu *et al.*, 1998, 2001).

Lysozymes are bacteriolytic enzymes that have been characterized from phages, bacteria, fungi, plants, and animals (Jollès and Jollès, 1984). They are responsible for weakening the cell wall, leading to eventual lysis of bacteria cells (Düring, 1996). Some lysozymes also have chitinase activity (EC 3.2.1.14) resulting from random hydrolysis in the chitin. Their mode of action is such that they act only as bactericides to gram positive bacteria (Boman and Hultmark, 1987). However, they have displayed activity against gram negative bacteria when they act in synergy with other antimicrobial peptides (cecropin or attacin) (Boman and Hultmark, 1987).

Hanke *et al.* (2000) transferred the *T4 lysozyme* gene using pSR8-36 to Pinova apple. This gene was able to induce increased resistance in some transgenic lines in the greenhouse. Ko *et al.* (1998) cloned the pSR8-36 chimeric *T4 lysozyme* gene containing the α -amylase signal peptide into pBIN19 under the control of the enhanced 35S promoter with the AMV translational enhancer. Ko *et al.* (1998) also combined this chimeric *T4 lysozyme* gene with *attE* under control of the Pin promoter. These two constructs were introduced into the Galaxy apple (Aldwinckle *et al.*, 1998; Ko *et al.*, 1998). Some T4 lysozyme transgenics of Galaxy have shown increases in fire blight resistance in preliminary growth chamber tests (Ko *et al.*, 2002). However, transgenic lines containing both attacin and *T4 lysozyme* genes were not significantly more resistant than Galaxy transgenic for attacin or T4 lysozyme alone, indicating that there was no *in planta* synergy

between these two genes with respect to resistance to *E. amylovora* (Ko *et al.*, 2002).

Inhibition of bacterial pathogenicity factors Expression by the plant of any product inhibiting bacterial pathogenicity or virulence factors should lead to resistance or reduced susceptibility. In *E. amylovora* several of these factors are well known, and this knowledge had only started to be exploited to develop strategies for engineering resistance to fire blight. To date in apple, only one of these pathogenicity factors has been inhibited, the capsular EPS. The capsular EPS plays an essential role in pathogenicity. Indeed, the capsule functions not only to bind water, ions, and nutrients, but also as a protective layer to reduce possible recognition and response by the host (Romeiro *et al.*, 1981). This EPS can be naturally degraded by protein depolymerases of some bacteriophages. Hartung *et al.* (1988) have isolated an *EPS-depolymerase* gene from phage ϕ Ea1h. This gene has been integrated into the genome of two apple genotypes, Pinova (Hanke *et al.*, 2002, 2003) and M.26 (Borejsza-Wysocka *et al.*, 2007). In the work of Hanke *et al.* (2003), 61 out of 83 transgenic apple lines carrying the ϕ Ea1h depolymerase gene appeared significantly less susceptible than nontransgenic Pinova, after inoculation of detached leaves *in vitro*. Three lines appeared less susceptible than the control after inoculation of plants in the greenhouse. The partial fire blight resistance observed in these transgenic apple lines could be explained by the mode of action of depolymerase. Indeed, in capsule degradation assays using purified depolymerase, totally unencapsulated *E. amylovora* cells required high enzyme concentrations (Kim and Geider, 2000). Thus, they assumed that the amount of depolymerase produced in most of these transgenic clones is below the necessary threshold for sufficient destruction of EPS capsules at a high concentration of inoculum, but partly effective when the concentration of inoculum is lower. To achieve better control of fire blight in very susceptible apple cultivars, improvements in this strategy might be obtained by directing the depolymerase protein fused with a signal peptide to the apoplast and the intercellular space and by up-regulating this gene with a translational enhancer (e.g., AMV). Preliminary results have shown that in transgenic “M.26” apple, increased

resistance was observed when depolymerase gene is secreted into the intercellular space (Borejsza-Wysocka *et al.*, 2007).

Enhancement of natural plant defense Plant defense mechanisms are activated during incompatible and compatible reactions. However, in the case of a compatible interaction, the speed and level of expression of defense genes are insufficient to prevent disease development in the plant. Some strategies depend on blocking the progress of the pathogen in order to allow adequate activation of defense pathways of the plant (Figure 1).

During plant–pathogen interactions, pathogens secrete compounds called elicitors that induce plant defense mechanisms at variable speeds. These compounds when isolated from bacteria or fungi might be used to protect plants against their pathogens. These inducing substances can be classified into two groups: those interacting with the plant following the gene for gene model of Flor (1956) (*avr* genes) and those inducing the defense pathways of the plant. A bacterial *avr* gene conferred almost total resistance to plants expressing the corresponding resistance gene; in the case of *Arabidopsis* expressing the transgene *avrB* (Gopalan *et al.*, 1996) or *avrRpt2* (Mc Nellis *et al.*, 1998), resistance was expressed as an HR. But it is thought to be more likely that this type of resistance will be bypassed by pathogens compared with strategies using an elicitor with direct (PopA, Harpin N) or indirect (pectate lyase) action. Genetic engineering of induced defense mechanisms through the introduction of an elicitor gene into the plant genome has already been tested to generate resistance to pathogenic microorganisms. One approach is to place an elicitor gene under the control of a strictly pathogen-inducible promoter, in order to create local and limited cell death at the point of infection. This has been successfully achieved to protect tobacco from the oomycete *Phytophthora cryptogea*, by expression of either an oomycete elicitor (elicitin, Keller *et al.*, 1999) or a bacterial elicitor (PopA, Belbarhi *et al.*, 2001) under the control of the *hsr203J* promoter. A similar strategy, based on the expression of the Harpin N_{Ea} from *E. amylovora* under the pathogen-inducible promoter *gstI*, was tested on nonhost plants of fire blight, and decreased the infection of *A. thaliana* by the oomycete *Peronospora parasitica* (Bauer *et al.*,

1999) and potato by the oomycete *Phytophthora infestans* (Li and Fan, 1999). In order to create novel mechanisms for fire blight resistance in apple, some transgenic apple lines transformed with a construct containing the elicitor harpin N_{Ea} from *E. amylovora* with the inducible promoter *gstI* or the constitutive promoter CaMV 35S, respectively, were produced (Abdul-Kader *et al.*, 1999). Stable integration of Harpin N_{Ea} with the constitutive CaMV 35S promoter did not cause any detectable damage in the transgenic apple (Abdul-Kader *et al.*, 1999). Two years of field testing of own rooted plants of transgenic lines of M.26, containing the *harpin N_{Ea}* gene with the *gstI* promoter, showed a significant reduction in susceptibility to fire blight (Malnoy *et al.*, in preparation). Two of these lines showed a level of resistance equivalent to that of the apple rootstock M.7, which is partially resistant to fire blight. These transgenic lines can also help promote a better understanding of the role of harpin N_{Ea} in the induction of fire blight resistance in the transgenic clones. It will also be interesting to look at the behavior of these transgenic lines in response to other pathogens of apple.

Broad-spectrum resistance might also be achieved through manipulation of defense signaling molecules that act downstream of pathogen recognition. A plethora of defense signals have been identified using genetic, biochemical, and physiological approaches (Feys and Parker, 2000; McDowell and Dangel, 2000). Key players in the network include reactive oxygen intermediates (ROIs), nitric oxide (NO), salicylic acid (SA), the hormones ethylene, and jasmonic acid, and several proteins with presumed or demonstrated regulatory roles. In principle, each component of the signaling network represents a potential switch for activating the defense arsenal. This approach could provide broad-spectrum resistance if based on master regulators that activate the entire arsenal of defense responses. With these considerations in mind the *NPRI* gene (also known as *NIM1*) has emerged as a good candidate to provide broad-spectrum resistance. Some experiments have indicated that this gene is a key mediator of SAR. The *NPRI* gene encodes a protein that contains ankyrin repeats and displayed limited overall sequence similarity to the IKB human immune response regulator (Cao *et al.*, 1994, 1997; Ryals *et al.*, 1997). Sheng-Yang He and Qiao Lin

Jin (Michigan State University) cloned an *NPR1* ortholog, *MpNPR1*, from apple. This gene and the *Arabidopsis AtNPR1* gene were overexpressed under the control of pin2 or CaMV 35S promoters in two apple cultivars, Galaxy and M26, with the goal of increasing resistance to fire blight (Malnoy *et al.*, 2007b, c). The overexpression of *MpNPR1* activated the expression of some PR proteins (PR2, PR5 PR8) and increased resistance to fire blight in growth chamber tests. Preliminary results demonstrated that the cv. Galaxy clones with an additional copy of *MpNPR1*, under the control of the inducible promoter pin2, had a ca. 40% reduction in symptoms compared to the control (Malnoy *et al.*, 2007b, c).

In the past decade, the molecular bases of the interactions that determine pathogen recognition by the host plant cell have been clearly demonstrated, resulting in the cloning of both the avirulence (*avr*) and the corresponding resistance (*R*) genes. *E. amylovora* secretes the DspE pathogenesis factor that has some similarities to an Avr protein. Its apparent function as an Avr protein in the plant pathogen interaction seems to indicate a key role for it in disease development. Using a yeast two hybrid screen, Meng *et al.* (2006) identified four similar, leucine-rich-repeat (LRR), receptorlike serine/threonine kinases, from apple. The genes encoding the four DspE-Interacting Proteins of *Malus* (*DIPM* genes) are conserved in all hosts of *E. amylovora* tested, but not in tested nonhost plants. Interaction between the *DIPMs* and DspE is thought to be involved in disease development. With the aim of silencing the *DIPM* genes and preventing disease-causing interaction with DspE, 400 bp sense sequences from nonconserved regions of each gene, with homology among each other of <50% were introduced into the genome of the apple cv. Galaxy (Borejsza-Wysocka *et al.*, 2006). In addition, three constructs containing the four 400 bp sequences in tandem, a full length sense sequence of one gene and a hairpin sequence of that gene were also introduced into Galaxy apple. Reverse transcriptase PCR (RT-PCR) assays of the transgenic plants for transcript expression of the target *DIPMs* have shown evidence of silencing at the mRNA level in some lines. Some of the transgenic lines have also been evaluated for resistance to fire blight by inoculation of shoots of own-rooted potted plants with the virulent strain Ea273 of

E. amylovora. Preliminary results indicate that some lines have increased resistance (Borejsza-Wysocka *et al.*, 2004, 2006).

Using the same yeast two hybrid screen, Beer *et al.* (2006) were able to identify one protein that interacts with the Hrp N elicitor factor of *E. amylovora*. This small protein (60 aa) has a functional signal peptide and is associated with plant plasma membranes. With the aim of silencing the gene encoding for the HrpN-Interacting Protein of *Malus* (*HIPM*), an RNai construct containing the full length of this gene was introduced into the apple cv. Galaxy. Preliminary results showed partial resistance to fire blight (M. Malnoy, personal communication).

2.2.2.2 Apple scab

Apple scab, which is caused by the ascomycete *V. inaequalis* (Cke.), is the most important fungal disease of apple in most apple-growing regions with high spring and summer rainfall. This fungus attacks the foliage and the fruit of the apple tree. The infection of immature apples causes severe fruit drop, resulting in reduced yield, and later infections result in unmarketable fruits. The control of this disease in commercial orchards can require up to 15 fungicide treatments per year. However, because of increasing fungicide resistance within the pathogen, use of modern specific fungicides must be strictly limited and/or alternated. An alternative approach is growing resistant cultivars that use scab-resistance genes from small-fruited wild species. However, the production of high quality resistant cultivars by classical breeding is difficult because of the long juvenile phase and self-incompatibility of apple. Starting with the wild species *Malus floribunda* 821 carrying the *Vf* gene for resistance to apple scab, breeders have developed many scab-resistant cultivars, but few have met with any measure of commercial success mainly because of lack of high quality and/or storage ability. The direct transfer of a scab-resistance gene offers a new alternative for cultivar improvement.

Several groups have attempted to improve the scab resistance of highly susceptible apple cultivars such as Gala (Jensen *et al.*, 2002; Belfanti *et al.*, 2004), Galaxy (Faize *et al.*, 2003, 2004; Malnoy *et al.*, 2007e), Jonagold (De Bondt *et al.*, 1999), and McIntosh (Bolar *et al.*, 2000;

Malnoy *et al.*, 2007e) or partially resistant cultivars such as Ariane (Faize *et al.*, 2003, 2004), by the integration of heterologous antifungal genes (*chitinases*, *Amp1*, *AFP2*, *puroindoline*, or *hordothionine*) or *Vf* gene orthologs.

The biocontrol fungus *Trichoderma atroviride* (previously *Trichoderma harzianum*) produces many chitinolytic enzymes, including endochitinase, which randomly cleaves chitin, a major component of the fungus cell wall. *Trichoderma* endochitinase encoded by the gene *ech42* inhibits spore germination and hyphal elongation. Bolar *et al.* (2000) obtained several lines of the susceptible McIntosh apple with varying levels of *ech42* expression. Some of the transgenic lines exhibited increased resistance to *V. inaequalis*. However, transgenic lines with high endochitinase activity had reduced vigor. Similar results were also observed by Faize *et al.* (2003) when the *ech42* gene was introduced into the apple cultivars Galaxy and Ariane. This reduction of growth appeared to be associated with lignin content, peroxidase, and glucanase activity in the transgenic lines (Faize *et al.*, 2003). Endochitinase from the *ech42* gene interacts synergistically with other chitinolytic enzymes of *T. atroviride* such as N-acetyl- β -glucosaminidase (exochitinase *nag70*). Bolar *et al.* (2001) and Faize *et al.* (2003) studied transgenic lines of three different apple cultivars expressing *ech42* and *nag 70* alone or in tandem and demonstrated *in planta* synergy between these enzymes. Indeed, the transgenic lines expressing these two genes were more resistant than plants expressing either single enzyme at the same level.

Significant increase of resistance to *V. inaequalis* was also observed with the expression of other antifungal genes. Expression of the puroindoline B gene, a member of the plant lipid transfer proteins (LTPs), in two apple cultivars (Ariane, which is resistant to races 1–5 and susceptible to races 6 and 7, and Galaxy, which is susceptible to all races of scab) induced reduction of symptoms after inoculation with *V. inaequalis* race 6 of 55% and 64% in the most resistant transgenic lines of Galaxy and Ariane, respectively (Faize *et al.*, 2004). Preliminary results showed that the *hordothionin* (Jensen *et al.*, 2002), *Ace-AMP1*, and *Rs-AFP2* (De Bondt *et al.*, 1999) genes have potential for increasing resistance of apple to *V. inaequalis*.

A scab-resistance locus *Vf* was identified in the wild apple species, *M. floribunda* 821, and has been

widely introgressed into susceptible apple cultivars (Korban, 1998). The *Vf* locus confers resistance to five races of *V. inaequalis*, but not to races 6 and 7, recently identified in Europe. A cluster of four receptorlike sequences has been identified at the *Vf* locus, which resembles the *Cf* resistance genes in tomato (Vinatzer *et al.*, 2001; Xu and Korban, 2002). Three of them, designated *Vfa1*, *Vfa2*, and *Vfa4*, have intact ORFs, whereas, the fourth one, *Vfa3*, is an obvious pseudogene. Differential expression has been observed among the four *Vf* orthologs during leaf development, whereby *Vfa1*, *Vfa2*, and *Vfa3* are highly expressed in immature leaves, but only slightly detectable in mature leaves; whereas, *Vfa4* is expressed in immature leaves, and highly expressed in mature leaves (Xu and Korban, 2002). Barbieri *et al.* (2003) and Belfanti *et al.* (2004) have proved that overexpression of the *Hcrvf2* (= *Vfa2*) gene, under the control of the constitutive promoter CaMV 35S, was sufficient to confer scab resistance to a susceptible apple cultivar. However, this resistance is specific to *V. inaequalis* races, effective toward races 1–5, but not toward race 6 (Silfverberg-Dilworth *et al.*, 2005b). Malnoy *et al.* (2007e) showed also that the gene *Vfa2* is sufficient to confer partial resistance to *V. inaequalis* in Galaxy and McIntosh when it is expressed under the control of its own promoter. They also showed that the *Vfa1* gene is able to confer partial resistance to a mixture of apple scab races (race 1–5). These two studies showed that the *Vfa1* and *Vfa2* genes are involved in the resistance of apple scab in apple. In contrast, the *Vfa4* gene is not involved in resistance to apple scab. Indeed, the Galaxy and McIntosh transgenic lines expressing the *Vfa4* gene were as, or more susceptible to or than the control (Malnoy *et al.*, 2007e). It will be interesting to transform some scab susceptible apple cultivars with the *Vfa1* and *Vfa2* genes in tandem, to determine their synergetic effect. It will also be informative to study the specificity to the different *V. inaequalis* races of Galaxy and McIntosh transgenic lines expressing *Vfa1* or *Vfa2*.

2.2.2.3 Other apple pathogens

Resistance in apple to two other diseases (crown gall and *Alternaria* blotch) and to an insect (lightbrown apple moth) has been reported to be increased by genetic engineering.

Lightbrown apple moth (LBAM), *Epiphyas postvittana*, is a serious pest of pome and stone fruits and of many other horticultural crops, including grape vine, citrus, kiwifruit, berry fruits, avocados, and some vegetable and flower crops in New Zealand (Wearing *et al.*, 1991). It commonly causes superficial fruit damage, and this, together with the presence of larvae, forms the basis of a quarantine problem in exported fruit. LBAM also occurs in Australia, New Caledonia, Hawaii, England, and other parts of Europe, where it is found on apples, pears, and oranges (Dickler, 1991). Markwick *et al.* (2003) produced some Royal Gala transgenic lines expressing the *avidin* or *streptavidin* gene. Enzyme-linked immunosorbent assay (ELISA) assays showed that *avidin* expression ranged from 1.9 to 11.2 μM and *streptavidin* expression ranged from 0.4 to 14.6 μM . Expressed at these levels, both biotin-binding proteins conferred a high level of insect resistance on transformed apple plants to larvae of the LBAM (Markwick *et al.*, 2003). Mortality of LBAM larvae was significantly higher ($P < 0.05$) on three *avidin*-expressing (89.6%, 84.9%, and 80.1%) and two *streptavidin*-expressing (90% and 82.5%) apple lines than on nontransformed control plants (14.1%) after 21 days. Weight of LBAM larvae was also significantly reduced by feeding on all apple shoots expressing *avidin* and on apple shoots expressing *streptavidin* at levels of 3.8 μM and above (Markwick *et al.*, 2003).

Crown gall is a significant agricultural problem worldwide. *A. tumefaciens*, a ubiquitous soil bacterium (Bouzar and Moore, 1987), causes this disease in a wide variety of plants including fruit and nut trees, grapevines, cane berries, chrysanthemum, rose, and other nursery crops (Pinkerton *et al.*, 1996). *A. tumefaciens* genetically transforms plant cells to grow as tumors; therefore, after a few hours of infection the disease will progress even if the tumor-inducing bacteria are killed with antibiotics. Thus, prevention is the only effective way to control crown gall. Tumors on stems and leaves result from excessive production of the phytohormones auxin and cytokinin in plant cells genetically transformed by *A. tumefaciens*. High phytohormone levels result from expression of three oncogenes transferred stably into the plant genome from *A. tumefaciens*: *iaaM*, *iaaH*, and *ipt*. The *iaaM* and *iaaH* oncogenes direct auxin biosynthesis, and the *ipt* oncogene causes

cytokinin production. In contrast to other tissues, roots do not respond to high cytokinin levels, and auxin overproduction is sufficient to cause tumor growth on roots. Inactivation of *iaaM* abolished gall formation on apple tree roots. Transgenes designed to express double-stranded RNA from *iaaM* and *ipt* sequences in Jonagold prevented crown gall disease on roots of transgenic apple trees (Viss *et al.*, 2003). Thus, silencing the *A. tumefaciens iaaM* oncogene provides a simple and effective means to prevent crown gall disease in crop plants such as apple trees.

Alternaria blotch, caused by *Alternaria mali*, is a serious disease of apple in Japan, Korea, and China. It also occurs in Zimbabwe and has been found in the United States. *A. mali* is responsible for the formation of lesions on leaves in late spring and summer. These lesions are small, round, blackish spots, gradually enlarging to 2–5 mm in diameter, with a brownish purple border. Fruit infections are uncommon, except in very susceptible cultivars. In order to increase the resistance to this disease, Kim *et al.* (2007) introduced the gene *Rab*, known to participate in general defense signaling pathways in plant cells, into the McIntosh apple genome. Preliminary data show that all the transformants have smaller disease lesions and spot size than the control (Kim *et al.*, 2007).

In conclusion, increased resistance to pathogens is possible via genetic engineering. The concept was proven first by using genes from different organisms such as insects, animals, and bacteria. Due to the concern of the public, the use of genes from *Malus* will be more appropriate. One strategy that can be used effectively for increased pathogen resistance is the use of specific apple genes, which confer resistance to several pathogens, such as the *NPR1* gene, which increased resistance of apple to fire blight, apple scab, and cedar apple rust (Malnoy *et al.*, 2007b, c).

2.2.3 Environmental stress resistance

Iron is an essential element for all living organisms. Although abundant in soils, iron often forms highly insoluble ferric-hydroxide precipitates with limited availability for plants, especially crops grown on calcareous soils (Guerinot and Yi, 1994). Apple trees grown on calcareous soils often

show chlorosis due to iron deficiency; this is the case with most apple trees grown in China. The chlorosis of apple trees grown under iron deficiency conditions can be easily avoided by grafting the scion on apple rootstocks with iron-deficiency tolerance. In higher plants iron is acquired from the soil through the coordinated action of H^+ -ATPases, membrane-bound Fe^{3+} -chelate reductase and Fe^{2+} specific cation transporters (Qu *et al.*, 2005). Several of these genes involved in iron assimilation have been cloned (Eckhardt *et al.*, 2001; Li *et al.*, 2006) and some have been used to improve iron deficiency tolerance (Goto *et al.*, 1998, 1999, 2000). Qu *et al.* (2005) reported that by introducing the iron transporter *LeIRT2* into the Balenghaitang rootstock they increased the tolerance of transgenic lines to iron deficiency. Plants in hydroponically cultivated transgenic lines weighted 21–34% greater than control plants.

During exposure to biotic and abiotic stresses, molecular oxygen can undergo reactions that result in the formation of ROIs. ROIs are highly destructive to lipids, nucleic acids, and proteins and the resulting injury is referred to as oxidative stress. In fruit crops, oxidative stress plays a role in freezing injury, sunburn, and physiological scald. Artlip *et al.* (2006) were able to increase the resistance of apple to heat and low temperature by overexpressing the cytosolic ascorbate peroxidase (*APX*) gene. Transgenic lines exhibited variability in their levels of *APX* enzyme activity compared to the control, and also displayed varying degrees of increased resistance to high and low temperature stress. Resistance to freezing injury was 1–3° greater, and resistance to acute heat stress up to 7° greater in transgenic lines (Artlip *et al.*, 2006).

2.2.4 Carbohydrate metabolism in apple (Table 2)

In addition to sucrose and starch, Sorbitol is accumulated by apple plants. Sorbitol, the sugar alcohol of glucose, is the predominant sugar found in apple. It is synthesized in mature leaves and translocated to fruit, where it is converted to fructose. The sugar alcohol sorbitol appears to be widely distributed in nature. It can be found in species of bacteria, insects, animals, yeasts, algae, fungi, and higher plants. However, certain woody members of the Rosaceae family, including

Malus, *Pyrus*, *Prunus*, and *Sorbus* spp. appear to be unique in the entire plant kingdom with respect to their ability to synthesize, accumulate, and degrade sorbitol. In apple trees, sorbitol is believed to be the major photosynthetic product, translocated from mature leaves to growing tissues, such as fruits and young leaves. At the biochemical level, sorbitol metabolism occurs as a result of two significant reactions: one results in the formation of sorbitol 6-phosphate from glucose 6-phosphate by aldose 6-phosphate reductase in photosynthetic tissues, referred to here as sorbitol 6-phosphate dehydrogenase (*S6PDH*); the second results in the conversion of sorbitol to fructose by sorbitol hydrogenase (*SDH*) in skin tissues. The *S6PDH* gene from apple was cloned by Kanayama *et al.* (1992) and its expression in transgenic tobacco was correlated with sorbitol synthesis and concentration (Tao *et al.*, 1995). When the *S6PDH* apple gene was overexpressed in the apple genotype Orin, transgenic lines with integration of several copies of the *S6PDH* showed a reduction in activity of this gene caused by cosuppression (Kanamaru *et al.*, 2004). This reduction of activity was accompanied with a reduction in sorbitol level in the transgenic lines but showed a sixfold to sevenfold increase in sucrose compared with the nontransgenic plants. However, these transgenic lines ceased growth in early summer compared to the control. This suggests that growth of the shoot apex may be stopped by a deficiency of sugar supply to the sink organ, because apple uses sorbitol rather than sucrose as the main translocated sugar (Kanamaru *et al.*, 2004). Conversely, transgenic Orin apple lines, which had integrated few copies of the *S6DPH* genes, showed increased *S6PDH* activity and had increased levels of sorbitol and sucrose content (Kanamaru *et al.*, 2004).

When the *S6PDH* apple gene was expressed in antisense in the apple Greensleeves, its activity was decreased as was the accumulation of sorbitol. However, such transgenic lines had an increased content of sucrose and starch at both dusk and predawn (Cheng *et al.*, 2005). The sorbitol to sucrose ratio in leaves is reduced by 90% and in phloem exudates by 75%. The fruit accumulated more glucose and less fructose, starch and maleic acid, with no overall difference in weight and firmness (Teo *et al.*, 2006). Silencing leaf sorbitol synthesis affects the fruit quality such as glucose,

fructose, starch, and malic acid accumulation (Teo *et al.*, 2006). In these transgenic lines the sorbitol dehydrogenase gene is down-regulated whereas the sucrose synthase activity is up-regulated in the shoot tips with decreased sorbitol synthesis, leading to homeostasis of vegetative growth (Zhou *et al.*, 2006).

Modification of the sorbitol pathway in apple may clarify understanding of the regulation of photosynthesis and carbon partitioning by sorbitol synthesis. In conclusion, these two studies show that the photosynthetic process in sorbitol synthesizing species has considerable plasticity. When sorbitol synthesis is decreased by antisense or sense inhibition of *S6PDH*, cytosolic fructose-1,6-biphosphatase activity is down-regulated to reduce the carbon flux to the cytosol, and starch synthesis in the chloroplast is consequently up-regulated.

Suleman and Steiner (1994) have proposed a model to explain the fire blight susceptibility of tissue based upon sorbitol concentration. According to the model, increases in sorbitol concentration result in increasingly negative solute potentials, which have negative effects on the growth of *E. amylovora*, thus rendering these tissues more resistant. However, this hypothesis has not been proved, and evidence indicates it may not be correct (J.L. Norelli, personal communication).

2.2.5 Ethylene biosynthesis in apple

Ethylene is an important plant growth regulator that affects diverse plant processes including fruit ripening, fruit flavor composition, senescence, and response to biotic and abiotic stress. The biosynthesis of ethylene occurs in two enzymatic steps; in the first reaction, S-adenosyl methionine is converted to ACC (1-aminocyclopropane-1-carboxyl acid) by ACC synthase (ACS), and then in a second reaction, ACC is catalyzed by ACC oxydase (ACO) to produce ethylene. During ripening, the expression of *ACS* and *ACO* genes and the activity of their encoded enzymes govern the rate of ethylene production (Dandekar *et al.*, 2004). ACS regulates the rate limiting step in ethylene production (Yang and Hoffman, 1984) but ACO can also play a regulatory role (Gray *et al.*, 1992). Both enzymes have been well characterized and have

been cloned from apple (Dong *et al.*, 1991, 1992). These two genes have been expressed in antisense configuration in the apple Greensleeves (Dandekar *et al.*, 2004; Defilippi *et al.*, 2004, 2005a, b) and the cv. Royal Gala (Hrazdina *et al.*, 2003) (Table 2). From these two studies apple trees with down-regulation of ethylene have been produced. Hrazdina *et al.* (2003) reported a reduction in softening in some Royal Gala transgenic apple trees in which ACS had reduced activity. Dandekar *et al.* (2004) confirmed that transgenic lines that had a higher or lower ethylene production rate than the control have a correspondingly altered storage potential for their fruit. The availability of these lines suppressed in ethylene biosynthesis allowed the identification of events under ethylene control, such as fruit softening, external color development, and ester accumulation. The Greensleeves transgenic apple with suppressed ethylene biosynthesis permitted Defilippi *et al.* (2004) to analyze the role of ethylene in regulating overall flavor of apple fruits. Flavor components were differentially regulated in response to the suppression of both ethylene biosynthesis. Headspace analysis of aroma production showed a reduction in ester and alcohol production in the ethylene suppressed lines (Defilippi *et al.*, 2004). However, no major differences were observed in the concentrations of aldehyde volatiles. Other flavor metabolites that showed an ethylene-dependent pattern were organic acids and sugar (glucose and fructose) (Defilippi *et al.*, 2004). As for aroma volatile-related enzymes, Defilippi *et al.* (2005a, b) found that the acyltransferase (ATT) enzyme is under ethylene regulation and seems to play a role in determining ester formation.

These transgenic apple trees suppressed in ethylene biosynthesis were helpful in understanding the role of ethylene biosynthesis in fruit ripening and aroma formation, and will provide further understanding about the role of ethylene.

2.2.6 Dwarfism and rooting ability in apple rootstocks

Dwarfing characteristics of fruit tree rootstocks are of great importance for commercial production. Production per area of high quality fruits is greatly increased by the best dwarfing rootstocks.

Dwarfing rootstocks are useful for high-yield fruit production because trees can be planted closely together and labor is reduced because the apples can be harvested without using a ladder. Rootstocks like M.9 (dwarf) have been developed for this purpose. Some rootstocks are too vigorous to be used for commercial purposes and several dwarf rootstocks have problems with adaptability (Zhu *et al.*, 2001a). Some current projects are being conducted to improve apple rootstocks by introducing genes able to increase the dwarfing effect without affecting their rooting ability (Zhu *et al.*, 2001a, b). Several genes that cause reduced growth have been identified (Jasik *et al.*, 1997). The *rol* rootling (*A*, *B*, *C*, and *D*) genes from *A. rhizogenes* and the plant phytohormones genes have been shown to exert dwarfing properties (Jasik *et al.*, 1997).

The *rolB* gene has been found to be the most effective of the *rol* genes in promoting improved root formation; this gene has been applied in genetic transformation to improve adventitious rooting of some recalcitrant plants (Rugini *et al.*, 1991; Nilsson *et al.*, 1997). It has been successfully expressed in several apple rootstocks: M.26 (Welander *et al.*, 1998), M.9 (Zhu *et al.*, 2001b), and Jork9 (Sedira *et al.*, 2001, 2005; Pawlicki-Jullian *et al.*, 2002) and in the apple cultivar Florina (Radchuck and Korkhovoy, 2005). Resulting transgenic apple rootstocks and cultivar showed enhanced rooting in general, and an increased number of roots per explant (Welander *et al.*, 1998; Sedira *et al.*, 2001; Zhu *et al.*, 2001b; Pawlicki-Jullian *et al.*, 2002; Radchuck and Korkhovoy, 2005). However, growth was reduced in some rootstocks compared to the controls (Zhu *et al.*, 2001b; Pawlicki-Jullian *et al.*, 2002). In contrast, the transgenic apple Florina does not show any phenotypical difference after several months of growth *in vitro* or in the greenhouse compared to the control (Radchuck and Korkhovoy, 2005). The possible explanation of the differential effect of *rolB* on growth may be that Radchuck and Korkhovoy (2005) grew plants for 2 years, whereas Zhu *et al.* (2001b) grew them for 4 months and under a limiting supply of nutrients compared with steady-state nutrient supply conditions (Zhu and Welander, 2000). The apple rootstocks expressing the *rolB* gene are more sensitive to auxin (IBA) compared to the controls (Zhu *et al.*, 2001b; Sedira *et al.*, 2005). Zhu *et al.*

(2001a) also reported that the expression of the *rolA* gene increased sensitivity to auxin of the transgenic M.26 lines compared to the controls. Expression of *rolA* altered the growth capacity of transgenic M.26 (Holefors *et al.*, 1998) and A2 (Zhu *et al.*, 2001a). Some of these transgenic rootstocks showed a reduction in length, leaf area, and dry weight. Similar results were observed with the expression of either the *rolC* or *phytochrome B* gene in the apple rootstocks Marubakaidou (Igarashi *et al.*, 2002) and M.26 (Holefors *et al.*, 2000), respectively.

A delay in flowering and strongly reduced fertility had been previously described for some *rolA* transgenic plants (Tepfer, 1984; Sun *et al.*, 1991). However, this was not observed in the apple cv. Gravenstein grafted on the *rolA*-transformed M.26 rootstock (Zhu *et al.*, 2001a). Gravenstein flowered on both the *rolA*-transformed rootstock and on the nontransformed rootstock in the second year after grafting, and flowers were normal in greenhouse (Zhu *et al.*, 2001a). In apple, the side effects of the *rolA* gene might not be transmissible to a scion cultivar, as shown by the preliminary results of Zhu *et al.* (2001a). Currently, Zhu *et al.* (2007) have reported that the transgenic rootstocks A2-*rolA*, M.26-*rolB*, and M9/29-*rolB*, grafted with different apple cultivars are already in field trials to evaluate effects of rootstocks on growth and development of scion cultivars, and the possible transport of the *rolB* protein from rootstock to scion cultivars. Their preliminary results showed that, for the same cultivar, no differences in bud break, flowering, and flower numbers were found between the transgenic and nontransgenic rootstocks. The plant height and stem diameter were reduced for the vigorous cultivars grafted on the *rolB* rootstocks compared to the nontransformed rootstocks (Zhu *et al.*, 2007). In addition, no transgene was found in the nontransformed scion cultivars (Zhu *et al.*, 2007).

2.2.7 Other fruit traits

A significant problem with fresh-cut or juice-processed apples is enzymatic browning, a widespread problem in the food industry. The browning reaction is due to enzymatic action, caused by the polyphenoloxidase (PPO). PPO is considered to be located in plastids and physically

separated from its phenolic substrates that are mainly located in vacuoles. When plant tissues are damaged by cutting, bruising, or invasion by parasites, this compartmentalization is lost and PPO comes into contact with phenolic substrates (Waughn and Duke, 1984). In apple, PPO is mainly located around the core of the fruit, the location corresponding to the most intense browning (Murata *et al.*, 1993). A PPO cDNA (complementary DNA) was isolated from apple peel by Boss *et al.* (1995), showing that the PPO mRNA accumulated in immature fruits and wounded tissues. Haruta *et al.* (1998) isolated two genomic DNAs encoding apple PPOs, and showed that PPOs in the Rosaceae family were very similar by both molecular and immunological criteria (Haruta *et al.*, 1999). By using antisense methods, Murata *et al.* (2000, 2001) were able to repress the expression of the apple PPO gene. The silencing of this gene induced in the transgenic apple has lower browning potential (Murata *et al.*, 2000, 2001). Antisense expression of the PPO will need to be targeted to fruit tissue to prevent interference with PPO expression elsewhere in the plant that may be important for disease/pest resistance.

Polyphenol constituents derived from fruits like apple are more effective antioxidants *in vitro* than vitamins C and E, and thus may be more valuable for protection *in vivo*. Therefore, the active ingredient in “an apple a day keeps the doctor away” may well be the phytochemical component of the apple fruit. The phytochemical components include flavonoids, phenylpropanoids, and phenolic acids and have been highlighted as important contributing factors in the antioxidant activity in our diet (Rice-Evans *et al.*, 1997; Lee and Lee, 2003; Heo *et al.*, 2004). In apple, flavonols like quercetin and flavones like rutin may be important (Rice-Evans *et al.*, 1997). Indeed, quercetin derived from apple has a potential anticarcinogenic and antineuroprotective effects (Lee *et al.*, 2005). Stilbene synthase is an enzyme responsible for the synthesis of phytoalexin resveratrol. The production of resveratrol is related to fungal infection (Liswidowati *et al.*, 1991) and abiotic stress (Chung *et al.*, 2003; Rudolf and Resurreccion 2005) such as UV light and ozone. Stilbenes, in general, and resveratrol, in particular, are biologically active compounds, which have antifungal activities against various pathogens (Adrian *et al.*, 1997), among them

V. inaequalis (Schulze *et al.*, 2005). In addition to these implications for disease resistance, resveratrol and its glycosides have attracted interest as health promoting agents because of their antiinflammatory, estrogenic antiplatelet, and anticarcinogenic activities (Bertelli *et al.*, 1995; Jang *et al.*, 1997; Manna *et al.*, 2000). The biological and pharmacological activities of resveratrol are thought to be due to strong antioxidant properties. Due to the fact that apple has long been recognized as a great source of antioxidants, the synthesis of resveratrol in transgenic apple would expand the antioxidant capacity and could therefore be regarded as an additional factor for improving the intrinsic quality of the fruit. Szankowski *et al.* (2003) have produced transgenic apple plants expressing the *Stilbene synthase* gene from grapevine under the control of its own wound-, pathogen-, and UV-inducible promoter. Under greenhouse conditions, these transgenic apple lines are phenotypically normal, and flowered within the first and second years after grafting (Ruhmann *et al.*, 2006). Transgenic apple fruit was phenotypically indistinguishable from nontransgenic fruit of the same cultivars (Ruhmann *et al.*, 2006). The *S. synthase* gene is expressed in the fruit skin and in the flesh after promoter induction. The introduction of this novel pathway did not dramatically influence the accumulation of other phenolic compounds naturally present in apple fruits (Ruhmann *et al.*, 2006). Resveratrol was modified by the addition of a sugar in both vegetative and fruit tissues. Because of the high antioxidant activity of resveratrol, its synthesis in apple will contribute to fruit quality and might also have positive effects on fruit stability during storage (Ruhmann *et al.*, 2006).

Polygalacturonases (PGs) are expressed in a wide range of tissues and developmental stages in plants and are encoded by relatively large gene families. PGs are associated with fruit ripening, cell separation processes such as leaf and flower abscission, pod and anther dehiscence, pollen grain maturation, pathogen defense, plant host interactions, and processes of cell expansion, growth, and xylogenesis (Hadfield and Bennett, 1998; Torki *et al.*, 1999). A PG enzyme in ripe apple fruit has been isolated and biochemically characterized as an endo-PG (Wu *et al.*, 1993). The corresponding cDNA (*MdPG1*) was isolated

from Golden Delicious (Atkinson, 1994) and was shown to hybridize to an mRNA present in ripe fruit but not in developing fruit or flowers (Atkinson *et al.*, 1998). This gene, *MdPG1*, was overexpressed in apple with the expectation that disruption of cell wall metabolism would occur in the ripening fruit (Atkinson *et al.*, 2002). However, Atkinson *et al.* (2002) observed that *MdPG1* overexpression in transgenic apple led to a range of novel phenotypes. These phenotypes included silvery leaves and premature leaf shedding due to reduced cell adhesion in leaf abscission zones. Mature leaves had malformed and malfunctioning stomata, disturbed water relations, and a brittle leaf phenotype (Atkinson *et al.*, 2002). The modification of apple trees by a single *PG* gene offered Atkinson *et al.* (2002) a new and unexpected perspective on the role of pectin and cell wall adhesion in leaf morphology and stomatal development.

Allergy to apple fruits is a common phenomenon in patients with birch pollen allergy. Approximately 90% of patients allergic to birch pollen have IgE antibodies against the birch pollen allergen Bet v 1 (Mari *et al.*, 2003). This allergen belongs to a group of pathogenesis-related proteins, more specifically the PR10 proteins (Pühringer *et al.*, 2000). Many plant foods, in particular fruits and tree nuts, contain homologous proteins that are recognized by the same Bet V 1-specific IgE antibodies. In apple, this allergen was designated Mal d 1 (Vanek-Krebitz *et al.*, 1995). Approximately 70% of patients allergic to birch pollen have been reported to have adverse reactions to apple as a consequence of the cross-reactive IgE antibodies (Ebner *et al.*, 1991). Although birch pollen-related apple allergy is almost exclusively mild and restricted to the oral cavity, most patients allergic to apples avoid the fruit in their diet (Gilissen *et al.*, 2005). Related fruits of the Rosaceae family, such as pear, cherry, and peach can also induce adverse reactions on the basis of the same cross-reactive IgE antibodies (Ortolani *et al.*, 1988). Therefore, avoidance often results in deprivation in the diet of a wide range of common plant foods that have important nutritional value. Production of an apple with a significant reduction of the overall expression of *Mal d 1* from existing economically successful cultivars seems to be an attractive approach.

Gilissen *et al.* (2005) chose the approach of RNA interference (RNAi) for post-transcriptional silencing of the gene *Mal d1*. They have isolated one *Mal d 1* gene from Gala and transformed the Elstar apple cultivar with an *Mal d 1* RNAi vector. Normally it takes 3–5 years to grow an apple fruit-producing tree from seed or *in vitro* culture. Because *Mal d 1* genes are expressed in leaves as well as in the apple fruit, Gilissen *et al.* (2005) were able to evaluate *Mal d 1* gene silencing in the leaves of young apple shoots growing *in vitro*. Their results showed a reduction of expression of the *Mal d 1* by immunoblotting. This translated into significantly reduced *in vivo* allergenicity (Gilissen *et al.*, 2005). These observations support the feasibility of production, by gene silencing, of apple cultivars hypoallergenic for *Mal d 1* (Gilissen *et al.*, 2005). These data will need to be confirmed further by analyzing the expression of *Mal d 1* in transgenic fruit and by testing their allergenicity. *Mal d 1* silenced plants must also be evaluated for undesirable reduction in disease resistance.

2.2.8 Flowering time

In the development of all woody plants from seed, there is a juvenile phase, lasting 3–7 years in apple (Visser, 1964), during which flowering does not occur and cannot be induced under normal conditions (Hackett, 1985). In the fruit industry, it is important to accelerate flowering by reducing the juvenile phase of the trees after planting in order to facilitate the earliest possible production of fruit. In fruit breeding, various practical techniques have been considered to accelerate the flowering of seedlings. The basic idea is to grow the seedling rapidly from the germination stage to the flowering stage (Visser, 1964; Zimmerman, 1971; Aldwinckle, 1975). In apple, grafting the seedling onto dwarfing rootstocks, such as M9 and M27, will usually bring earlier flowering by 1 or 2 years (Visser, 1973). Using some of these techniques, Zimmerman (1971) produced the first flowering in crabapple seedlings within 13 months, and Aldwinckle (1975) obtained flowering 16–20 months after germination of seeds of apple cultivars at above node 120 from the base of the plants. Yao *et al.* (1999), by incorporating techniques used for reducing the juvenile period into the

containment greenhouse management practice, were able to promote rapid flowering, after 2 years, of the transgenic Royal Gala line, for subsequent analysis of the transgene expression. However, under normal conditions, it would be difficult to reduce the juvenile phase of an apple seedling to less than 4 years even if they are grafted on dwarfing rootstocks. Significant correlations have been found between the length of the juvenile phase and parent characteristics, such as growth rate and the length of the vegetative phase (Lavi *et al.*, 1992).

The most striking advances of the genetic control of flowering times come from studies in *Arabidopsis* (Levy and Dean, 1998; Pineiro and Coupland, 1998). Several genes that control flowering time have been isolated, namely *LEAFY* (*LFY*) (Weigel *et al.*, 1992), *APETALA1* (*API*) (Mandel *et al.*, 1992), *TERMINAL FLOWER 1* (*TFL1*) (Ohshima *et al.*, 1997), and *FLOWERING LOCUS* (*FT*) (Kardailsky *et al.*, 1999). In transgenic *Arabidopsis*, overexpression of *LFY*, *API* or *FT* shortens the juvenile phase and causes early flowering (Mandel *et al.*, 1992; Weigel and Nilsson, 1995; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999), whereas *TFL1* overexpression causes late flowering (Ratcliffe *et al.*, 1999). *TFL1* plays a key role in the maintenance of the inflorescence meristem by preventing the expression of *LFY* and *API* in the shoot apical meristem (Liljegren *et al.*, 1999). Apple orthologs of several of these genes, such as *AFL1*, *AFL2*, *MdAPI* (*MdMADS5*), and *MdTFL1* have been isolated and characterized (Kotada *et al.*, 2000, 2002; Kotada and Wada, 2005). Kotada *et al.* (2006) were able to reduce the juvenile period in apple by silencing the *MdTFL1* gene. Indeed, depending on the copy number of the *MdTFL1* antisense gene integrated in the apple genome, the first flower appeared between 8 and 25 months after transfer of the transgenic lines in the greenhouse, whereas the first flower in the nontransformed control appeared 69 months after transfer in the greenhouse. Most flowers of the transgenic lines had functional reproductive organs, resulting in normal fruit that contained several seeds. The pollen and the seed of these plants are viable.

An alternative approach to the silencing of the *MdTFL1* gene is to induce flowering by the overexpression of genes involved in the formation

of floral meristems, such as the *LFY*, *API*, or *FT* genes. Hanke *et al.* (2007a) chose this approach and transformed the apple cultivars Pinova with the *BpMADS4* gene of silver birch, which is similar to the *FT* gene. Some apple transgenic lines, constitutively expressing this gene, showed development of flowers *in vitro*. For example, some transgenic lines showed a solitary flower *in vitro* 13 weeks after transformation (Hanke *et al.*, 2007a). Most of these flowers appeared morphologically normal, but some were abnormal (Hanke *et al.*, 2007a).

The further use of these transgenic lines should be useful in breeding, cultivars production, and basic research, for example, molecular and physiology studies on the effects of genes in fruit development and disease resistance.

2.2.9 Self-fertility

Fruit production in many tree fruit crops is dependent on cross-pollination between cultivars. This is due to the existence of a self-incompatibility (SI) mechanism, which is a widespread intraspecific system to prevent self-fertilization that is controlled by a single *S*-locus. Cross-pollination between compatible cultivars depends on insects as pollen vectors during flowering, and their activity is impaired by inclement weather. Suboptimal pollination efficiencies are one of the factors contributing to low fruit yields that may occur in some years in commercial orchards (Goldway *et al.*, 1999). There is a strong interest in the self-fertile character in many fruit and nut tree crops because self-pollination could ensure more consistently high production compared to cross-pollination. This has been apparent in sweet cherry and almond, where dysfunctional *SI* genes have been obtained through mutagenesis and interspecific crosses. Although the severity of the *SI* reaction varies between cultivars and pollination conditions, truly self-fertile apple cultivars are commercially nonexistent (Broothaerts *et al.*, 2004a). Moreover, because high crop yields often create lower quality fruit and stimulate a biennial bearing tendency, it is unclear whether self-fertility would be beneficial for apple production. Broothaerts *et al.* (2004a) addressed this concern by silencing the *SI* mechanism in apple. Controlled self- and cross-pollination of the

flowers of the transgenic Elstar and nontransgenic apple trees over a 3-year period showed that transgenic lines produced normal levels of fruit and seeds after selfing. In contrast, the controls produced much less fruit following self-pollination compared to cross-pollination (Broothaerts *et al.*, 2004a). Further work should be undertaken to study the behavior of the transgenic trees under natural growth conditions to find out to what extent self-fertility affects crop yield under various environmental conditions. The self-fertile trees may also be used as a tool to study inbreeding depression, or for the development of homozygous breeding lines.

2.2.10 Selectable markers

Selectable marker genes are widely used for the efficient transformation of crop plants. In most cases, selection is based on antibiotic or herbicide resistance. These marker genes are preferred because they tend to be most efficient (e.g., in apple up to 80% transformation rate with the *nptII* gene for kanamycin resistance Table 2). Due to concerns about acceptability of antibiotic resistance genes in fruit cultivars, considerable effort is being put into developing a suite of strategies (site-specific recombination, homologous recombination, transposition, and co-transformation) to eliminate the marker gene from the nuclear or chloroplast genome after selection (Miki and McHugh, 2004). Current efforts are emphasizing systems in which the marker genes are eliminated efficiently soon after transformation (Schaart *et al.*, 2004) by using the cre-lox system. Schaart *et al.* (2004) were able to produce some transgenic Elstar containing no selectable marker. However, these methods are tedious and total elimination may be questioned. Alternatively, transgenic apple can be produced by the use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Examples are (Table 2) phosphomannose isomerase (Flachowsky *et al.*, 2004; Zhu *et al.*, 2004; Degenhardt *et al.*, 2006; Szankowski and Degenhardt, 2007) and Vr-ERE (Chevreau *et al.*, 2007). Only Degenhardt *et al.* (2006) reported the regeneration of transgenic apple lines using the *pmi* gene as selectable marker

with a rate of transformation from 1% to 24%. The other report shows the expression of the reporter gene in the transformed leaves but they did not regenerate into plants. Other methods based on the transformation of apple without any selectable marker have also been reported (Malnoy *et al.*, 2007a). Here, genetically modified apples are produced by *Agrobacterium* inoculation followed by regeneration of shoots without the use of a selectable agent. This produces many plants, the majority of which are nontransgenic. However, depending on the regeneration and gene transfer frequencies, some plantlets are transgenic and have to be identified by PCR screening. A prerequisite is a regeneration/transformation protocol of high efficiency. So far, this method has been limited to model plants and few specific crop cultivars, for example, in potato. Due to the very high efficiency of transformation of the apple genotypes Galaxy and M.26, Malnoy *et al.* (2007a) have been developing this technique in apple. They reported regeneration via this procedure for both genotypes with an efficiency of transformation close to 12% for Galaxy and 25% for M.26.

These methods are generally of lower efficiency than that achieved using *nptII*, but are promising for production of marker-free cultivars for commercial use.

2.3 Stability of Inheritance of the Transgene, Yield, and Quality

The technology for efficient production of transformed apple lines has now been developed, and transformed plants and fruiting trees can be obtained relatively quickly. Some transformed lines of apple have shown an increase in resistance to fire blight in field trials, and the fruit of these lines has shown no difference in quality, size, or yield compared to the control (Aldwinckle *et al.*, 2003b). Ruhmann *et al.* (2006) showed some similar results that the expression of the *S. synthase* gene does not affect the quality of the fruit.

With the respect to the release of transgenic woody plants the stability of the integrated transgenes and their long-term expression is one of the main objectives in monitoring and risk assessment of genetically engineered plants. Stable integration and expression of transgenes is important for fruit trees, which have an

exceptionally long productive life compared with annual crops. Several laboratories have studied the stability of expression of transgenes in transgenic apple. James *et al.* (1995, 1996) showed the stable expression and Mendelian expression of transgenes in transgenic Greensleeves. They showed a 1:1 segregation of the *nos* gene in the R₁ transgenic apple progeny. In addition, they reported the evidence of stable expression of both *nos* and the cotransformed gene *nptII* in the fruit flesh of the apple fruit some 7 years after the initial transformation. Briviba *et al.* (2004) reported stable expression of the *S. synthase* and the *bar* gene in apple 3.5 years after transformation, in all the transgenic lines except one. In contrast, Reim and Hanke (2004) reported that in 34% of the transgenic lines examined that the transgene, the selectable marker, or either gene was not detected, after several years in greenhouse.

2.4 Regulatory Measures Adopted

Like other genetically engineered (GE) crops in the United States, GE apple is regulated by the Institutional Biosafety Committee (IBC), the Animal and Plant Health Inspection Service (APHIS) of USDA, the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA). There is no internationally accepted regulatory protocol for field trial or commercialization of apple.

The IBC is an internal responsibility of each research institution. It monitors potentially hazardous biological research and ensures compliance with biological safety procedures. APHIS must determine whether a GE plant cultivar is likely to become a pest, i.e., to have a negative agricultural or environmental effect. APHIS regulates the import, transportation, and field testing of GE plants through notification and permitting procedures. For the commercialization of GE plants, APHIS requires extensive data on the introduced gene construct, effects on plant biology, and effects on the ecosystem, including spread of the gene to crops or wild relatives. After the GE crop is on the market, APHIS has the authority to halt its sale, if there is evidence that the plant is becoming a pest. FDA determines the safety of foods or food ingredients. EPA regulates GE plants that are engineered for pest resistance.

A major concern for apple growers is that nonscience-based tactics would be used by nongovernment organizations (NGOs) to raise public concern about GE cultivars, and by extension about apples in general. Such concerns include the possible spread of antibiotic resistance, used for the selection of GE cultivars, to pathogens of humans and livestock; the use of genes from other organisms, especially animals; and the unstable insertion of the CaMV 35S promoter. The spread of antibiotic resistance persists as a concern despite a panel of international experts concluding that the probability of gene transfer to gut bacteria is extremely remote (WHO, Joint FAO/WHO Expert Consultation on Food Derived from Biotechnology; topic Gene Transfer: Mechanisms and Food Safety Risks, http://www.who.int/fst/Gmfood/consultation_May2000/biotech_00_14.pdf). To alleviate some of these concerns, techniques avoiding use of selectable markers during the process of transformation, or techniques eliminating such markers after transformation, have been developed and have begun to be used for the production of a new generation of GE crops.

The testing and release of GE organisms, especially GE plants, is tightly regulated internationally with the intention of preventing any negative effects on the environment or human health. However, these regulations are based on transgenic organisms and do not discriminate between transgenic plants and intragenic plants, although we believe that there are fundamental differences between them. Although transgenesis and intragenesis both use the same GE techniques namely the introduction of one or more genes and their promoters into a plant, intragenesis involves only genes from the species itself or from an interfertile relative. Such intragenes could be transferred by traditional breeding techniques. If the current GE regulations, which are mainly based on the process of transferring transgenes, continue to fail to differentiate between intragenic and transgenic plants, the use of intragenesis could be unintentionally hampered. Only Canada now has a product-based rather than a process-based regulation system, and therefore has the legal possibility to control intragenic plants less strictly than transgenic plants. Nielsen (2003) differentiated between intragenic and transgenic plants, and Schouten *et al.* (2006) argue that this

type of GE plant (termed cisgenic in their paper) should be treated differently under GE regulations.

In the case of an intragenic plant, the gene of interest has been present in the gene pool of the species and its interfertile relatives for evolutionary time periods. Therefore intragenesis does not alter the gene pool of the recipient species. An example is the introduction of the apple scab resistance gene *Vf* from a wild species into the domestic apple, which began as early as the 1950s (Schmidt and van de Weg, 2005). Despite more than 50 years of traditional breeding programs, the new apple cultivars carrying this gene have yet to acquire the same fruit quality in terms of taste and texture as the popular commercial susceptible cultivars, because of linkage drag. As the *Vf* gene has recently been cloned (Belfanti *et al.*, 2004; Malnoy *et al.*, 2007d), its transfer into elite cultivars using intragenesis should lead to more useful results in a considerably shorter time. However, whether this technique will develop into a powerful new tool strongly depends on several factors, including especially how intragenic plants are treated by existing legal frameworks, and the consumer acceptance of such products. Although consumer acceptance is beyond the control of lawmakers and regulators, it would be sensible to regulate intragenic plants differently than transgenic plants. Self-evidently, intragenic plants should still be tested to confirm that they contain only the intended changes and no foreign genes, such as a backbone gene from a plasmid.

3. FUTURE ROAD MAP

Much methodological progress has been made in the field of genetic engineering of apple in the last 10 years, and this has already resulted in production of new plant genetic material, which is currently being evaluated. However, progress is still needed in order to adapt techniques to a larger range of varieties and rootstocks, and to develop selection techniques, which avoid the need for antibiotic resistance transgenes. In the coming years, more diverse horticultural traits will certainly be amenable to genetic engineering, with increased knowledge of the genetic mechanisms underlying the traits of interest. The recent announcement of the funded project to sequence the apple genome will soon result in availability

of the sequence of many useful apple genes (R. Velasco, personal communication). However, before transformed apple can be commercialized, several important issues need to be addressed. Risk assessment studies must be thoroughly conducted to evaluate the potential toxicity or allergenicity of the transformed products and the environmental consequences of an eventual gene flow to natural populations of wild *Malus*. More research efforts must be devoted to search for regulatory sequences enabling targeted and controlled expression of the genes, and stability of expression of the gene throughout the tree life should be ensured. Practical use of transformed apple varieties will not be possible without general public acceptance.

One of the recurring themes of the debates concerning the application of genetic transformation technology has been the role of intellectual property rights (IPR). This term covers both the content of patents and the confidential expertise usually related to methodology and referred to as "Trade secrets" (Dunwell, 2005). Although few of the patents of this area have any real commercial value, there are small number of key patents that restrict the "freedom to operate" of new companies seeking to exploit the methods. Over the last 20 years, these restrictions have forced extensive cross-licensing between ag-biotech companies and have been one of the driving forces behind the consolidation of these companies, although, such issues are often considered of a little interest to the academic scientists working in the public sector. During the period since the production of the first transformed plants, a wide diversity of patents have been sought on all aspects of the process, ranging the underlying tissue culture methods through the means of introducing the heterologous DNA, and also the composition of the DNA construct so introduced (Kesan, 2000).

Recent advance that may circumvent the IPR limitation to *Agrobacterium* technology is the development of gene transfer techniques using other bacteria, such as *Sinorhizobium* (Broothaerts *et al.*, 2005). It is claimed that these methods may offer an open source alternative to the established transformation technologies.

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Pears

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1. INTRODUCTION

Pears are fruit trees belonging to the genus *Pyrus*, and are native to temperate Europe and to temperate and subtropical Asia. Pears are now cultivated in all temperate regions of the world. Modern cultivars of the European pear are considered by many people of western origin to be one of the most delectable of tree fruits, combining a buttery juicy texture with rich flavor and aroma. The pears of China, Korea, Japan, and elsewhere in eastern Asia are preferred there for their crisp texture and sweet flavor.

1.1 History, Origin, and Distribution

The genus *Pyrus* belongs to the apple subfamily *Maloideae* of the rose family *Rosaceae*, and contains eight widely recognized primary species, all indigenous to Europe, temperate Asia, and the mountainous areas of northwestern Africa (Westwood, 1982). The genus probably evolved during the Tertiary period in the foothills of the Tian Shan mountains in Xinjiang Province in western China, and spread eastward throughout temperate and subtropical Asia and westward throughout Europe, with isolation and adaptation leading to speciation. Vavilov (1951) identified

three centers of diversity for the genus: China, Central Asia, and the Near East/Asia Minor. The Near Eastern center, and specifically the Caucasus Mountains, is believed to be the center of origin of domesticated forms of the European pear, *Pyrus communis*.

The earliest written record of pear culture in Europe is that of Homer, who wrote in Greece about 1000 BC that pears were one of the “gifts of the gods” (Hedrick *et al.*, 1921). By the time of Theophrastus (371–286 BC), pear culture was well established in Greece and cultivars with distinct names were propagated by grafting and cuttings. Great strides in selection of the buttery fleshed cultivars, including “Beurré Bosc” and “Doyenné du Comice”, were made in the 18th century Belgium and France. Selection of edible fruit types from wild populations in China began about 3300 years ago, and pears have been cultivated in China for more than 2000 years.

1.2 Botanical Description

The pear genus (*Pyrus L.*) can be divided into two sections. The *Pashia* Koehne section includes the species of Eastern Asia and includes the small-fruited species of *P. armenicifolia* Yu, *P. betulifolia* Bunge, *P. calleryana* Decne, *P. kawakamii* Hayata,

and *P. xerophila* Yu, and the medium- to large-fruited *P. pyrifolia* Burm., *P. pashia* D. Don., and *P. ussuriensis* Maxim. There are several naturally occurring interspecific hybrid taxa, including *P. × bretschneideri* Rehd. (probably, a hybrid of *P. pyrifolia* and *P. ussuriensis*), which includes many important cultivars, and *P. sinkiangensis* Yu, a complex hybrid group of cultivars thought to be derived from *P. ussuriensis*, *P. × bretschneideri*, and *P. communis*. Section *Pyrus* includes the species of the secondary diversity centers of Central Asia, *P. salicifolia* Pall. and *P. regeli* Rehd., of the circum-Mediterranean, *P. amygdaliformis* Vill., *P. elaeagrifolia* Pall., *P. syrica* Boiss., and *P. cossonii* Redh., and of Europe, *P. caucasica* Fed., *P. nivalis* Jacq., and *P. pyrastrer* Burgsd., and the cultivated species, *P. communis* L.

The plants of all species are trees or large shrubs, and are deciduous except for *P. kawakamii*, which is semi-evergreen and is native to Taiwan and Southeast Asia. Climatic and edaphic adaptation is wide, with most species occurring naturally in temperate mixed hardwood forests, at altitudes up to 2200 m above sea level. Tree habit can be strongly upright to spreading, reaching a height of more than 24 m and trunk diameter of 1 m or more. The pome fruits vary considerably in size, but have juicy flesh, with variable amounts of stone cells, especially in the East Asian species. Fruit shape varies from obovoid or subglobose to pyriform in *P. communis*, *P. × bretschneideri*, and *P. sinkiangensis* Yu, obovoid or round in *P. pyrifolia* and *P. ussuriensis*, to round in *P. calleryana*, and *P. betulifolia*. Fruit skin color in *P. communis*, *P. pyrifolia*, and *P. × bretschneideri* can be yellow to light green at maturity, or covered with a tan to brown russet. Flesh texture of the latter two Asian species is crisp, while that of *P. ussuriensis* and *P. sinkiangensis* tends to be soft. Most existing cultivars of *P. communis* have melting, buttery flesh, but cooking cultivars with hard, coarse texture exist and, in fact, were more common prior to the 1700s.

The genus has a basic chromosome number of 17, and may have arisen as an amphidiploid of two primitive forms of the *Prunoideae* ($x = 8$) and *Spiraeoideae* ($x = 9$) (Bell *et al.*, 1996). All species of *Pyrus* that have been examined are diploid ($2n = 34$, $x = 17$), but occasional triploids such as “Beurre Amanlis”, “Cure”, “Minister Dr. Lucas” and “Buerre Bedford” and tetraploids

such as “Parberton Bartlett”, “Double Williams”, “Improved Fertility”, “Large Winter Nelis”, and “Trevu Tetra” can be found. Diploid DNA content is approximately 0.55–0.63 pg (picogram), depending on species (Arumuganathan and Earle, 1991; Dickson *et al.*, 1992), with total map length estimated at approximately 950 cM (Yamamoto *et al.*, 2002). The chloroplast DNA is circular and 156 kb in length (Katayama and Uematsu, 2003).

1.3 Economic Importance

Pears rank second to apples in the amount of worldwide production of deciduous tree fruit species. Pears are grown in 63 countries throughout the temperate and subtropical regions of the world. Cultivars of *P. communis* are the main edible pears in Europe, the Middle East, western Asia, North America, and mountainous areas of Central America. The snow pear, *P. nivalis* Jacq., is also grown to a limited extent in France and the United Kingdom for making the alcoholic beverage called perry. In Asia, *P. pyrifolia* is the main cultivated species in central and southern China, Korea, Japan, Taiwan, and other countries of Southeast Asia. In northern China, *P. ussuriensis*, *P. × bretschneideri*, and *P. sinkiangensis* are grown. Selections of *P. pashia* are cultivated in southern China and South Asia. Hybrids of *P. pyrifolia* and *P. communis* are grown in southern temperate and subtropical regions of North and South America, and in Egypt where chilling hours to overcome bud dormancy fall below 800 h. Species used for ornamental purposes include the small-fruited *P. calleryana* Decne., *P. fauriei* Schneid., *P. betulifolia* Bunge, *P. salicifolia* Pall., and *P. kawakamii* Hayata. Seedlings and clonal selections of *P. communis* are generally used as rootstocks for European pear scion cultivars, while *P. betulifolia*, *P. calleryana*, *P. pyrifolia*, *P. ussuriensis*, and *P. × bretschneideri*, are used as rootstocks in Asia, and *P. pyrastrer* Burgsd., *P. amygdaliformis* Vill., and *P. elaeagrifolia* Pall. are occasionally used in Asia Minor and Central Asia (Lombard and Westwood, 1987). Quince (*Cydonia oblonga* L.) is also used as a rootstock for European pears in regions where lack of cold hardiness is not a limiting factor.

The remarkable increase in world pear production from 1990 to 2005 was mainly due to the growth of area harvested which increased

from 1.1 million ha to 1.9 million ha, with much of the reported increase due to China, now at 1.2 million ha. In Europe, there was a strong expansion in pear growing in the 1980s, followed by a small reduction to 220 000 ha. The other major producing regions include South Africa (46 000 ha), North America (40 000 ha), South America (38 000 ha), and Oceania (10 000 ha).

Production in all countries in 2005 totaled 19.5 million metric tons (mt) (FAOSTAT, 2007) with an annual growth rate of nearly 5% in the last decade. More than 65% of the world production is concentrated in Asia, with Southern Europe ranking second with a 12% share of total world production. China produced some 11.5 mt. The rate of change in production in China from 1990 to 2005 was 300%. Other major producing countries were Italy (926 540 mt), the United States (747 250 mt), Spain (671 000 mt), Argentina (535 420 mt), and Turkey (450 000 mt). In Europe, pear production totals ca. 3.5 mt, with 87% of this production in six countries: Italy 28%, Spain 22%, Germany 14%, France 9%, Belgium 8% and The Netherlands 6% (USDA/FAS, 2006).

Economic usage of *Pyrus* species has been reviewed by Bell *et al.* (1996). Most of the fruit crop is utilized as fresh fruit, but significant amounts are processed and marketed as canned pears, dried fruit, puree, blended juices, or pure nectar, and alcoholic beverages such as perry, and still and sparkling pear wine. As noted, several east and mid-Asian species, especially the callery pear, *P. calleryana*, are widely used as ornamentals.

On the basis of 100 g of the edible portion, including skin, European pears provide 58 kcal of energy (USDA, 2004). They consist of 78–86% water, 1.6–3.6% crude cellulose, 0.4 g of protein, 0.12 g fat, 15.5 g of total carbohydrate, 9.8 g of total sugars, two-thirds of which are fructose, and 3.1 g of fiber. Of the major mineral nutrients, there are 8 mg of calcium, 11 mg of phosphorus, 0.3 g of iron, 2 mg of sodium, and 130 mg of potassium. There are 20 IU of vitamin A, 0.02 mg of thiamine, 0.04 mg of riboflavin, 0.1 mg of niacin, and 4 mg of ascorbic acid (vitamin C). Of the major temperate tree fruits, pears are highest in fiber, average to low in mineral elements, and lowest in vitamin A. For the remaining vitamins, pears rank low to average. Values for *P. pyrifolia* are similar. The oxygen radical absorbance capacity of pears ranks just below tomatoes, less than 10% of the amount

in strawberry, and is also only moderate for the total phenol antioxidant index.

1.4 Traditional Breeding

Pear culture in Europe dates from ancient Greece and Rome, and led to a very great diversity of local cultivars throughout Europe. European pear breeding reached its “golden age” between 1750 and 1850 in Belgium, France, and England (Bell *et al.*, 1996). Selection was performed among open-pollinated seedlings of common cultivars of *P. communis*. Several cultivars released during this period still constitute the basis of the current European pear production: “Conference” (England, 1885), “Williams Bon Chrétien” (England, 1796), “Abbé Fétel” (France, 1866), “Docteur Jules Guyot” (France, 1870), “Doyenné du Comice” (France, 1849) (Doré and Varoquaux, 2006). Controlled hybridization was probably used by pear breeders since the second half of the 19th century. Documents from the Brno monastery indicate that Gregor Mendel himself studied pear taste and ripening time, and performed controlled hybridization between several pear varieties (Vavra and Orel, 1971).

In Asia, the consumption of European pears is low, and most of the pear production is from Asiatic species (*P. pyrifolia*, *P. ussuriensis*, *P. × bretschneideri*) and their hybrids. Until the 20th century, most of the cultivars grown in this part of the world were chance seedlings. Pear breeding programs in Asia have been recorded since 1915 in Japan, 1920 in Korea, and 1956 in China (Bell *et al.*, 1996).

1.4.1 Modern breeding objectives

Pear trees are grown on rootstocks, belonging to the genera *Pyrus* and *Cydonia*, and different breeding aims are documented for scion and rootstock cultivars. There are numerous objectives of European and Asian pear scion genetic improvement. Until the middle of the 20th century, breeding was mainly focused on improving tree vigor, productivity, and fruit appearance. However, increasing importance of economics in fruit production, evolution of marketing, and necessity of environmentally friendly production systems

extended breeding objectives to the characteristics including tolerance or resistance to pest and diseases, adaptability to environmental factors, extension of the harvesting period, extension of storage and shelf life, and taste and nutritional characteristics (Bellini and Nin, 2002).

Resistance to pests and diseases is a major objective of most pear breeding programs in Europe and northern America. Fire blight, caused by the bacterium *Erwinia amylovora*, is considered the most devastating disease in pear. Two important breeding programs have already released tolerant or resistant varieties from Harrow (Canada) and Kearneysville (USA) (Lespinasse and Aldwinckle, 2000). Resistance to pear psylla (*Cacopsylla pyricola*), which is the insect vector of pear decline phytoplasma, pear scab (*Venturia pirina*), and powdery mildew (*Podosphaera leucotricha*), is also sought in European pears. Resistance to black spot caused by *Alternaria alternata* and resistance to pear scab caused by *Venturia nashicola* are important for Asian pear breeding. Adaptability to environmental factors is focused mostly on cold hardiness in northern parts of Europe, Asia, and America, but also includes adaptation to hot and humid tropical climates in countries such as India and Taiwan. Growth habit modification goals are generally toward compact size and rapid cropping. In France, particular attention is given to tree bearing and branching to facilitate a modern orchard management system with reduced labor cost (Costes *et al.*, 2004). Many pear scion breeding programs share the common aim of extension of the harvest season, either for early ripening pear or for late ripening “winter” pear with long storage ability. Improvement of fruit quality is a complex objective, which includes fruit size, appearance, flesh quality, storage ability, nutritional value, and adaptation to various industrial uses (canning, baby food). Attributes of good quality differ between European and Asian pears, and some breeding programs now attempt to create completely new pear types by hybridizing between European and Asian pears (White and Brewer, 2002).

European pears are grown on clonal rootstocks belonging either to *P. communis* or to *C. oblonga* (quince rootstocks). *P. communis* rootstocks generally exhibit good graft compatibility, tolerance to fire blight and iron chlorosis, and average fruit size and ability to propagate, but they are often

too vigorous for intensive pear orchards. Quince rootstocks confer dwarfing-induced vigor, good fruit size, and good ability to propagate but they can lead to graft incompatibility and susceptibility to iron chlorosis. Asian pears are grown on seedlings of *P. communis*, *P. pyrifolia*, *P. pashia*, *P. calleryana*, *P. ussuriensis*, and *P. betulaefolia* (Wertheim, 1998). The principal goal of pear rootstock breeding today is to combine dwarfing-induced vigor with good compatibility and ability to propagate. Traits of particular importance for pear rootstocks also include tolerance to drought, waterlogging, and lime-induced iron chlorosis (Wertheim and Webster, 2005). Incompatibility of many pear scion cultivars with quince rootstocks led modern pear rootstock breeding programs to investigate the use of *Pyrus* species. One of the main drawbacks in developing *Pyrus* rootstocks is the rare coincidence of dwarfing potential and ease of propagation (Bell *et al.*, 1996).

1.4.2 Tools and strategies

Fruit tree breeding methods generally involve three major steps: creation of genetic variation, selection of elite material, and extended trials of promising preselections before market release (Schmidt and van de Weg, 2005). Crossing is still by far the most widely used method of creation of genetic variability for pear. The genetic pool available for the pear breeder is wide. Most species of *Pyrus* are diploid ($2n = 34$; $x = 17$) and no significant interspecific cross-incompatibility is known to exist. Crossing programs rely on the availability of appropriate genetic resources, which can comprise recent breeding selections, old and new cultivars, and various species of the genus *Pyrus*.

Mutation induction, although efficient for some characteristics such as skin color, compact growth, or tetraploidy, has never played a major role in pear breeding. The occurrence of chimera, which is often unstable, is one of the major obstacles to the use of spontaneous or induced mutations for pear breeding (Chevreau *et al.*, 1989a, b). Recently, *in vitro* systems for pear mutation breeding have been developed to decrease the risk of obtaining chimeric plants, either by using adventitious regeneration or by applying rapid cycles of micropropagation to separate

mutated from nonmutated sectors (Predieri and Zimmerman, 2001).

The contribution of *in vitro* methods to create novel genetic variability in pear has advanced considerably during the two last decades. Haploidization via *in situ* parthenogenesis induced by irradiated pollen and *in vitro* rescue of the haploid plantlets have been successfully developed for pear (Bouvier *et al.*, 1993). Techniques of adventitious bud regeneration from *in vitro* leaves have been developed for several genotypes of European and Asian pears and for quince. So far, applications of these techniques for the induction of somaclonal variation have been very limited. The occurrence of somaclonal variation has been demonstrated for fire blight resistance and iron-chlorosis tolerance, albeit at low frequency (Chevreau and Bell, 2005). Protoplast technology has also been applied to pear since 1986 by Ochatt and Caso (1986). However, somatic hybridization between *Pyrus* and an incompatible genus, *Prunus*, has been reported only once (Ochatt *et al.*, 1989) and has not been confirmed.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

1.5.1 Conventional breeding accomplishments

Many new European pear scion cultivars have been developed from hybridization programs and released within the last 20 years with major improvements in the quality, storage, and shelf life of early season pears, as well as in late season pears. Important progress has also been made in the field of disease resistance, particularly concerning fire blight. Countries with the highest number of pear scion releases since 1990 have been the United States, Germany, France, Russia, and Italy. However, very few of these novel cultivars have the potential to replace the classical pear cultivars whose agronomic and commercial limits are already well known and which have gained a solid market share (Bellini and Nin, 2002). Successes of induced mutagenesis for pear improvement are still limited. Three mutants of Asian pear (“Gold Nijisseiki”, “Kotobuki Shinsui” and “Osa Gold”) have been released. They were obtained after chronic γ -irradiation of plants and were selected

for increased resistance to black spot disease, caused by *A. alternata* (Yoshioka *et al.*, 1998).

Rootstock breeding programs are considerably longer term than scion breeding programs, because no reliable laboratory technique is available so far to preselect for vigor induction and compatibility before testing rootstock/scion combinations in the orchard. Despite hybridization programs in several countries and release of several new rootstocks, there is still a lack of compatible dwarfing pear rootstocks of *Pyrus* type, combining a good propagation ability with disease resistance, and adaptation to difficult environmental conditions (cold, drought, or lime-induced chlorosis) (Wertheim, 2002).

1.5.2 Progress toward marker-assisted selection

One of the main limiting steps toward pear genetic improvement by hybridization is the length of the juvenile period. The majority of pear seedlings flower between the 5th and the 7th year after crossing. No reliable correlation is known between fruit characteristics and vegetative traits. Apart from a few disease resistance traits from which preselection through seedling inoculation is efficient (scab, fire blight, psylla), selection can only start when the trees start cropping. In conventional pear breeding, only one to five seedlings are retained per 1000 per generation, therefore the cost of orchard management for pear hybrid selection is very high.

Important efforts have been devoted to the development of various types of molecular markers for pear. These include isozymes, restriction fragment length polymorphism (RFLP) markers of mitochondrial DNA, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and selectively amplified microsatellite polymorphic loci (SAMPL). But so far they have been used mostly for cultivar identification or phylogenetic studies. Integration of molecular markers into pear genetic studies and breeding requires additional knowledge about the linkage between molecular markers and the desired traits. Three traits have been linked to molecular markers in Asian pears: a major gene for resistance to black spot disease, the *S*

locus of self-incompatibility, and two ACC (1-Aminocyclopropane-1-carboxylate) synthase genes (Chevreau and Bell, 2005). Recently, genetic linkage maps of both European and Asian pears have been constructed based on an interspecific cross (Yamamoto *et al.*, 2002). Position of the resistance allele for pear scab (*Vn*) and the susceptible allele for black spot (*A*) were mapped in different linkage groups in Asian pear (Iketani *et al.*, 2001), as well as the first quantitative trait loci (QTLs) for fire blight resistance in European pear (Dondini *et al.*, 2004). The high degree of conservation between apple and pear genomes should also speed up the localization of important agronomical traits. Recently, the pear scab resistance gene, *Vnk*, from the Japanese pear “Kinchaku” was located on the same linkage group as the apple scab resistance gene *Vf*, but in a different genomic region (Terakami *et al.*, 2006). Despite this progress, marker-assisted selection (MAS) will take place only very gradually in the pear hybrid selection process.

1.5.3 Rationale for transgenic pear breeding

Marketing of pears both within and among nations is characterized by a ceiling on the number of cultivars. In Europe, less than 10 cultivars constitute 75% of the pear production (Deckers and Schoofs, 2005). Over 80% of the North American pear production can be accounted for by three cultivars (Seavert, 2005). In China, three cultivars account for about 65% of the national production (Saito *et al.*, 2005). Both producers and consumers prefer the old traditional cultivars, which are unique and clearly recognized. Therefore, acceptance of new hybrid cultivars is very slow.

In this context, gene transfer offers pear breeders new tools to directly improve existing elite cultivars without changing their main recognizable characteristics. An improvement in any of these few important cultivars should necessarily have a significant impact on production. However, one should not imagine that gene transfer is a simple process that will speed up the selection of new cultivars. After the selection of a promising transgenic clone, much time is needed to test the stability of the expression of the transgene

and of the associated desired new trait, to check the maintenance of the other traits of the original cultivar, and to establish the safety of the fruits produced. Also at the moment, the European public does not seem to accept genetically engineered (GE) processed food, and might be even more reluctant to accept GE fresh products, such as fruits. However, acceptance of transgenic pear cultivars may be easier in other parts of the world, especially the United States and Canada. GE pear rootstocks should be much more easily commercialized, as the risk of dissemination of the transgene through pollen flow is absent, as well as the concerns about food safety.

Even though the direct use of gene transfer for pear breeding seems a long-term goal, the various possibilities offered by gene transfer to overexpress or silence a precise gene constitutes a unique tool for the progress of genetic knowledge of this species. With the increased speed of gene discovery in fruit species, gene transfer will become a necessary tool to demonstrate the function of these genes. Finally, combination of conventional breeding aided by molecular markers and transformation techniques to alter expression of *Pyrus* genes rather than using genes from other organisms will also offer a very attractive approach.

2. DEVELOPMENT OF TRANSGENIC PEARS

2.1 Development of a Pear Transformation Procedure

Development of an effective system for gene transfer in pear depends largely on the availability of tissue culture techniques that permit efficient DNA delivery, regeneration of shoots, selection of transformants, and propagation of transformed plants. In many instances, the lack of efficient regeneration systems is the major limiting factor preventing the development of gene transfer technologies for perennial crops (Dandekar, 1992).

Development of a genetic manipulation system for the production of transformed fruit trees greatly depends on the establishment of a reliable and effective regeneration and transformation system. Adventitious shoot formation from leaf

explants has been reported for a limited number of mostly commercial genotypes, including *P. communis* cultivars (Chevreau *et al.*, 1989b, 1997; Chevreau and Leblay, 1993; Predieri *et al.*, 1989; Abu-Qaoud *et al.*, 1991; Leblay *et al.*, 1991), *P. pyrifolia* cultivars (Lane *et al.*, 1998), *P. syriaca* Boiss. genotypes (Shibli *et al.*, 2000), wild pear *P. communis* var. *pyraster* L. (Caboni *et al.*, 1999), and quince rootstocks (Dolcet-Sanjuan *et al.*, 1991; Baker and Bhatia, 1993). In all cases, leaves from *in vitro* grown plants have been used as explants and have been exposed to a sequence of dark (2–4 weeks) and light exposure. The optimum culture conditions for adventitious regeneration are summarized in Table 1. These techniques involve a very limited callus phase at the site of wounding, and bud regeneration occurs in 3–6 weeks.

Although sufficient regeneration levels were achieved in some of these genotypes, the regeneration of transformed plants remains a difficult task and seems to be strongly genotype dependent.

The first successful gene transfer in the pear was in 1996 (Mourgues *et al.*, 1996) some years after the first transformation in the apple (James *et al.*, 1989), and to date, several transgenes (*attacin*, *rolC*, and *rolB*) have been introduced into pear cultivars such as Passe Crassane (Reynoird *et al.*, 1999a), Beurre Bosc (Bell *et al.*, 1999), and *Pyrus* rootstocks. Although transformation of several pear cultivars has been reported (Table 2), the transformation rates were generally 1–4% with the exception of an impressive efficiency of up to 42% in Conference and up to 59% in Burakovka (Lebedev *et al.*, 2002a, b). In most of these cases, obtaining transformed shoots took at least 6 months, whereas Gao *et al.* (2002) obtained transformed shoots within 1.5 months due to the use of juvenile explants. Using cotyledons, the authors concluded that transformants of woody fruit trees can be produced quickly when no specific cultivar is considered. The method of Gao *et al.* (2002) involved two pieces of cotyledon from the same seed that were treated individually to produce transformed and nontransformed plants, thereby overcoming the disadvantage of transformants originating from different cotyledons that would differ both genetically and physiologically, because individual cotyledons are genetically heterogeneous. However, this technique does not maintain the genetic background of the

transformed cultivars or rootstock and has limited potential for pear breeding.

The techniques of transformation used for these different pear genotypes are quite similar (Table 2). Due to the fact that all genotypes do not respond similarly to the same procedure of transformation, some variations have been reported.

- *Nature of the explants.* In 75% of the procedures published, young expanded pear leaves were used as explants. The regeneration of transformed lines from leaves has proved to be enhanced by placing the leaf segment abaxial side up on the medium, possibly due to increased oxygen exchange since stomata are located abaxially. The other explants used are the cotyledon, internode, and axillary shoot meristem.
- *Inoculation procedures.* Two inoculation procedures have been used. The first consists of wounding the leaves with a scalpel dipped in the inoculum (Mourgues *et al.*, 1996). The second is to bathe leaves in the inoculum for a few minutes (Gao *et al.*, 2002).
- *Strains of *Agrobacterium tumefaciens* used.* 65% of the pear transformation procedures were based on use of *A. tumefaciens* EHA compared to *A. tumefaciens* strain A281 (5%) (Merkulov *et al.*, 1998), C58C1 (15%), CBE21 (5%) (Lebedev *et al.*, 2002a, b), or AK10 (5%) (Kaneyoshi *et al.*, 2001) for the T-DNA integration.
- *The selectable marker used.* In 95% of cases, kanamycin has been used as selectable marker. Concentration of kanamycin used for the regeneration of transgenic apple varied according to the cultivar. Another antibiotic tested as a selectable marker was hygromycin (Lebedev *et al.*, 2002a, b).
- *Other modifications.* Other modifications have been made to these procedures to increase the transformation efficiency. These modifications included the use of a plant phenolic compound (acetosyringone) to induce the expression of several virulence genes in *A. tumefaciens*, the gelling agent, the growth regulators (Table 1), the source of carbon (sucrose or sorbitol), the binary vector, and the antibiotics used (cefotaxime, timentin).

Although some modifications to the initial pear transformation procedure of Mourgues *et al.*

Table 1 Adventitious regeneration from adult pear and quince explants

Genotype	Mineral medium	Growth regulators	Other conditions tested	Maximal rate of regeneration	References
<i>Pyrus communis</i>					
Seckel	Nitsch and Nitsch	TDZ 3 μM + NAA 5.4 μM	Mother plant on BA (2.2 μM) Leaves from apical part of the shoot	42–74%	Abu-Qaoud <i>et al.</i> , 1991
Louise Bonne	Modified Lepoivre	TDZ 2.5 μM + NAA 1 μM		90%	Chevreau and Leblay, 1993
Passe Crassane					
Doyenné du Comice					
Passe Crassane	Modified Lepoivre	TDZ 2.5 μM + NAA 1 μM	Agar/Phytigel Cefotaxim + timentin kanamycin	47–95%	Chevreau <i>et al.</i> , 1997
Doyenné du Comice					
Conférence					
Seckel					
Williams					
Old Home					
OHF 333					
Harrow Sweet					
La France	MS	TDZ 30 μM + NAA 2 μM	Cotyledon	40% 75%	Gao <i>et al.</i> , 2002
Bartlett		TDZ 30 μM + NAA 8 μM			
La France	Nitsch and Nitsch	TDZ 5 mg l ⁻¹ NAA 0.2 mg l ⁻¹	Sucrose 35 Gellangum 0.4% Leaf placed abaxial site down	17–77%	Hennayake <i>et al.</i> , 2003
Max Red Bartlett					
Winter Nelis					
Conférence					
Deveo					
Seigneur d'Esperin					
Précoce					
Doyenné du Comice					
Spadona	AP	TDZ 5 mg l ⁻¹ + NAA 0.2 mg l ⁻¹	Sorbitol 3% Sucrose 1.5% Adenine hemisulfate 160 mg l ⁻¹	80%	Yancheva <i>et al.</i> , 2006
Silver Bell	Nitsch and Nitsch	TDZ 5 mg l ⁻¹ + NAA 0.2 mg l ⁻¹	Sucrose 3% Sulbenicillin	Nd	Matsuda <i>et al.</i> , 2005
La France	Quoirin and Lepoivre	BA 8.8 μM + NAA 1 μM	Leaves wounded by 3 cuts	40–64%	Caboni <i>et al.</i> , 1999
Var pyraister					
Burakovka	Quoirin and Lepoivre	BA 2 mg l ⁻¹ , IBA 0.2 mg l ⁻¹ , Ga ₃ 0.3 mg l ⁻¹	Leaves adaxial side down	30–36% 13–27%	Lebedev <i>et al.</i> , 2002c
Pamyat'Yakovleva					
GP217			Explant placed in liquid regeneration media for 40–50 min	30–5%	
BP10030	Modified Lepoivre	TDZ 15 μM + NAA 1 μM	Used sorbitol instead of sucrose for OHF333	98% 66%	Zhu and Welander, 2000
OHF333		TDZ 1 μM + NAA 1 μM			
<i>Pyrus pyrifolia</i>					
Chojuro	B5 (Gamborg)	TDZ 1–5 μM + GA ₃ 0.25 μM	Older leaves Incubation in dark	<20%	Lane <i>et al.</i> , 1998
Kosui					
Hosui					
Nijisseiki					
Shinchu					
Okusankichi					

Table 1 (Continued)

Genotype	Mineral medium	Growth regulators	Other conditions tested	Maximal rate of regeneration	References
Housui Kousui Shinsei	$\frac{1}{2}$ MS	TDZ 5 mg l ⁻¹ NAA 0.2 mg l ⁻¹	Sucrose 35 Gellangum 0.4% Leaf placed abaxial site down	20–57%	Hennayake <i>et al.</i> , 2003
<i>Pyrus syriaca</i> Endemic genotype	MS	TDZ 2 µM	Sucrose 0.15 M Leaves of intermediate age	76%	Shibli <i>et al.</i> , 2000
<i>Pyrus betulaefolia</i> Bunge	$\frac{1}{2}$ MS	BA 5 mg l ⁻¹ + NAA 20 mg l ⁻¹	Cotyledon Cefotaxime	75%	Kaneyoshi <i>et al.</i> , 2001
<i>Cydonia oblonga</i> East Malling A	MS N ⁺	TDZ 32 µM + NAA 0.3 µM		78%	Dolcet-Sanjuan <i>et al.</i> , 1991
	MS	TDZ 1.5 µM + NAA 2.5 µM	3 weeks of dark incubation Sucrose 3% Leaves abaxial side down	85%	Baker and Bhatia, 1993

(1996) have been developed for each pear cultivar, the general structure of the procedure is the same (Figure 1).

2.2 Genetic Engineering in Pear

2.2.1 Disease resistance

2.2.1.1 Fire blight

The most important disease of pear is fire blight, a disease of American origin. It was first reported in the Hudson Valley of New York in 1784. It has been known as the most destructive bacterial disease of the *Maloideae* (pear, apple, and quince) and other members of the Rosaceae family for over 200 years and in fact is the first plant disease proved to be of bacterial origin (Norelli *et al.*, 2003). This disease is caused by the necrogenic endobacterium *E. amylovora*, which is capable of infecting blossoms, fruits, vegetative shoots, woody tissues, and rootstock crowns. Upon entering plants through natural openings or wounds, the bacterium causes necrosis that develops quickly along the shoots to the main branches, producing the characteristic symptom of this disease: a necrotic shoot blight

that can kill a tree in one season (Thomson, 2000). In nonhost plants, such as *Arabidopsis thaliana* or tobacco, *E. amylovora* elicits a hypersensitive response (HR) characterized by a rapid and localized collapse of tissues.

E. amylovora, is a gram negative bacterium and its anatomy, physiology, and serology have been well described (Paulin, 2000). Twenty years ago, factors involved in its pathogenicity and virulence have been identified. Transposon mutagenesis led to the identification of three key groups of genes responsible for pathogenicity: (1) The cluster of *dsp* genes (disease specific gene, Boucher *et al.*, 1987) is strictly required for pathogenicity (Gaudriault *et al.*, 1997; Bogdanov *et al.*, 1998b). Two *dsp* genes were isolated, *DspA* or *E*, which encodes a pathogenicity factor homologous to the *Avr E* gene of *Pseudomonas syringae*. The *Dsp B* or *F* gene encodes a protein similar to a chaperone protein of pathogenic bacteria such as *Yersinia* and *Shigella* (Gaudriault *et al.*, 1997; Bogdanov *et al.*, 1998a, b); (2) The *ams* gene (amylovoran synthesis gene) is required for synthesis of extracellular polysaccharides (EPS) (Belleman and Geider, 1992); (3) The *hrp* (hypersensitive reaction and pathogenicity gene) cluster is necessary for pathogenicity on host plants and HR elicitation on nonhost plants

Table 2 Genetically transformed pear cultivars

	Explants used	Inoculation procedure	<i>Agrobacterium</i> strains	Transgenes	Selectable marker	Efficiency of transformation	References
<i>Pyrus communis</i>							
<i>Cultivars</i>							
Ballade	Leaf	Scalpel cut + immersion for 10–15 min	EHA 101	<i>FT</i>	Nd	0.9%	Matsuda <i>et al.</i> , 2006
Bartlett	Cotyledon	Immersion for 15 min	EHA 101	<i>ACC Oxidase</i>	Kanamycin 50 mg l ⁻¹	2.7% 5.2%	Gao <i>et al.</i> , 2002
Beurre Bosc	Leaf	Nd	Nd	<i>Gus</i>	Kanamycin increasing from 15 mg l ⁻¹ to 50 mg l ⁻¹	0.2–6.3%	Bommineni <i>et al.</i> , 2000
	Internode	Nd	Nd	Nd	Kanamycin 80 mg l ⁻¹ for 4 weeks only	1.4–44%	Bell <i>et al.</i> , 1999
Burakovka-	Leaf	Scalpel cut	EHA 101	<i>Gus</i>	Kanamycin 100 mg l ⁻¹	12–42%	Mourgues <i>et al.</i> , 1996
	Leaf	Scalpel cut	EHA 101	<i>Gus</i>	Kanamycin 100 mg l ⁻¹	12–42%	Mourgues <i>et al.</i> , 1996
Conférence Doyenné du Comice	Leaf	Scalpel cut + immersion for 40–50 min	CBE21	<i>roIC</i>	Kanamycin 25 mg l ⁻¹	0.5%	Gao <i>et al.</i> , 2002
	Leaf	Scalpel cut	EHA 101	<i>Defensin Thaumatin II</i>	Kanamycin 100 mg l ⁻¹	1.3%	Matsuda <i>et al.</i> , 2005, 2006
La France	Cotyledon	Immersion for 15 min	EHA 101	<i>ACC Oxidase</i>	Kanamycin 50 mg l ⁻¹	2.7% 5.2%	Mourgues <i>et al.</i> , 1996
	Auxillary shoot meristem	Scalpel cut + immersion for 10–15 min	EHA 101	<i>Gus</i>	Kanamycin 30 mg l ⁻¹	1–3.2%	Matsuda <i>et al.</i> , 2005, 2006
Passe Crassane Silver Bell	Leaf	Scalpel cut	EHA 101	<i>Gus</i>	Kanamycin 100 mg l ⁻¹	12–1.3%	Mourgues <i>et al.</i> , 1996
	Leaf	Scalpel cut + immersion for 10–15 min	EHA 101	<i>Gus</i>	Kanamycin 30 mg l ⁻¹	3.2%	Matsuda <i>et al.</i> , 2005
Spadona	Leaf	Scalpel cut + immersion for 20 min	EHA 105	<i>Gus</i>	Kanamycin 5 mg l ⁻¹	0.3–0.8%	Yancheva <i>et al.</i> , 2006
Vyzhinta	Leaf	Immersion	A281	<i>GFP</i>	Kanamycin 50 mg l ⁻¹	3–4%	Merkulov <i>et al.</i> , 1998
Rootstocks BP10030	Leaf	Wounded with forceps + immersion	C58C1	<i>Gus</i>	Kanamycin decreasing from 100 mg l ⁻¹ to 50 mg l ⁻¹	0.33–1%	Zhu and Welandar, 2000
	Leaf	Wounded with forceps + immersion	C58C1	<i>Gus</i>	Kanamycin decreasing from 100 mg l ⁻¹ to 50 mg l ⁻¹	0.33–1%	Zhu and Welandar, 2000
OHF333	Leaf	Immersion	EHA 105	<i>Gus</i>	Kanamycin 25 mg l ⁻¹	0.4–3.1%	Lebedev and Dolgov, 2002
GP217	Leaf	Scalpel cuts + 2 drops of inoculum added on the leaves	EHA 105	<i>Gus</i>	Hygromycin 5 mg l ⁻¹	6.2–11.5%	Lebedev and Dolgov, 2002
<i>P. pyraister</i>	Cotyledon	Immersion	AKE10	<i>Gus</i>	Kanamycin 100 mg l ⁻¹	Nd	Caboni <i>et al.</i> , 2002
<i>P. betulaefolia</i> Bunge	Cotyledon	Immersion	AKE10	<i>Gus</i>	Kanamycin 20–50 mg l ⁻¹	0.9–2.5%	Kaneyoshi <i>et al.</i> , 2001

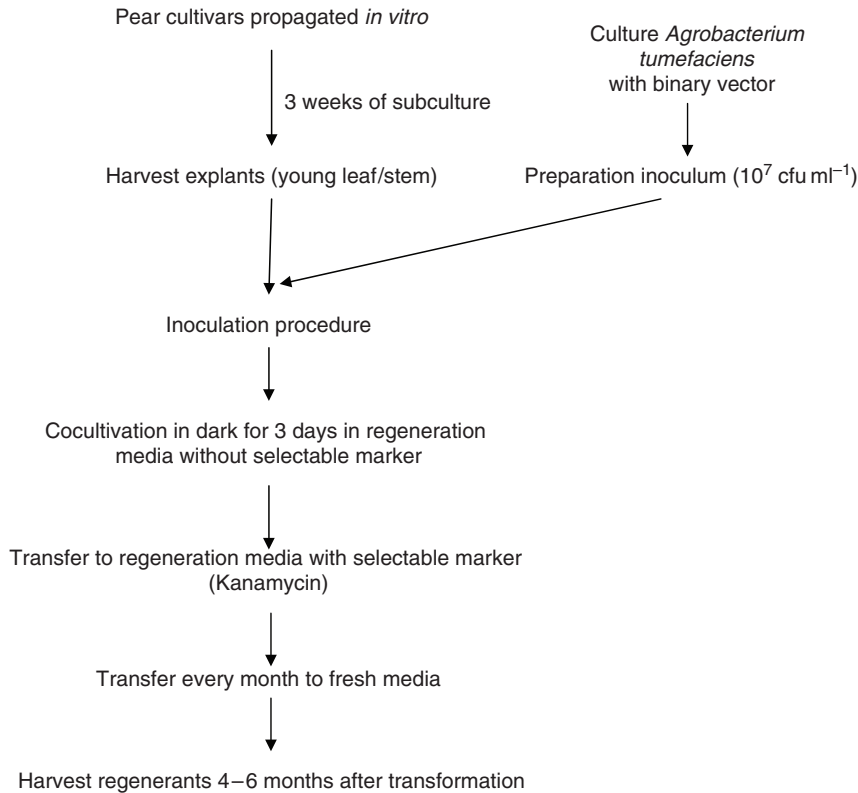


Figure 1 Representation of the pear transformation procedure

(Barney *et al.*, 1990; Kim and Beer, 2000). The *hrp* gene cluster has been characterized in several other pathogenic bacteria such as *Xanthomonas* and *Ralstonia* (Lindgren, 1997). This cluster encodes components of a Type III secretion pathway (Hueck, 1998), and regulatory and secreted proteins. Today, at least seven proteins of *E. amylovora* are known to be secreted via the Hrp pathway: DspA/E, OrfB, HrpW, HrpA, HrcQ, HrpJ, and HrpN_{Ea} (Kim and Beer, 2000). Harpin N_{Ea} was the first secreted protein characterized by molecular analysis (Wei *et al.*, 1992). This protein is a 44-kDa glycine-rich protein, which lacks cysteine, is heat stable, and is able to induce an HR when purified and infiltrated into tobacco leaves. Besides three key pathogenicity factors, *E. amylovora* produces a siderophore belonging to the desferrioxamine family, which acts as virulence factor (Dellagi *et al.*, 1998).

The economic impact of fire blight is difficult to determine, as losses are rarely recorded when

they are low (a few flower clusters on a few trees killed within a season) and a single outbreak can disrupt orchard production for several years. However, in recent years, fire blight has caused serious losses around the world (Norelli *et al.*, 2003). While outbreaks are sporadic in occurrence, they often result in significant losses when they do occur, in terms of trees damaged and killed, loss of millions of dollars (Aldwinckle *et al.*, 2003). In 2000, southwest Michigan experienced a severe fire blight epidemic and reported a \$42 million economic loss for the region that resulted in the removal of 300 000 apple trees covering approximately 2000 acres. Losses and costs in many other states and countries, such as New Zealand, Italy, and Washington State in 1998 were also very substantial.

The strategies of ribosomal DNA (rDNA) technology to improve the resistance to fire blight are aimed at producing incompatible interactions between the plant and the pathogen, by means

of restricting the multiplication of the pathogen in the plant after infection. These outcomes can be obtained in different ways: (1) production of antimicrobial proteins; (2) inhibition of bacterial pathogenicity factors; and (3) enhancement of natural plant defense.

Production of antimicrobial protein Antibacterial proteins are important components of the overall antimicrobial defense mechanisms of many groups of animals, including arthropods, amphibians, and mammals (Mourgues *et al.*, 1998). Probably acting in synergy, they have a bactericidal action on a large range of gram-negative and gram-positive bacteria. Genes encoding antimicrobial proteins that have been cloned and expressed in apple and pear in an attempt to confer resistance to fire blight disease include attacin E, cecropin, and lysozyme (Table 3).

Attacins and cecropin are antimicrobial peptides found in the hemolymph of *Hyalophora cecropia* in response to bacterial infection (Hultmark *et al.*, 1980). These two peptides have no known enzymatic activity. They are active against the membrane of gram-negative bacteria. Attacin causes an increase in the permeability of the outer membrane (Carlsson *et al.*, 1991) and cecropin induces pore formation in the bacterial membrane (Flink *et al.*, 1989), both effects resulting in death of the bacterial cells. Attacin E and synthetic analogs of cecropin (SB-37 and Shiva 1) under the control of inducible (Pin2) and constitutive (CaMV 35S) promoters have been introduced into the Passe Crassane genotype to enhance resistance to *E. amylovora* (Table 3). Some of the transgenic Passe Crassane lines expressing these genes show partial resistance to fire blight *in vitro* (Reynoird *et al.*, 1999a, b; Malnoy *et al.*, 2000). The level of resistance was significantly greater in greenhouse and *in vitro* when the lysozyme gene was integrated into the Passe Crassane genome (Malnoy *et al.*, 2000). Lysozymes are bacteriolytic enzymes, which have been characterized from phages, bacteria, fungi, plants, and animals (Jollès and Jollès, 1984). They are responsible for weakening the cell wall, leading to eventual lysis of the bacteria (Düring, 1996). Some lysozymes also have chitinase activity (EC 3.2.1.14) resulting from random hydrolysis in the chitin. Their mode of action is such that they only act as bactericides to gram-positive bacteria

(Boman and Hultmark, 1987). However, they have displayed activity against gram-negative bacteria when they act in synergy with other antimicrobial peptides (cecropin or attacin) (Boman and Hultmark, 1987). A construct that associated the *T4 lysozyme* gene with *attE* under control of the Pin promoter was made by Ko *et al.* (2002), and integrated into the genome of Passe Crassane (Malnoy *et al.*, 2000). However, transgenic lines containing both attacin and T4 lysozyme genes were not significantly more resistant than the Passe Crassane transgenics for attacin or T4 lysozyme alone, indicating that there was no *in planta* synergy between these two genes with respect to resistance to *E. amylovora* (Malnoy *et al.*, 2000).

Inhibition of bacterial pathogenicity or virulence factors Expression by the plant of any mechanism inhibiting bacterial pathogenicity or virulence factors should lead to resistance or reduced susceptibility. However, even though several of these factors are well known for many bacterial plant pathogen species, in particular for *E. amylovora*, this knowledge has only started to be exploited to develop strategies for engineering fire blight resistance. Two strategies to inhibit pathogenicity or virulence factor of *E. amylovora*, have been reported in pear: degradation of the capsular extracellular polysaccharide (EPS) amylovoran, and competition for assimilation of iron through the siderophore.

Degradation of the capsular exopolysaccharide (EPS) The capsular EPS plays an essential role in pathogenicity: bacterial mutants that do not produce EPS fail to colonize host plants (Belleman and Geider, 1992; Bernhard *et al.*, 1993). Indeed, the capsule functions not only to bind water, ions, and nutrients, but also as a protective layer to reduce possible recognition and response by the host (Romeiro *et al.*, 1981). A protective role of EPS against oxidative stress had also been proposed by Kiraly *et al.* (1997). However, recent studies on the interaction between pear and *E. amylovora* have shown no role of the capsule in the elicitation of the oxidative burst or in the protection of the bacteria against active oxygen species (Venisse *et al.*, 2003).

The composition and sugar linkages of EPS are characteristic of a bacterial species (Geider,

Table 3 Traits expressed in transformed pear cultivars

Trait	Pear cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
Bacterial resistance						
Fire Blight resistance (<i>Erwinia amylovora</i>)	<i>Passe</i> <i>Crassane</i>	<i>Sliva</i> <i>SB37</i> (+/- sp)	Synthetic peptide	CaMV 35S	Some transgenic lines with partial resistance <i>in vitro</i>	Reynoid <i>et al.</i> , 1999b
		<i>Attacin E</i>	<i>Hyalophora cecropia</i> (giant silk moth)	CaMV 35S	Some transgenic lines showed partial resistance <i>in vitro</i> . No correlation with the level of attacin expression	Reynoid <i>et al.</i> , 1999b
		<i>Attacin E</i> , <i>T4 lysozyme</i>	<i>Hyalophora cecropia</i> (giant silk moth)	Pin2 CaMV 35S	Significant reduction of symptoms <i>in vitro</i> for the transgenic expressing the T4 Lysozyme	Malnoy <i>et al.</i> , 2000
		<i>Lactoferrin</i>	T4 Bactertophage Bovine	CaMV 35S	Most of the transgenic clones demonstrated significant reduction in susceptibility to <i>E. amylovora in vitro</i> (17%) and in the greenhouse (60%)	Malnoy <i>et al.</i> , 2003a
					These transgenic clones also exhibited a significant reduction of symptoms when inoculated with two other bacterial pathogens of pear: <i>Pseudomonas syringae</i> pv. <i>syringae</i> and <i>A. tumefaciens</i>	
					Only 2 of 15 transgenic clones consistently showed a decrease in fire blight susceptibility <i>in vitro</i> and in the greenhouse	Malnoy <i>et al.</i> , 2005a
Fungal resistance	<i>Burokovka</i>	<i>Hrp N</i> Supersweet protein thaumatin II Defensin <i>Rs-AFP2</i>	<i>Erwinia amylovora</i> <i>Thaumatococcus daniellii</i> Radish	CaMV 35S CaMV 35S	Transgenic plants under evaluation	Lebedev <i>et al.</i> , 2002b, c
Insect resistance	Bartlett	Synthetic lytic peptide <i>D5C1</i>		CaMV 35S	Transgenic plants under evaluation	Lebedev <i>et al.</i> , 2002a
<i>Psylla</i> resistance				CaMV 35S	Fourfold reduction in psylla population levels	Puterka <i>et al.</i> , 2002
Resistance to herbicide	GP217	<i>Bar</i>	<i>Streptomyces</i>	CaMV 35S	No damage of tree in greenhouse treated with 1% solution of the herbicide Basta	Lebedev <i>et al.</i> , 2002a

(Continued)

Table 3 Traits expressed in transformed pear cultivars (*Continued*)

Trait	Pear cultivars	Gene introduced	Gene origin	Promoter	Principal results	References
Rooting ability / dwarfism	Beurre Bosc	<i>ro1C</i>	<i>A. rhizogenes</i>	<i>Ro1C</i> promoter	Plant in greenhouse had reduced height, number of nodes, and leaf area	Bell <i>et al.</i> , 1999
	BP10030	<i>ro1B</i>	<i>A. rhizogenes</i>	<i>Ro1B</i> promoter	ro1B has increased the rooting ability of the dwarfing rootstock BP10030	Zhu <i>et al.</i> , 2003
Flowering time	La France	<i>FT</i>			Transgenic lines had a shortened stem length	Matsuda <i>et al.</i> , 2006
Promoter	Ballade				<i>In vitro</i> flowering between 1 and 25 months after regeneration	
	Conference		Tobacco	Sgd24 Str246 Hsr203J CaMV 35S	Sgd4 promoter was locally activated in response to pathogens but not in response to abiotic stress Str246C promoter was systemically activated in response to pathogens and abiotic stress Hsr203J was not functional	Malnoy <i>et al.</i> , 2003b
Selectable marker	Conference	<i>Vr-ERE</i>	<i>Vigna radiata</i>	CaMV 35S	Benzaldehyde (BA) is able to inhibit adventitious bud regeneration BA can be used instead of kanamycin but the efficiency of the selection pressure is not yet proven	Chevreau <i>et al.</i> , 2007

2000). The major EPS of *E. amylovora*, called amylovoran, is composed of repeated units of three galactose residues, a side chain of glucuronic acid, and another galactose residue, which carries acetyl groups and a pyruvate residue (Nimtz *et al.*, 1996). Amylovoran biosynthesis requires a large number of enzymes encoded by the *ams* (amylovoran synthesis) cluster of genes located adjacently in the 17 kb region of the bacterial chromosome (Bugert and Geider, 1995). This EPS can be naturally degraded by a protein depolymerase of some bacteriophages. Hartung *et al.* (1988) have isolated an EPS-depolymerase gene from phage ϕ Ea1h. The encoded enzyme, when applied topically, reduced fire blight symptoms in pear slices (Kim and Geider, 2000). The purified protein has an optimal activity at low pH, which is similar to the acidic conditions encountered in damaged plant cells during infection.

The depolymerase gene from phage ϕ Ea1h was constitutively expressed in pear (Malnoy *et al.*, 2002, 2005a). In Passe Crassane expression of the depolymerase gene in 15 independent transgenic clones led, in general, to very low depolymerase activity, although relatively high expression at transcriptional and translational levels was observed. Only two of these transgenic clones consistently showed a decrease in fire blight susceptibility *in vitro* and in the greenhouse. These clones were also among the highest expressers of depolymerase at the RNA and the enzyme activity levels. These results in pear are comparable to the preliminary results reported on transgenic apple expressing the same depolymerase gene (Hanke *et al.*, 2003). The partial fire blight resistance observed in these transgenic pear and apple lines could be explained by the mode of action of the depolymerase. Indeed, in capsule degradation assays using purified depolymerase, obtention of totally uncapsulated *E. amylovora* cells required high enzyme concentrations (Kim and Geider, 2000). Thus, the authors assumed that the amount of depolymerase produced in most of these transgenic clones is below the necessary threshold for sufficient destruction of EPS capsules at a high concentration of inoculum, but partly effective when the concentration of inoculum is lower. To achieve better control of fire blight in very susceptible pear cultivars, improvements in this strategy might be obtained by directing the depolymerase protein fused with a signal peptide

to the intercellular spaces and by upregulating this gene with a translational enhancer (e.g., AMV, alfalfa mosaic virus).

Decreasing iron availability in plants *E. amylovora* produces a siderophore belonging to the desferrioxamine family, which is a virulence factor in bacteria (Dellagi *et al.*, 1998). This siderophore allows the pathogen to overcome conditions of low iron availability encountered in host tissues and has a protective effect against toxicity of active oxygen species (Expert *et al.*, 2000; Venisse *et al.*, 2003). Thus, decreasing iron availability in transgenic plants might be an attractive approach to limit bacterial invasion in the host tissue (Expert *et al.*, 1996).

Lactoferrin, found in mammalian milk, is a member of a family of iron-binding glycoproteins. Lactoferrin genes from various sources (human and bovine) show significant homology and encode a single polypeptide chain of about 700 amino acids (molecular mass of approximately 80 kDa) with an iron binding site in each of the similar amino and carboxy terminal regions (Pierce *et al.*, 1991). Their mode of action against bacteria is not only bacteriostatic but also bactericidal (Kalman and Arnold, 1988; Borthier *et al.*, 1989). A potential bactericidal domain exists in a short peptide sequence, termed lactoferricin, near the N-terminal region of the lactoferrin molecule, which can be released by proteolytic cleavage (Bellamy *et al.*, 1992a, b). Expression of a human lactoferrin gene transferred into the tobacco genome has shown that the extract from transgenic calli has a potent activity against a large spectrum of microorganisms including gram-positive and gram-negative bacteria (Mitra and Zhang, 1994). Moreover, expression of this gene conferred increased resistance to *Ralstonia solanacearum* (Zhang *et al.*, 1998). Malnoy *et al.* (2003b) have examined the ability of bovine lactoferrin, which is slightly more effective than human lactoferrin when controlled by the *CaMV* 35S promoter, to reduce fire blight susceptibility in pear. Most of their transgenic clones demonstrated significant reduction in susceptibility to *E. amylovora in vitro* (17%) and in the greenhouse (60%). These transgenic clones also exhibited a significant reduction of symptoms when inoculated with two other bacterial pathogens of pear, *P. syringae* pv. *syringae* and *A. tumefaciens*. Moreover, this

increase in bacterial resistance was correlated with an increase in root ferric reductase activity and leaf iron content. Despite negative effects on the growth of a few clones, their results indicate the potential of lactoferrin gene transformation to protect pear from fire blight through increased iron chelation. To increase the level of fire blight resistance, they proposed to use the more efficient iron-chelator, plant ferritin, which is able to chelate 4000 iron atoms (Goto *et al.*, 1998), whereas lactoferrin chelates only two iron atoms. Different ferritin genes have been overexpressed in transgenic plants for increased nutritional quality (Goto *et al.*, 1999), for bioremediation of heavy-metal-polluted environments (Vansuyt *et al.*, 2000), and for tolerance to oxidative damage and pathogens (Deak *et al.*, 1999). However, the constitutive expression of transgenic ferritin also has profound pleiotropic effects, which can be either positive (e.g., enhanced growth of transgenic lettuce reported by Goto *et al.* (2000)) or negative (e.g., reduced growth and chlorophyll content of transgenic tobacco reported by Van Wuytswinkel *et al.* (1998)). In order to avoid the potential negative effects of ferritin overexpression in pear, E. Chevreau placed this gene under the control of the pathogen inducible promoter *sgd24*, which is locally and specifically activated by pathogens in pear (personal communication). This promoter also displays an average level of expression that is much lower than the constitutive expression driven by the CaMV 35S promoter (Malnoy *et al.*, 2003a).

Enhancement of natural plant defenses Plant defense mechanisms are activated during incompatible and compatible reactions. However, in the case of a compatible interaction, the speed and level of expression of defense genes are insufficient to prevent disease development in the plant. Some strategies depend on blocking the progress of the pathogen in order to allow adequate activation of defense pathways of the plant.

During plant–pathogen interactions, pathogens secrete compounds called elicitors that induce plant defense mechanisms at variable speeds. These compounds when isolated from bacteria or fungi might be used to protect plants against their pathogens. These inducing substances can be classified into two groups: those interacting with the plant following the gene-for-gene model of

Flor (1956) (*avr* genes) and those inducing the defense pathways of the plant. A bacterial *avr* gene conferred almost total resistance to plants expressing the corresponding resistance gene; in the case of *Arabidopsis* expressing the transgene *avrB* (Gopalan *et al.*, 1996) or *avrRpt2* (Mc Nellis *et al.*, 1998), resistance was expressed as an HR. But it is more probable that this type of resistance will be bypassed by pathogens compared with strategies using an elicitor with direct (PopA, Harpin N) or indirect action (pectate lyase).

Harpin N_{Ea} is also a known inducer of systemic acquired resistance (SAR) in plants. In *A. thaliana*, Dong *et al.* (1999) demonstrated that harpin elicits resistance to *Peronospora parasitica* and *P. syringae* pv. *tomato*, accompanied by induction of the SAR genes *PR-1* and *PR-2*. Harpin N_{Ea} acts through the *NIM1*-mediated SAR signal transduction pathway in a salicylic acid-dependent fashion. The site of action of harpin in the SAR regulatory pathway is upstream of salicylic acid. Similar induction of SAR was demonstrated in cucumber following treatment with harpin P_{SS} (Strobel *et al.*, 1996). The practical potential of harpin N_{Ea} as an elicitor of defense reaction has been tested on several plant species challenged with various disease agents (Wei and Beer, 1996). This work led to the registration of a new biological pesticide, Messenger, whose active ingredient is harpin N_{Ea}. The elicitors appear to trigger a common network of signaling pathways that coordinate the defense responses of plants.

In order to create novel mechanisms for fire blight resistance in pear, some pear (Malnoy *et al.*, 2005b) lines transformed with a construct containing the elicitor harpin N_{Ea} from *E. amylovora* with constitutive promoter CaMV 35S was employed. Stable integration of Harpin N_{Ea} with the constitutive CaMV 35S promoter did not cause any detectable damage in the transgenic Passe Crassane clones. Integration of harpin N_{Ea} in the genome of Passe Crassane significantly increased the *in vitro* fire blight resistance of 10 clones out of 17. Compared to the nontransformed control, the reduction of symptoms was 36% and 58% with an inoculum concentration of 10⁷ and 10⁶ cfu ml⁻¹, respectively. These results are encouraging, because Passe Crassane is one of the pear genotypes most susceptible to fire blight. These transgenic lines can also help promote a better understanding of the role of harpin N_{Ea} in the induction of fire blight resistance in the

transgenic clones. It will also be interesting to look at the behavior of these transgenic lines in response to some other pathogens of pear.

2.2.1.2 *Pear psylla*

Pear psylla, *C. pyricola* Foerster, is a major pest of pear in the United States that feeds primarily on phloem tissue of pear. This pest is host specific to certain *Pyrus* spp. (Bell and Stuart, 1990). Changes in pear plant chemistry, physiology, or morphology brought on by the expression of foreign genes could impact the biology and behavior of pear psylla. The biology and behavior of pear psylla, *C. pyricola* Foerster, on a transgenic clone of Bartlett pear, containing a synthetic antimicrobial gene, *D5C1*, was compared with that of a nontransgenic parental clone to determine whether there were any nontarget effects. Short-term studies (<7 days) indicated pear psylla adults preferred to settle and oviposit, and nymphs fed more and developed slightly faster, on transgenic pear compared with nontransgenic pear. In contrast, a long-term study (32 days) on psylla colony development showed considerably fewer eggs, nymphs, and adults were produced on transgenic pear. Although adults reared on transgenic pear did not show weight reduction, females produced fewer eggs and nymphal hatch was significantly reduced on the transgenic pear clone. The fourfold reduction in psylla population levels that resulted on this disease resistant transgenic pear line would be an added benefit to a pear integrated pest management (IPM) program (Puterka *et al.*, 2002).

2.2.2 Dwarfism and rooting ability in rootstocks

Dwarfing characteristics of fruit tree rootstocks are of great importance for commercial production. Dwarf rootstocks are useful for high yield fruit production because trees can be planted close together; labor is reduced because the pears can be harvested without using ladders. Rootstocks have been developed for this purpose, but these dwarf rootstocks are more difficult to propagate from cuttings compared to vigorous ones. Several genes causing reduced growth have been identified

in apple (Jasik *et al.*, 1997). The *rol* (*A*, *B*, *C*, and *D*) genes from *Agrobacterium rhizogenes* and the plant phytohormone genes have been shown to exert dwarfing properties (Jasik *et al.*, 1997). The three genes (*rolA*, *rolB*, and *rolC*) derived from the Ri plasmid of *A. rhizogenes* were used to improve the dwarfing ability of several apple rootstocks.

The *rolB* gene has been found to be the most effective among the *rol* (root loci) genes in promoting root formation; this gene has been applied in genetic transformation to improve adventitious rooting of some recalcitrant plants (Rugini *et al.*, 1991; Nilsson *et al.*, 1997). This gene has been successfully expressed in the rootstock BP10030 (Zhu *et al.*, 2003). The transgenic rootstock expressing the *rolB* gene has an increased rooting ability and a shortened stem length. The significant improvement in the rooting ability of the transformed lines BP10030 makes mass production of this rootstock possible at a low cost. Since BP10030 is a dwarfing pear rootstock of *Pyrus* type, the use of the *rolB* transgenic plants of this rootstock will brighten the perspective for commercial pear production in the near future. One concern in using transgenic plants is the stability of the altered trait in offspring. However, this may not apply to pear rootstocks because fruit tree rootstocks are propagated vegetatively. The risk of losing the transgene *rolB* is thus very low, compared to annual crops where the segregation occurs after crossing and selection (Zhu *et al.*, 2003). Nevertheless, before any commercialization of these transgenic lines, Zhu *et al.* (2007) are investigating the influence of these lines on growth and development of pear cultivars grafted onto them to evaluate the stability of transgene expression in greenhouse and in the field. Their preliminary results show that, for the same cultivar, no differences in bud break, flowering, and flower numbers were found between the transgenic and nontransgenic rootstocks. The plant height and stem diameter were reduced for the vigorous cultivars grafted on the *rolB* rootstocks compared with the untransformed rootstocks (Zhu *et al.*, 2007), and no transgene was found in the untransformed scion cultivars (Zhu *et al.*, 2007). Integration of the *rolC* gene also gave similar results in Beurre Bosc (Bell *et al.*, 1999). Their preliminary observations in the greenhouse showed that the transgenic line expressing the *rolC* gene had reduced height, number of nodes, and leaf area.

2.2.3 Flowering time

In the development of all woody plants from seed, there is a juvenile phase, lasting at least 4 years in pear (Visser, 1976), during which flowering does not occur and cannot be induced under normal conditions (Hackett, 1985). In the tree fruit industry, it is important to accelerate flowering by reducing the juvenile or vegetative phase of the trees after planting in order to facilitate the earliest possible production of fruit. In fruit breeding, various practical techniques have been considered to accelerate the flowering of seedling. The most striking advances of the genetic control of flowering time come from studies in *Arabidopsis* (Levy and Dean, 1998, Pineiro and Coupland, 1998). Several genes that control flowering time have been isolated, namely, *LEAFY* (*LFY*) (Weigel *et al.*, 1992), *APETALA1* (*API*) (Mandel *et al.*, 1992), *TERMINAL FLOWER 1* (*TFL1*) (Ohshima *et al.*, 1997), and *FLOWERING LOCUS* (*FT*) (Kardailski *et al.*, 1999). Overexpression of the citrus homolog *CiFt* gene in two pear cultivars La France and Ballade induced *in vitro* flowering in 13 lines (Matsuda *et al.*, 2006). Early flowering phenotype was closely correlated with *CiFT* expression level. Similar work is currently under way in Israel, where silencing of *TFL1* by RNA interference (RNAi) induced early flowering in the pear cultivar Spadona (M. Flaishman, personal communication).

2.2.4 Control of transgene expression

The genetic construct integrated into the pear genome to control the expression of the gene of interest must contain a DNA sequence encoding the active protein and several regulatory sequences. These sequences control when, how, and where the protein of interest will be expressed in the plant. Among these regulatory sequences, the promoter identifies the coding region and allows messenger RNA (mRNA) transcription, and also determines when, and in some cases, under what conditions transcription is initiated. The CaMV 35S promoter from cauliflower mosaic virus has been used widely in transgenic plants; in pear it controls 80% of transgenes integrated in the pear genome because it provides continuous expression regardless of developmental stage

in most plant tissues (constitutive expression) (Table 2). Duplication of the upstream (5') DNA sequences of the 35S promoter results in elevated levels of transcription (Kay *et al.*, 1987). With such promoters, a gene is expressed in the majority of tissues during most phases of plant growth and development. This limited temporal and spatial regulation may be suitable for proof of concept experiments, but presents a number of potential drawbacks for use in genetically improved crops. For example, the presence of transgenes driven by constitutive promoters may result in homology dependent gene silencing, particularly when the promoter is also highly active (Vaucheret *et al.*, 1998). Thus, gene expression under the control of inducible promoters (tissue-, development stage-, pathogen-specific) is preferred in any strategy to produce transgenic plants with transgene-mediated improvements in resistance to pathogens. This targeted gene expression could be advantageous in providing resistance where and when needed, limiting the amount of transgenic protein in fruit, reducing the plant's energy cost for transgenic protein synthesis, and reducing unnecessary exposure of nontarget organisms to the protein (Norelli and Aldwinckle, 2000).

Knowledge of genes involved in plant pathogen interactions is still limited in pear. Therefore, different inducible promoters isolated from tobacco and potatoes have been used in experimental studies in response to *E. amylovora* infection and different abiotic stresses, and may be useful in commercial cultivars.

Two gene families have been identified in tobacco, whose expression in response to bacterial pathogens is rapid and intense: the "*hsr*" (hypersensitivity related) and "*str*" (sensitivity related) gene families (Marco *et al.*, 1990). The *hsr203J* (Pontier *et al.*, 1994), *aig1* (Reuber and Ausubel, 1996), and *hin1* genes (Gopalan *et al.*, 1996) belong to the *hsr* family. These genes are specifically (or preferentially) activated by signal molecules generated during the hypersensitive reaction (HR). Among these genes, only the *hsr203J* promoter has been fully characterized (Pontier *et al.*, 1994, 1998, 2001). *Hsr203J* expression occurs rapidly and locally when tobacco leaves are inoculated with the phytopathogenic bacterium *R. solanacearum* (Pontier *et al.*, 1994). The *hsr203J* promoter is neither developmentally nor tissue-specifically regulated and does not respond to

potential inducers such as environmental stimuli or endogenous plant signal molecules (Pontier *et al.*, 1998). In pear (Malnoy *et al.*, 2003a) and apple (Reynold *et al.*, 2000), the *hsr203J* promoter is activated after *E. amylovora* infection, but at a level insufficient (2% of the level of expression obtained with the CaMV promoter in pear) to be used to provide effective expression of antibacterial transgenes. Genes from the “*str*” family are activated during the early stages of compatible interaction (disease) as well as in incompatible (HR, resistance) interaction. The *str246C* gene isolated from tobacco belongs to the *str* family (Froissard *et al.*, 1994). The promoter of this gene is not only regulated by pathogens and elicitors, but also by other environmental and developmental stimuli, such as hormones, wounding, and heavy metal treatments (Gough *et al.*, 1995). Furthermore, it harbors a regulatory region (184 bp fragment of *str246C* promoter: *sgd24* promoter), which is sufficient for the induction of promoter activity in response to bacteria (Gough *et al.*, 1995). The *str246C* promoter was rapidly activated in pear during compatible and incompatible interactions, by wounding and following the application of several elicitors (capsicin, cryptogein, and harpin) and phytohormones (salicylic acid and methyl jasmonate) (Malnoy *et al.*, 2003a). The *sgd24* promoter, a deletion derivative of *str246C*, exhibited a low level of expression after bacterial inoculation, was weakly activated by wounding and elicitors, and was not activated by phytohormones (salicylic acid and methyl jasmonate) (Malnoy *et al.*, 2003a). Interestingly, the *sgd24* promoter was locally activated in pear, whereas the *str246C* promoter was activated systemically from the infection site. Both promoters, *str246C* and *sgd24*, are less active than the *CaMV* 35S promoter in pear, but their pathogen responsiveness makes them alternatives to use to drive the expression of transgenes to promote bacterial disease resistance.

2.2.5 Selectable marker

Selectable marker genes are widely used for the efficient transformation of crop plants. In most cases, selection is based on antibiotic or herbicide resistance. These marker genes are preferred because they tend to be most efficient (e.g., in

pear up to 80% transformation rate with the *nptII* gene for kanamycin resistance). Due to concerns about acceptability of antibiotic resistance genes in fruit cultivars, considerable effort is being put into developing a suite of strategies (site-specific recombination, homologous recombination, transposition, and co-transformation) to eliminate the marker gene from the nuclear or chloroplast genome after selection (Miki and McHugh, 2004). Some current efforts are emphasizing systems in which the marker genes are eliminated efficiently soon after transformation (Schaart *et al.*, 2004). Alternatively, transgenic pear can be produced by the use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Chevreau *et al.* (2007) have shown a promising effect of the *Vr-ERE* gene for the selection of transgenic lines. The benzaldehyde (BA), substrate of *Vr-ERE*, is able to inhibit adventitious bud regeneration. BA can be used instead of kanamycin but the efficiency of the selection pressure is not yet proven.

2.3 Testing of Activity and Stability of Expression of Transgenes, Yield, and Quality

With respect to the release of transgenic woody plants the stability of the integrated transgenes and their long-term expression is one of the main objectives in monitoring and risk assessment of genetically engineered plants. Stable integration and expression of the transgenes is important for fruit trees, which have an exceptionally long production time compared with annual crops. Different groups have been studying the stability of the expression of the transgenes in transgenic apple. James *et al.* (1995) showed stable expression and Mendelian inheritance of the transgenes in transgenic Greensleeves. They showed a 1:1 segregation of the *nos* gene in the R₁ transgenic apple progeny. In pear, the study of the stability of the expression of the transgene in transgenic pear is being undertaken by several groups. A first report published by Hao *et al.* (2005) established by polymerase chain reaction (PCR) and Southern analysis the stable maintenance of the *gus* gene in transgenic pear (*P. pyrifolia* var. Okusankichi) recovered from slow-growth

culture or cryopreservation. No data have yet been reported on transgene stability in transgenic pears in the field.

2.4 Regulatory Measures Adopted

Regulatory measures adopted and risks concerning pear GE crops are the same as those described in the chapter of apple transformation (see Apple).

3. FUTURE ROAD MAP

Concerning the nature of the actual genes functioning in GE crops, the regulatory agencies, growers, consumers, and perhaps nongovernmental organizations (NGOs) are thought more likely to accept the GE product if promoters and genes from the host family are used without selectable markers. The production of GE pear cultivars transformed with genes from different organisms was the first generation of GE cultivars to prove that the concept of increasing resistance by genetic engineering is effective. The next generation of GE pear lines will not contain antibiotic resistance selectable marker genes and will rely on altered expression of native pear genes for their improved disease resistance, architecture, and quality.

One of the recurring themes of the debates concerning the application of genetic transformation technology has been the role of intellectual property rights (IPR). This term covers both the content of patents and the confidential expertise usually related to methodology and referred to as “trade secrets” (Dunwell, 2005). Although few of the patents in this area have any real commercial value, there are small number of key patents that restrict the “freedom to operate” of new companies seeking to exploit the methods. Over the last 20 years, these restrictions have forced extensive cross licensing between ag-biotech companies and have been one of the driving forces behind the consolidation of these companies. Although such issues are often considered of a little interest to the academic scientist working in the public sector, they are critical for practical use of the technologies they develop. During the period since the production of the first transgenic

plants, a wide diversity of patents have been sought on all aspects of the process, ranging from the underlying tissue culture methods through the means of introducing the heterologous DNA, to the composition of the DNA construct so introduced (Kesan, 2000).

A recent advance that may circumvent the IPR limitation to *Agrobacterium* transformation technology is the development of gene transfer techniques using other bacteria, such as *Sinorhizobium* (Broothaerts *et al.*, 2005). It is claimed that these methods may offer an open source alternative to the established transformation technologies.

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Peach

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Peaches were probably the first fruit crop domesticated in China about 4000 years ago (Hesse, 1975). Peaches were moved to Persia (Iran) along silk trading routes. In fact, the epithet *persica* denotes Persia, which is where Europeans thought peaches originated.

Alexander the Great brought the peach to Greece. Greeks and especially Romans spread the peach throughout Europe and England starting in 300–400 BC (Hedrick, 1917). Peaches were brought to America with explorers of the 16th–17th centuries, with the Portuguese introducing it to South America and the Spanish to the northern Florida coast of North America. Due to the fact that stone fruits have the seed encased in a hard, lignified structure (stone) obviates special storage conditions thus facilitating their dispersion over long distances.

1.2 Botanical Description

Peach and nectarine, *Prunus persica* (L.) Batsch belong to *Prunus* genus, subfamily Prunoideae. This is the most economically valuable genus in the Rosaceae family after *Malus*. Fruits of the genus *Prunus* are described as “stone fruits” due to the

encasement of the seed within a lignified stonelike endocarp. Common *Prunus* fruits include, other than peach and nectarine, European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.), apricot (*P. armeniaca* L.), and almond (*P. dulcis* Miller). The flesh (mesocarp) of the almond fruit is hard and leathery and the seed is the edible portion.

All commercial varieties of peach and nectarines are *P. persica*, a diploid ($2n = 16$) (Jelenkovic and Harrington, 1972).

Tree growth habits include columnar, dwarf, spreading, and weeping (Faust and Timon, 1995). Peach flowers are borne singly on short peduncles (almost sessile) from lateral buds on 1-year-old wood; usually 1–2 flower buds/node, with few spurs forming, although spur-type trees have been reported (Scorza, 1987). Flowers exhibit cleistogamy, pollinating themselves prior to opening.

The fruit is a drupe with a thin epidermis, fleshy mesocarp, and a stony endocarp, that together with the seed forms a propagation unit comparable to botanical seed surrounded by its protective testa. Fruits can be beaked, round, flat shaped, yellow, white, red, melting or nonmelting fleshed; clingstone or freestone (describing adherence of the flesh to the stone); and with smooth or pubescent epidermis. This last characteristic is the only difference between

peaches and nectarines, a character that segregates as a simple trait presumably controlled by a single gene or a few closely linked genes. Trees are precocious, producing some fruit in the second year after planting. Peaches require extensive thinning (80–95% of flowers) for proper fruit size development. Fruit thinning is usually done by hand 30–45 days after full bloom, leaving about 1 fruit per 15 cm of 1-year-old shoot length. The first commercial crop is generally harvested in year 3, with maximal yields reached by year 5–7.

1.3 Economic Importance

In temperate regions, the family Rosaceae is very important in economic terms. Its commercially valuable members include fruit producing (e.g., stone fruits, apples, pears, brambles, and strawberries), nut producing (almond), lumber producing (e.g., black cherry), and ornamental (e.g., roses, flowering cherry, quince) species.

Peach is grown on all continents except Antarctica. Generally, commercial production lies between latitudes 30° and 45°. The major limiting factors for expansion of commercial production areas are extreme cold temperatures below –35 °C to –40 °C or insufficient length of cold temperature to satisfy dormancy requirements. Table 1 shows the worldwide production and harvest area for peaches and nectarines in the last 3 years.

Peaches have few marketing niches other than fresh and canned fruit. Melting flesh freestone peaches are sold fresh, and nonmelting flesh peaches are usually canned. The utilization in the United States is about 98% of nectarines marketed fresh, 45–50% of the commercial peach crop is marketed fresh, 40–43% canned, 4–8% frozen, 1–2% dry, and 2–3% others (as jam, baby food, etc.).

Like all members of the genus *Prunus*, peach leaves, flowers, and especially seeds and bark contain cyanogenic glycosides, such as amygdalin and prunasin. These compounds are toxic or lethal in large doses. However, in plant tissues, cyanide is low enough in concentration to be considered therapeutic, particularly for cancer treatment, and has been used for this purpose since at least 25 BC. In fact it has been shown that peach flower extracts can prevent ultraviolet-induced carcinogenesis in humans (Heo *et al.*, 2001).

1.4 Traditional Breeding

Hybridization has been used to develop most peach and nectarine cultivars and it remains as the dominant technology. Uncontrolled hybridization involves little more than collecting seed from trees that may be self- or cross-pollinated or a mixture of both. Controlled hybridization is the technique of applying pollen of the selected male parent

Table 1 Most important producer countries of peaches and nectarines during the years 2003–2005^(a)

	2003			2004			2005		
	Hectares ^(b)	Metric tons ^(c)	% ^(d)	Hectares ^(b)	Metric tons ^(c)	% ^(d)	Hectares ^(b)	Metric tons ^(c)	% ^(d)
WORLD	1389	14 901	100.0	1403	15 300	100.0	1430	15 674	100.0
China	610	6179	41.5	603	5829	38.1	613	6030	38.5
Italy	97	1175	7.9	97	1700	11.1	96	1740	11.1
USA	74	1390	9.3	74	1 429	9.3	74	1369	8.7
Spain	78	1270	8.5	72	916	5.9	89	1130	7.2
Greece	25	249	1.7	52	791	5.2	50	681	4.3
Turkey	27	470	3.2	26	372	2.4	26	485	3.1
France	19	347	2.3	19	387	2.5	19	425	2.7
Iran	26	390	2.6	26	390	2.5	26	390	2.5
Egypt	31	303	2.0	32	361	2.4	32	360	2.3
Chile	20	304	2.0	20	311	2.0	21	315	2.0

^(a) Source: FAOSTAT, 2006

^(b) Area expressed in thousand of hectares

^(c) Production expressed in thousand of tons

^(d) Percentage of the whole production worldwide

to the receptive stigma of the selected female parent. In the case of self-compatible species, such as peach, covering trees with an insect-proof material ensures self-pollination. While simple in concept, controlled pollination requires in-depth knowledge of crop biology and relies on exacting techniques for maximizing the number of hybrid progeny. Many of these techniques are described in Moore and Janick (1975).

Once a desirable phenotype is selected by the breeder, it can be reproduced indefinitely by vegetative propagation through the rooting of cuttings or more often through graftage of a scion cultivar onto a rootstock. This process makes possible the development of rootstocks selected for root characteristics (adaptation to specific soils, resistance to soil-borne diseases, insects, nematodes, etc.) and scion cultivars selected for fruit and production characteristics.

1.4.1 Rootstocks

The most important goals of peach rootstock breeding are resistance to diseases (*Armillaria* spp., *Phytophthora* spp., and *Verticillium dahliae*), insects (*Myzus* spp.), and nematodes (*Meloidogyne* spp., *Criconebella xenoplax*, *Pratylenchus* spp., *Mesocriconema xenoplax* and *Xiphinema americanum*), adaptation to heat and/or cold, vigor reduction of the scion, and good scion–rootstock compatibility (Reighard, 2002).

There are relatively few rootstock options for peach compared to other temperate tree fruits and, most rootstocks used are grown from seed. They are fairly uniform due to the self-pollinating, homogeneous nature of the peach. Several nematode tolerant rootstocks, rootstocks adapted to calcareous soil, and cold-hardy rootstocks have been released worldwide (Layne, 1987; Reighard, 2002).

Root-knot nematode (*Meloidogyne* spp.) resistant rootstocks are recommended for peach where this nematode is endemic. “Nemaguard”, “Nemared”, and “Guardian” are resistant to *Meloidogyne* (Nyczepir, 1991; Nyczepir *et al.*, 1999). *C. xenoplax*, the ring nematode and bacterial canker (*Pseudomonas syringae*) are involved in the peach tree short life (PTSL) syndrome (Beckman *et al.*, 2002; Okie and Scorza, 2002). PTSL causes significant losses in peach

in the southeastern region of the United States. Peach rootstocks have been screened for resistance to *C. xenoplax* (Okie *et al.*, 1987; Westcott and Zehr, 1991). “Guardian” is the only PTSL tolerant rootstock that is available (Okie *et al.*, 1994). The peach rootstocks “Bailey”, “Guardian”, “Rubira”, and “GF 305” are resistant to lesion nematodes (*Pratylenchus vulnus* and *Pratylenchus penetrans*) and dagger nematodes (*X. americanum*) (Reighard, 2002). Rootstock germplasm has also been screened for resistance to Cytospora canker caused by *Leucostoma* spp. (Scorza and Pusey, 1984; Chang *et al.*, 1989), and brown rot (*Monilinia fructicola*) (Gradziel and Wang, 1993) but no resistant rootstocks are available.

There are no compatible dwarfing stocks currently widely available, although *Prunus tomentosa* and *Prunus besseyi* have been used for dwarfing in research plantings, they are short lived. Several rootstocks with dwarfing potential have been released in Europe and the United States but none of them have been adequately tested to date (Scorza and Sherman, 1996; Perry *et al.*, 2000; Reighard, 2002; DeJong *et al.*, 2005).

1.4.2 Fruit cultivars

In general, the major breeding objectives for all commercial *Prunus* cultivars are high productivity and fruit quality, and resistance to pests and diseases (Scorza and Sherman, 1996). Cold hardiness, late blooming, and delayed softening are also important characteristics (Callahan *et al.*, 1991). In order to reach these goals, most major peach producing countries have active breeding programs and, at least, 60–70 new cultivars are released annually worldwide (Byrne, 2002).

Peaches are susceptible to many pests and diseases and only a few genotypes show some degree of resistance (Mehlenbacher *et al.*, 1990; Scorza and Okie, 1990). This lack of natural resistance to pests and diseases makes it difficult to obtain new resistant cultivars.

Peaches are widely adapted throughout their range, and except for extreme low or high chilling types, cultivars developed in one growing area are often utilized in many production regions. Therefore, in northern regions greater winter hardiness of both flower buds and whole trees is a major breeding consideration as it is the

most important factor limiting production in mid-continent and northern climates (Hesse, 1975; Mehlenbacher *et al.*, 1990; Scorza and Okie, 1990). Flower bud hardiness in peach has been shown to be a complex quantitative trait (Mowry, 1964).

The inheritance of peach fruit quality traits has been studied (Hesse, 1975; Scorza and Sherman, 1996). Breeding programs have produced very high quality fruits at maturity, however, for storage and shipping of fruit to nonlocal markets, these varieties must be picked earlier than full maturity resulting in fruits of lesser flavor and aroma in the market place. Therefore, increased firmness of ripe fruit is one of the major objectives in peach breeding. Fruit softening in peach has been studied and is related, at least in part, to endopolygalacturonase levels (Callahan *et al.*, 1989; Callahan *et al.*, 2004).

Architecture of trees is also another important breeding objective for some programs. Control of tree growth habit can reduce the need for pruning and may allow increased productivity in high-density systems (Scorza, 1984). Peach trees with columnar growth habit and high fruit quality have been produced and are under test in the United States and in Italy (Scorza *et al.*, 1989, 2000).

1.5 Limitations of Conventional Breeding: Use of New Tools, Biotechnology

Conventional breeding of temperate fruit trees is constrained by their long reproductive cycle, complex reproductive biology, and high degree of heterozygosity. In most cases, the characteristics that are desirable for commercial cultivars, including large fruit size, high coloration of the fruit epidermis, and firmness of the flesh, are recessive and/or multigenic (Bailey and French, 1949). Therefore, integration of adaptive traits from germplasm requires several rounds of introgressive backcrossing to fix the new trait and regenerate the high quality value traits in the original cultivated parent.

In addition to the time required for developing new cultivars, a significant amount of labor is required for pollen collection from male parents; individual hand emasculation of flowers of female parents; hand pollination; collection of seed from fruit developed from hybridization; seed stratification and germination; greenhouse

or nursery culture of seedlings; field planting of the seedlings; and selection and testing of superior phenotypes. Peach, a crop with a relatively short generation time of 3–4 years, has generally required from 10 years to 20 years from first fruiting to cultivar release. The relatively large areas necessary to grow segregating populations also add considerable expense to breeding programs.

The potential advantages of transformation for genetic improvement lie in the ability to target specific improvements in an otherwise known genotype, this process may require less time, labor, and field space. Perhaps most significantly transformation may provide for genetic improvements that would otherwise be impossible using traditional breeding.

The most important characteristic of genetic transformation lies in the fact that using this technique a single or a few genes can be transferred into an established genotype. Once a useful transformant is isolated (assuming stability of transgene expression) vegetative propagation, the normal method of multiplying fruit trees, provides unlimited production of the desired transgenic line. Fixation through the sexual cycle is unnecessary and inconvenient if commercially accepted cultivars are transformed.

2. DEVELOPMENT OF TRANSGENIC PEACHES AND NECTARINES

2.1 Methods Employed

There are many different techniques that allow the insertion of genetic material into plant cells and these can be divided into two main categories: direct (electroporation, microinjection, ultrasounds, liposomes, biolistic) or nondirect (*Agrobacterium* spp., viruses) with this latter technique requiring a living vector.

In peach, the only techniques used until now are biolistic (Ye *et al.*, 1994) and *Agrobacterium tumefaciens* (Hammerschlag *et al.*, 1989; Scorza *et al.*, 1990; Smigocki and Hammerschlag, 1991; Pérez-Clemente *et al.*, 2004).

The biolistic technique or DNA bombardment consists of shooting DNA coated micro particles (gold or tungsten) into plant tissues (Sanford, 1990). In the nuclei, the DNA can be inserted into

the genome by recombination. One disadvantage of this method lies in the fact that it is very aggressive and damages tissues. This can dramatically reduce regeneration. Also, it is not very efficient because the probability of the DNA insertion into plant chromosomes by recombination is low.

The method most utilized for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants is *A. tumefaciens* mediated. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of plant cells where it is then stably integrated into the host genome and transcribed. In the natural state, this process causes crown gall disease (Nester *et al.*, 1984) but for plants genetic engineering the disease-causing genes are removed from the plasmid. Gene transfer from the bacteria to the plant is a complex process and, using genetic engineering, *Agrobacterium* can be used to transfer any foreign gene (see review, de la Riva *et al.*, 1998).

2.2 Transgenes: Designing and Traits

Correct expression of the transgene is important for producing a desired phenotype. Sometimes the introduced genes are bacterial genes such as *cry* genes from *Bacillus thuringiensis* (*Bt*) and normally the eukaryotic cells cannot properly express them. So, it is necessary to modify the regulatory sequences. The most important regulatory sequence is the gene promoter, but also the terminator.

The most commonly used promoters reported are the constitutive promoters 35S, from the cauliflower mosaic virus (CaMV), and Nos, from *Agrobacterium* spp. with their respective terminators.

Alternatively, inducible promoters have been used. These are expressed in response to specific cues such as the presence of a substrate, pH level, or expression limited to a specific tissue and/or stage of development. In apple, for example, there are some advances with the use of tissue specific promoters (Gittins *et al.*, 2000, 2001).

Another constitutive promoter often used is the *Ubiquitin 1* promoter from corn (*Zea mays*). Besides its moderate constitutive activity, heat stress-induced expression has been reported in monocots such as maize (Christensen *et al.*, 1992) and rice (Takimoto *et al.*, 1994).

The dominant traits of commercialized transgenic plants are herbicide tolerance and *Bt*-based insect resistance (James, 2005). The traits introduced in fruit trees have for the most part been related to pest and diseases resistance (see review, Petri and Burgos, 2005). Currently, the only transgenic fruit tree that has been commercialized is papaya (*Carica papaya* L.) resistant to PRSV (papaya ringspot virus) (Fitch *et al.*, 1992; Lius *et al.*, 1997) (for further information about commercialized transgenic plants and their traits see www.agbios.com).

The only gene other than marker genes that has been successfully inserted into peach is isopentenyl transferase (*ipt*) from *Agrobacterium rhizogenes* (Smigocki and Hammerschlag, 1991). This gene is involved in cytokinin synthesis and produces transformed plants with increased branching (Medford *et al.*, 1989; Smigocki and Owens, 1989).

2.3 Selection of Transformed Tissues: Marker Genes

In a transformation procedure only a few cells will stably incorporate the transgene(s). Marker genes allow for the recognition and selection of transformed cells (Miki and McHugh, 2004). These marker genes are introduced with the gene of interest. There are two different classes of marker genes: selection and reporter genes.

2.3.1 Selection marker genes

These confer an advantage to the transformed cells in presence of a selective agent. The most common selective agents are antibiotics or herbicides.

The most common selectable marker gene, and used in *Prunus* almost exclusively, is neomycin phosphotransferase (*nptII*). This gene confers resistance to aminoglycoside antibiotics, like kanamycin, inactivating the phytotoxic antibiotic by phosphorylation.

In *Prunus*, there are a few reports of the use of other selection marker genes. These include hygromycin phosphotransferase (*hpt*) (Dolgov and Firsov, 1999) and the herbicide “Basta” resistance gene (*bar*) (Druart *et al.*, 1998). Selection in which nontransformed cells are inhibited from growing or killed is termed “negative selection” versus

“positive selection” in which transgenic cells can metabolize substances in the medium such as mannose for growth while untransformed cells cannot (Sigareva *et al.*, 2004). In peach, there is only one report of a positive selection-based protocol. In this case, using a media without cytokinins, the *ipt* gene acted as a selective marker gene and only the transformed cells were able to undergo organogenesis (Smigocki and Hammerschlag, 1991).

Theoretically, if the inhibitory concentration of a determined selective agent for any cultivar and any tissue is established, its addition to the medium should allow for the selection of the transformed cells and inhibit the development of nontransformed cells in the case of negative selection. However, sometimes the addition of the selective agent in the inhibitory concentration is prejudicial to the regeneration of transgenic plants and the timing of exposure and a stepwise increase in selective agent concentration is necessary. In these cases, it is necessary to establish an efficient selection strategy.

In *Prunus*, early selection, immediately following co-cultivation with the agrobacteria, has been used successfully (Gonzalez-Padilla *et al.*, 2003; Pérez-Clemente *et al.*, 2004; Song and Sink, 2006) but sometimes only a late selection or a progressive selection has allowed regeneration of transgenic plants (Scorza *et al.*, 1994, 1995b; Miguel and Oliveira, 1999; Yancheva *et al.*, 2002; Ramesh *et al.*, 2006). It has been suggested that massive necrosis in nontransformed tissues, which may occur under high selection pressure, could inhibit regeneration even from transformed cells (Szankowski *et al.*, 2003).

2.3.2 Reporter genes

These genes confer a trait that allows for identification of transformed cells or tissues. They encode enzymes that have chromogenic, fluorogenic, luminescence, or radioactive substrates, making it possible to visually identify the transformed cells.

The most commonly used reporter gene is β -glucuronidase (*uidA* or *gus*) isolated from *Escherichia coli*. This enzyme hydrolyses β -glucuronides (Jefferson *et al.*, 1987). A blue precipitate appears in the transformed cells after the incubation of the tissue with the substrate

5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc). Other substrates such as X-Gal or 4-MUG can be used to determine the activity of this enzyme.

Currently, most laboratories report the use of a chimeric version of *uidA* containing a plant intron that avoids gene expression by *Agrobacterium* eliminating false positive assays (Vancanneyt *et al.*, 1990).

Another reporter gene that has been used is the green fluorescent protein, *gfp* (Chiu *et al.*, 1996). Several *gfp* versions exist, but *sgfp* is the one has been used in *P. persica* (Pérez-Clemente *et al.*, 2004; Padilla *et al.*, 2006). The *sgfp* gene protein product fluoresces under blue light (optimum at 490 nm). The maximum fluorescence peak is at 509 nm and it has a secondary one at 540 nm (Elliot *et al.*, 1999). This protein is a modification from the original by changing a serine by a threonine in the 65 position, producing a 100-fold higher fluorescence than the wild-type protein (Chiu *et al.*, 1996).

The major advantage of green fluorescent protein (GFP) versus GUS lies in the fact that GFP detection is a nondestructive procedure and fluorescence can be measured *in vivo*. GUS assays cause death of the analyzed cell.

2.4 Regeneration of the Whole Plant (State of Art in Peach)

The lack of adventitious regeneration systems is the major limiting factor preventing the development of gene transfer technologies for fruit trees, especially in peach. Although regeneration protocols from different peach tissues have been reported (Hammerschlag *et al.*, 1985; Mante *et al.*, 1989; Pooler and Scorza, 1995; Gentile *et al.*, 2002), there are only a few reports of transgenic peach plant regeneration (Table 2).

Ye *et al.* (1994) optimized biolistic parameters for this specie. They obtained 65 putative transformed lines, 19 of these produced shootlike structures. Bombardment was applied to different tissues, but transformation was stable only from embryo-derived calli. With the other tissues only transient expression was obtained.

Peach has been shown to be amenable to *A. tumefaciens*-mediated transformation (Scorza *et al.*, 1990). Zimmerman and Scorza (1996)

Table 2 Transformation in *Prunus persica* L

Scion/rootstock	Method	Construction (strain)	Genes	Explant	T.E. ^(a) (%)	Reference
14DR60, Tennessee natural, PER 2D Redhaven	<i>A. tumefaciens</i>	pGA472 (A281)	<i>nptII</i>	Embryogenic callus, leaves, immature embryos	0	Scorza <i>et al.</i> , 1990
	<i>A. tumefaciens</i>	pTiA6 (tms328::Tn5)	T-DNA (<i>ipt</i>)	Shoots, immature embryos, embryonic axes	0	Hammerschlag <i>et al.</i> , 1989
Lovell	Bombardment	pBI505, pBI426	<i>nptII</i> , <i>gus</i>	Embryo callus, immature embryos, cotyledons, leaves, and shoot tips	–	Smigocki and Hammerschlag, 1991
Miraflores	<i>A. tumefaciens</i>	pBin19-sgfp (C58C1/pMP90)	<i>nptII</i> , <i>gfp</i>	Embryos	0	Ye <i>et al.</i> , 1994
S 6699, Oro A	<i>A. tumefaciens</i>	p35SGUSIntron (C58C1/pGV3850)	<i>nptII</i> , <i>gus</i>	Leaves	3.6	Pérez-Clemente <i>et al.</i> , 2004
Bailey, Lady Nancy, Harrow Beauty	<i>A. tumefaciens</i>	pLC101 (LBA4404, EHA105, GV3101, CG937, CG1052, CG1059)	<i>nptII</i> , <i>gfp</i>	Cotyledons, embryonic axis, hypocotyl slices, callus, internodes, leaves	0	Piagnani <i>et al.</i> , 2004
KV930465, KV930408, KV930303	<i>A. tumefaciens</i>	pBin19 (LBA4404, EHA105)	<i>nptII</i> , <i>gus</i>	Cotyledons, embryonic axis, hypocotyl slices, callus, internodes, leaves	0	Padilla <i>et al.</i> , 2006
KV939455, KV930478, KV930311	<i>A. tumefaciens</i>	pBISNI, pGA482Ggi (LBA4404)	<i>nptII</i> , <i>gus</i>	Cotyledons, embryonic axis, hypocotyl slices, callus, internodes, leaves	0	Padilla <i>et al.</i> , 2006

^(a)Transformation efficiency (nonspecified by authors when not written)

reported on the success of a procedure combining biolistics and *A. tumefaciens* for the transformation of tobacco meristems and the production of transgenic plants. However, when tested on peach a significant mortality rate was encountered due to the mechanical damage and desiccation during dissection to expose the meristems. Also bacteria growth was difficult to control Scorza *et al.* (1995a).

An interesting procedure utilizing a “shooty mutant” strain of *Agrobacterium* was reported in peach (Smigocki and Hammerschlag, 1991). This strain carries a Ti plasmid with a functional *ipt* gene but a Tn5 transposon-inactivated auxin biosynthesis (*iaaM*). The infection with a “shooty mutant” strain induces the development of tumors, from which transgenic shoot regeneration occurs. Peach tissues transformed with the *ipt* gene allowed selection of transformed shoots on a medium low in growth regulators, and were the first transformed peaches reported. *In vitro* assays of these plants demonstrated delayed senescence on cytokinin-free medium as compared with nontransformed controls. The resulting peach plants were shorter in stature than controls and one line exhibited greater branching (Hammerschlag and Smigocki, 1998) presumably due to the effect of expression of the *ipt* transgene.

The second report of transgenic peach plants is that of Pérez-Clemente *et al.* (2004). Using longitudinal mature embryos slices as the explant source they regenerated several transgenic plants expressing the marker genes *nptII* and *gfp* with a transformation efficiency of $3.6 \pm 1\%$. This protocol improved upon the preceding report of Smigocki and Hammerschlag in that mature embryos are available year round while immature embryos are available for only a limited time each year. Both reports suffer from the fact that there are no publications successfully duplicating these techniques and producing transgenic peaches in other laboratories. Therefore, the general applicability of the methods is not established.

Although transformation of seed-derived material is of limited use for improving vegetatively propagated peach scion cultivars, it could have an impact on the development of new seed-propagated rootstock varieties, and also it allows for the introduction of novel genes into the peach germplasm and could be used in conventional breeding programs. The relatively rapid turn over

of peach varieties suggests the usefulness of seed-based transformation for peach improvement.

2.5 Specific Regulatory Measures Adopted

While genetic transformation or genetic engineering holds promise to allow for the targeted improvement of peach varieties and for the utilization of unique genes not otherwise available in peach germplasm resources, there are major difficulties to overcome in the utilization of this technology. Notwithstanding the two reports of transgenic peach production (a total of perhaps four plants), both regeneration of peach and gene transfer appear to be problematic. Additional research is needed in order to develop reliable transformation techniques. The absence of transgenic peach plants with improved traits of interest precludes a practical discussion of regulatory matters. Clearly, this will be a matter for the future. With that stated, it can be speculated that transgenic peach plants will perhaps require some regulatory considerations that differ from those required for field crops such as soybean, maize, and cotton. The deregulation of papaya ringspot virus resistant transgenic papayas in 1998 and perhaps the more relevant current work on the deregulation of a plum pox virus (PPV) resistant transgenic plum (*P. domestica* L.) variety (Scorza *et al.*, 2007) will provide some guidelines for the regulation of transgenic peaches. Under the current US regulatory framework three agencies exercise regulatory jurisdiction over the testing and commercialization of transgenic crops. The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) regulates the planting of genetical engineered (GE) plants. The US Food and Drug Administration (FDA) has jurisdiction over GE plants used as food. The US Environmental Protection Agency (EPA) regulates GE crop plantings of over 10 acres (a total of 10 acres over the entire United States) and regulates GE plants that produce molecules that protect plants against pests. Since peaches are a food crop and would be planted on over 10 acres GE peaches would be regulated by all three agencies. Nonfruiting ornamental or rootstock peaches might be regulated by APHIS and EPA only or by APHIS only if they did not contain genes whose products acted to control pests or diseases.

3. FUTURE ROAD MAP

3.1 Expected Products

Few peach cultivars show significant resistance or tolerance to pests and diseases, including devastating diseases such as sharka or PPV (Decroocq *et al.*, 2005). Sharka affects plums, peaches, and apricots producing losses in fruit quality and quantity and weakening infected trees. It is probably the most important disease in *Prunus*. Currently, the only control of the disease is removing infected trees and widespread application of insecticides against aphids. This heavy application of insecticides to reduce PPV dissemination in orchards can lead to the selection of aphid populations that are resistant to the pesticides, which makes sustainable management of fruit orchards difficult (Decroocq *et al.*, 2005). Isolation and incorporation into *P. persica* of sharka-resistance genes would be of significant economic and environmental benefit. Expression of viral proteins in transgenic plants may confer resistance against the virus. *P. domestica* hypocotyl slices have been transformed with the PPV coat protein gene (Scorza *et al.*, 1994) producing GE plum trees with high quality fruit that have maintained a high level of PPV resistance for over 8 years under field conditions of heavy infection pressure (Malinowski *et al.*, 2006).

Managing root diseases is a considerable challenge. Over 500 cationic membrane-acting proteins with antimicrobial and antifungal activities have been identified to date (Theis and Stahl, 2004). Recently, tobacco plants have been transformed with the gene of a monocot mannose-binding lectin *Gastrodia* antifungal protein (GAFP, *gastrodianin*) (Cox *et al.*, 2006). Transgenic plants showed resistance to fungi and also to nematodes. The introduction and expression of such genes in peach rootstocks could be very interesting in order to obtain rootstocks resistant to important diseases such as PTSL or oak (*Armillaria*) root rot. Plums (*P. domestica*) transformed with GAFP showed increased resistance to *Phytophthora* root rot (Schnabel *et al.*, 2007).

In peach, the extension of the ripening period is an important objective. In tomato, fruit ripening has been delayed expressing antisense genes in the plant (Gray *et al.*, 1992). This system partially blocks or delays fruit ripening. In the peach

cultivar “Redhaven” three complementary DNAs (cDNAs) coding for different endo- β -1,4-glucanases, enzymes involved in fruit ripening and softening, have been reported (Trainotti *et al.*, 1997). Lester *et al.* (1996) and Callahan *et al.* (2004) reported on the role of endopolygalacturonase genes in producing melting and nonmelting flesh types. 1-aminocyclopropane-1-carboxylate (ACC) oxidase is one of the enzymes responsible for regulating the expression of ethylene, which is active in promoting fruit ripening and softening. The ACC oxidase gene has been sequenced and cloned. Transgenic plums with the peach antisense ACC oxidase gene were obtained and, for some lines, ethylene production and fruit softening were delayed (Callahan and Scorza, 2007). The manipulation of these genes and other genes involved in peach fruit ripening and softening through antisense or gene silencing technologies and perhaps utilizing inducible promoters to control the timing of expression could provide for tree ripe fruit in the market, which is desired by consumers, with firmness sufficient for handling and storage.

Climate changes are now seen to pose potential problems for agriculture, as most temperate plants are exquisitely sensitive to their environment. Peach trees adapt to the short days and cold winter temperatures through the process of dormancy and resume growth as long days and warm temperatures return. Knowledge of and the ability to manipulate genes important in these adaptive processes may be critical in the future for improving the adaptation of particular peach cultivars or germplasm. The evergrowing (*EVG*) gene (Rodriguez-A *et al.*, 1994), which is sensitive to and regulates responses to the environment (day-length), has been cloned and sequenced (Bielenberg *et al.*, 2004). The effects of this gene, as it is manipulated in transgenic plants, on growth and development and responses to the environment, are currently under investigation (R. Scorza, personal communication).

3.2 Addressing Risks and Concerns

The two major concerns surrounding GE peaches would focus on human health and the environment. The effects on human health would be directly related to the particular gene engineered into peach and its established record of safety or

the lack of safety data. It is difficult to imagine that a gene product such as a known allergenic protein would be engineered into peach and it is difficult to imagine that such an engineered peach would be approved for release. Therefore, the establishment of safety would focus on those genes and the proteins that they produce that are not normally part of the human diet, directly or indirectly. Humans consume a vast array of genetic material and proteins that sustain a healthy life. For example, viral-derived nucleotide sequences in a GE peach whether expressed as RNA, protein, or present only as nucleotides may, on the surface, not be considered part of the human diet yet viruses, their genetic material and the proteins that they produce are consumed on a regular basis in virus infected crops (Bradford *et al.*, 2005). Several of the most commonly used marker genes, such as *NPTII* and *GUS*, have been approved for use in foods in the United States (59 Federal Register 49353, Sept. 28, 1994 and 66 Federal Register 42957-42962, August 16, 2001). GE crops containing virus- and/or insect-derived genes and marker genes have been approved and are currently in the market (www.agbios.com). GE peaches may also be developed containing transgenes derived from genes already found in peach or closely related, sexually compatible species. There is no established difference in safety between GE plants containing, as a class, transgenes derived from the transgenic species or its relatives, or GE plants containing transgenes from unrelated species or phyla. Currently, GE plants are evaluated for safety on an event basis that considers the species transformed, the gene, gene construct, and particular insertion event.

The ecological or environmental risks of GE crops are related to their potential for weediness and their effects on nontarget organisms and how these effects might upset the ecological balance. Peaches are not considered a weedy or invasive species and the likelihood of a GE peach becoming weedy appears to be remote. The effects of a transgenic peach on nontarget species such as beneficial insects would be a function of the specific transgene and the protein that it produces and under current regulations in the United States and other countries would be evaluated on an event basis. The fact that peaches generally do not spread beyond the orchard, are not native in much of the world, and do not naturally produce fertile

hybrids with many other *Prunus* species suggests the potential for negative ecological impacts of GE peaches is minimal.

3.3 Expected Technologies

The improvement of peaches through gene transfer utilizing those gene transfer technologies currently available will rely on the development of efficient transformation and regeneration protocols. Without critical breakthroughs in these areas peach improvement through gene transfer will be thwarted. Currently, the most promising systems for peach appear to be seed based. These systems are not ideal because while they will allow for the addition of novel traits such as PPV resistance into peach germplasm and this germplasm can be used in traditional breeding programs, seed-based systems do not allow for the improvement of existing cultivars. Ideally, genotype-independent procedures will be developed. These may be based on the transformation of meristematic cells with high regeneration potential and or the use of regeneration-promoting genes (Petri and Burgos, 2005).

To date, there is only one report showing adventitious regeneration in peach using somatic tissues (leaves) but the regeneration rates were still low, 28.3 ± 1.7 the highest, and it was limited to a few genotypes (Gentile *et al.*, 2002). Although, it has been tried before unsuccessfully (Scorza *et al.*, 1995a), efficient meristem-transformation protocols could possibly resolve the genotype problem because *de novo* adventitious bud regeneration would not be required and this process appears to be highly genotype dependent.

Transformation of antibiotic-sensitive species, like peach, may be improved by using positive selection based on providing the transformed cells a metabolic advantage rather than in killing nontransformed cells (Joersbo, 2001). Recently, the use of a positive selection in almond resulted more efficient than the usual negative selection with kanamycin (Ramesh *et al.*, 2006).

The marker genes are another obstacle. The public concern with the introduction of antibiotic resistance into foods and the restrictions imposed by European Union mandates that do not allow deliberate release of plants transformed with antibiotic resistance genes and their commercialization (Directive 2001/18/EEC of the European

Parliament and the Council of the European Union), will place a priority on the development of methods to avoid the use of antibiotic selection or to allow elimination of marker genes from transformed plants postselection.

3.4 Specific Details for Intellectual Property Rights (IPR), Public Perceptions, Industrial Perspectives, Political and Economical Consequences

The application of genetic engineering to peach holds many of the same promises for genetic improvement in common with other species (biotic and abiotic stress resistance, growth control, improved fruit quality, etc.). GE peaches will also be affected by the same IPR issues, regulatory constraints, and public perceptions. As GE peaches enter into testing concerns related to gene flow, nontarget effects, and food safety must be addressed. Long-term field evaluation of the stability of the engineered trait will be necessary. All of these issues will be compounded in breeding programs and will affect the level and rate of progress that can be achieved. If GE technologies are to be realistically utilized then the genetic improvement of peaches and other fruit species must be undertaken by genetic improvement programs composed of scientists trained in classical and molecular breeding working as teams and with long-term institutional support.

SEE ALSO

Almond
Apple
Cherry
Pears
Plum

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Plums

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The word plum is derived from the word “plume” (old English), which is a modification of *prunum* (Latin) or *prounmom* (Greek) (Merriam-Webster Dictionary). It represents over 50 different species of *Prunus* that bear small fruits, which are generally larger than cherries and are unique relative to cherries by the presence of a suture on one side, no terminal bud, and a relatively flat stone. Plums can be differentiated from peaches by the lack of a terminal bud, multiple flowers from a bud, and an elongated pedicel (Okie and Weinberger, 1996). Plum trees are grown mainly for their fruits, which come in a variety of colors and shapes, and are used as *Prunus* rootstocks and as ornamentals. Their origins have been traced to three different continents, Europe, Asia, and North America, and they appear to have been domesticated very early (Okie and Weinberger, 1996; Faust and Suranyi, 1999).

The European plums, which are primarily used as dried plums (formerly known as prunes), are thought to have originated in the area between southern Europe and Asia Minor (Cullinan, 1937; Crane and Lawrence, 1952). Stones from *Prunus insititia*, *Prunus domestica*, *Prunus spinosa*, and *Prunus cerasifera* hybrids have been found in archeological digs dated as early as the Neolithic age (4000–2500 BC) in Germany, Ukraine, and Poland, and late Neolithic age (2500 BC) in Switzerland, Germany, Austria, and Cyprus, and the Bronze/Iron age (1400 BC) in England (Faust

and Suranyi, 1999). They were thought to be cultivated by the Assyrians and then bred by the Romans. Writings with mentions of plums are found from 7th century BC (reviewed in Faust and Suranyi, 1999) including writings of Archilochus, Xenophon, Vergil, Horace, and Ovid. Plums were well established as a cultivated fruit crop in Roman times and plums of various characteristics are mentioned in Pliny the Elder’s (1st century). Many types of plum stones were found in Roman archeological excavations all over Europe, and appear to have been planted wherever Roman armies had been (reviewed in Faust and Suranyi, 1999). A second wave of plum establishment in Europe followed the Crusades and by the Renaissance and later various plums were imported and grown all over Europe. Types, such as the Prune d’Agen (*P. domestica*), were named after the town of Agen in France where the plum resided in the monastery garden. “Reine-Claude” (*P. domestica*), which was introduced to France from Italy and named for Queen “Claude”, represents the “green gage” plums. The name “gage” comes from a single surviving plum sent to the “Gage family” in England from a monastery in France.

The Japanese plums, primarily used as fresh fruit, are represented by *Prunus salicina* Lindl. and *Prunus simonii* Carr. actually originated in China. *P. salicina* originated in the Yangtze river basin and *P. simonii* is from northern China. The history of one cultivar, “Zhui Li” (*P. salicina*), can be traced over 2000 years. Plums were brought to Japan a long time ago as plum stones have been found in archeological excavations in Japan dated

to the Yayoi Era or 300 BC. Plums have also been mentioned in Japanese literature and poetry as early as 750–800 AD. It is not clear though if it is a true plum or *Prunus mume*, an apricot (Okie and Weinberger, 1996; Faust and Suranyi, 1999).

The last group, the North American plums, is used as dried, cooked, or fresh fruit. There are many species including *Prunus americana* Marsh., *Prunus angustifolia* Marsh., *Prunus hortulana* Bailey, *Prunus maritima* Marsh., *Prunus nigra* Ait., and others. When Europeans came to North America, the native Americans had already cultivated plums, but most of the species are found only in small areas with the exception of *P. angustifolia* Marsh. Only a few plum stones have been found in archeological excavations, one dated from 250 BC to 400 AD and a second in Arkansas from the 1st to 5th century. A number of American plums were selected and propagated during the 1800s including “Miner”, *P. hortulana*, from Alabama and “Wild Goose”, *Prunus munsoniana* from Tennessee (Okie and Weinberger, 1996; Faust and Suranyi, 1999).

Plums originated in three distinct areas of the world, and breeding and crossbreeding took place mainly in the European plums as people moved back and forth throughout Europe and Eurasia. As people came to North America, plums spread out. Writings mention that European plums, Damsons, were brought from England and France in the late 1600s to New England and Canada and later to North Carolina. In the late 1700s plums were mentioned in nursery catalogs including plums described as greengage and egg plums. Orchards and nurseries offered both the European plums and American plums. In 1856, the “Prune d’Agen” plum was brought to San Francisco and established in a nursery in the Santa Clara Valley and in commercial orchards. Production was increased with the introduction of *P. cerasifera*, myrobalan, as a rootstock (Okie and Weinberger, 1996; Faust and Suranyi, 1999). This was the beginning of the California dried plum industry.

Japanese plums were brought to the United States in 1870 to Vacaville, California, and introduced into commercial orchards there. Luther Burbank established a breeding program with Japanese plums as well as American plums. In 1885, he imported a plum, “Blood Plum of Satsuma”, which had a red flesh that he used to produce a cultivar known as “Santa Rosa”, which

was thought to be the result of hybridization between *P. simonii* and *P. americana*. This along with other releases formed the basis of the California “Japanese” plum industry (Dreyer, 1985; Byrne, 1989; Okie and Weinberger, 1996; Faust and Suranyi, 1999). Burbank was awarded one of the first plant patents, no. 12, for one of these releases.

1.2 Botanical Description

Plum was first used to describe any dried fruit. Now it represents a number of different species, all from the division Magnoliophyta, class Magnoliopsida, order Rosales, family Rosaceae, genus *Prunus* L. The genus has been divided into seven subgenera based on how the leaves in buds are rolled, flower characteristics, and various characteristics of the fruit following a scheme of Strasburger (OECD, 2002). The subgenus *Prunus* includes the European and Japanese species, such as *P. domestica*, *P. salicina*, *P. cerasifera*, and the North American species, such as *P. americana* and *P. nigra*.

Plums are typically small- to mid-size trees, the Japanese plum trees being somewhat smaller and less upright than the European ones (<http://www.uga.edu/fruit/plum.html>). The Japanese trees are also more disease resistant, have a shorter juvenile period, and more abundant blooms. Bloom time for the Japanese varieties is earlier, which makes them more susceptible to spring frosts. Japanese varieties require cross-pollination while approximately half of the European plums are self-fertile. Commercially, all plums are grown on rootstocks of which many different species are used including *P. cerasifera* (myrobalan) and hybrids with native American plums.

Plum fruit is classified as a drupe and tends to be round in Japanese plums and more oval in the European plums (<http://www.uga.edu/fruit/plum.html>). The fruit takes between 2 and 6 months to ripen in European plums while only about 3 months in the Japanese plums. The fruit is also handled differently because of the high sugar/soluble solids of the European fruits; they can remain on the tree until they attain 25–35% sugar and then can be stored for long periods. The Japanese plums are more like peaches in that they are harvested earlier and have a limited

storage life of up to 2 weeks before they need to be consumed.

Like other *Prunus*, the base chromosome number (x) is 8, with a majority of the Japanese and North American species, including *P. salicina* and *P. cerasifera* being diploid with a chromosome number of $2n = 2x = 16$. *P. spinosa* L. is tetraploid having 32 chromosomes and *P. domestica* is hexaploid with 48 chromosomes (OECD, 2002). Table 1 lists a number of plum species, their distribution, and chromosome numbers.

The major commercial species of plum—*P. domestica* and *P. salicina*, as well as *P. americana*, *P. simonii*, *P. syriaca*, and *P. italica*—are not found

in the wild, suggesting that they were cultivated very early in three different areas (OECD, 2002). This presents an interesting riddle as to their origin and relationship to existing wild species. There is much speculation especially on the origin of the hexaploid *P. domestica* as well as the contribution of different species to many of the modern cultivars. It has been postulated (Crane and Lawrence, 1952) that *P. domestica* (hexaploid) was the result of a combination of *P. cerasifera* (diploid) and *P. spinosa* (tetraploid). This was based on the chromosome numbers; the fact that *P. cerasifera* and *P. spinosa* grow in the same region in the Caucasus, that triploids could be found in

Table 1 Summary of plum species^(a)

Species	Common name	Main use ^(b)	Total number of chromosomes	Origin	Distribution wild species
European					
<i>P. spinosa</i> L.	Sloe, blackthorn	Frt/Orna/Rtstk	32 ^(c)	Europe/Asia	Europe/North Africa/North Turkey
<i>P. cocomilia</i> Ten.	Italian plum/Mock apricot	Fruit	16	Europe	South Balkans/Turkey
<i>P. cerasifera</i> Ehrh.	Myrobalan/cherry plum	Frt/Orna/Rtstk	16 ^(d)	West and Central Asia/Euro	Balkans to Caucasian MT/SW Asia
<i>P. domestica</i> L.	Common garden plum Italian prune French prune (Agen) German plum Reine-Claude/Green Gage Yellow Egg plums	Frt/Orna/Rtstk	48	Euro/Asia	No wild form known
<i>P. insititia</i> L.	<i>P. domestica</i> subspecies? Damson Bullace Mirabelle St. Julien	Frt/Orna/Rtstk	48	Western Asia	No wild form known
Japanese					
<i>P. salicina</i> Lindl.	Japanese plum	Frt/Rtstk	16	China	No wild form known
<i>P. simonii</i>	Simon or apricot plum	Frt		North China	No wild form known
American					
<i>P. americana</i> Marsh.	Common wild plum	Frt/Rtstk	16	Central/East US	MA to GA to Gulf of Mexico to UT to NM
<i>P. nigra</i> Ait.	Canada plum	Frt	16	US/Canada	New Brunswick, New England, NY, MI, WI, OH
<i>P. angustifolia</i> Marsh.	Chickasaw plum	Frt	16	Southern US	DE to FL and TX/sandy soils
<i>P. hortulana</i> Bailey	Hortulana plum	Frt	16	Central US	Central KY, TN, IO, OK
<i>P. munsoniana</i> Wright and Hedr.	Wild Goose plum	Frt/Orna	16	South-Central US	KY/TN/MI/TX/MN/KS

^(a)Reproduced from Watkins (1976), Okie and Weinberger (1996), Faust and Suranyi (1999), and OECD (2002)

^(b)Frt, fruit; Orna, ornamental; Rtstk, rootstock

^(c)16/32/48/64/96 found

^(d)32, 48 found

these species, that one could obtain a hexaploid from a cross between these species, and that the variation in fruit ground color and anthocyanins in *P. domestica* could be accounted for by the additive properties of *P. cerasifera* and *P. spinosa*. Zohary (1992) disputed those arguments because the generation of the hexaploid was not followed up to determine how the chromosomes paired and how it crossed to *P. domestica*. It appeared that variation in *P. cerasifera* could be the predecessor as there was much more phenotypic variation and geographical locations, which overlapped with other *P. domestica* varieties. In addition, crosses between *P. domestica* and *P. cerasifera* resulted in some stable tetraploids implying that the chromosomes were balanced. Reynders-Aloisi and Grellet (1994), from restriction fragment length polymorphism analyses of ribosomal RNA genes, suggested that *P. domestica* originated through polyploidization of *P. cerasifera* and not from *P. spinosa*. Resolving the origin of *P. domestica* could help us not only to understand how the various species evolved but also to identify broader germplasm crosses that might yield promising traits. The development of techniques that differentiate isozymes, and now molecular markers to determine polymorphisms among species and even among cultivars, have allowed a new look into the relationships of the diverse plums.

Mowrey and Werner (1990) studied genetic similarity among 34 species of *Prunus* using 11 isozymes. As expected, most of the European, Japanese, and the American plums clustered into three distinct groups. But between the American and European plums two cherry species were grouped. Those two species, *Prunus besseyi* and *Prunus pumila*, have been reported to cross-hybridize with plums, yielding fertile F_1 progenies. Two American plums, *Prunus umbellata* Ell. and *Prunus maritima* Marsh., grouped closer to the Japanese and European plums than to the other American plums or cherry. This suggested that they might have come from a European introduction to North America (Mowrey and Werner, 1990).

Random amplified polymorphic DNA (RAPD) markers were used to group plums in general, determine species composition, and distinguish cultivars as well as rootstocks (Bellini *et al.*, 1998; Casas *et al.*, 1999; Shimada *et al.*, 1999; Boonprakob and Byrne, 2003; Liu *et al.*, 2006).

These groups found similar results in that the Japanese species grouped together as did the European species. In a rootstock study (Casas *et al.*, 1999), *P. insititia* grouped amongst *P. domestica* cultivars suggesting a common ancestry as was suggested by Crane and Lawrence (1952) that both the hexaploid species were derived from *P. spinosa* and *P. cerasifera*. Bellini *et al.* (1998) found that *P. cerasifera* Ehrh grouped within the *P. domestica* suggesting that *P. cerasifera* could have been an ancestor as suggested by Crane and Lawrence (1952). Shimada *et al.* (1999) found that *P. cerasifera* and *P. spinosa* grouped with *P. domestica* but were genetically distinct suggesting that more markers were needed to determine how closely related they were to *P. domestica*. Shimada *et al.* (1999) also suggested that the “Burbank” Japanese plum releases that were purported to have North American plums as ancestors grouped with the Japanese plums and even between Japanese and European plums but not with the North American plums. Boonprakob and Byrne (2003) confirmed the analyses of the “Burbank” Japanese plum founding clones and concluded that “Mariposa” and “Gaviota” were derived from *P. salicina* and *P. simonii* with maybe some *P. cerasifera*, and “Santa Rosa” and “Eldorado” were derived from all three species. Liu *et al.* (2006) suggested from their use of RAPDs that *P. simonii* is possibly a variant of *P. salicina* rather than a species, and even though *P. cerasifera* and *P. spinosa* are related to the European plum group it is unlikely that *P. domestica* originated from a hybrid of these two species.

Amplified fragment length polymorphism (AFLP) and intersimple sequence repeat analyses provided more molecular markers to differentiate species (Goulao *et al.*, 2001; Aradhya *et al.*, 2004). These separated the Japanese plums from the European plums. In addition, Aradhya *et al.* (2004) was able to group *P. cerasifera* separate from *P. domestica*. In an even finer analyses using microsatellite markers (Ahmad *et al.*, 2004; Rohrer *et al.*, 2004), the North American species grouped as expected with the exception that the more widely dispersed cultivars of *P. americana* did not group together but interspersed with the other North American plums (Rohrer *et al.*, 2004). Ahmad *et al.* (2004) separated apricots, Japanese plums, pluots, and plumcots. The plumcots were between the plums and apricots while the pluots were grouping with the plums.

Molecular markers, RAPDs and AFLPs, and now simple sequence repeats, have helped to determine the source of the modern germplasm with the goals of expanding the germplasm. Because of the ease in which many of the diploid species are able to cross-hybridize, background has always been a little unsure. One of the mysteries that remain has been how the hexaploid species *P. domestica* arose. It had been postulated that since no hexaploid species were found resembling *P. domestica* it must have come from the hybridization of a *P. spinosa* and a *P. cerasifera*. Recent evidences also do not support this idea. It appears from marker analyses that *P. domestica* is not closely related to *P. spinosa* and may more likely be the result of hybridization between different ploidy numbers of *P. cerasifera*. There has also been a report of a hexaploid plum that resembles *P. domestica* found in China. The marker analyses have also been helpful in determining the makeup of several of the Japanese plums that appear to be the result of interspecific breeding.

1.3 Economic Importance

Plums are grown in temperate zones worldwide. There is a fresh fruit market, a dried fruit market, and an emerging juice market. China is by far the largest grower of plums with over 4.6 metric tons of fruit in 2005 worth approximately one billion US dollars with Serbia and Montenegro a distant second with 0.5 metric tons of fruit worth about 140 million US dollars. The biggest exporter of fresh plums is Chile with a value of 76 million US dollars. The United States is the biggest exporter of dried plums with a value of 135 million US dollars. Table 2 presents a list of the top 20 countries in plum production, exports, and imports (FAOSTAT, 2000/2004/2005). It may be noted that several countries have a decline in production (in bold font), most notably the United States. Table 3 lists the area in hectares of plums that are harvested from the top 20 plum producing countries for 2004 and 2000 (FAOSTAT, 2000/2004). Again the area reflects the yields with China having the largest area cultivated and the decrease in the United States' production with decreased acreage.

The two major uses of plums are as a fresh market product (*P. salicina*) and a dried

fruit product (*P. domestica*), both for direct consumption. There are many other uses; the most notable is for the production of a type of plum brandy known as slivovitz, Mirabelle, rakia, zwetschgenwasser, or quetsch. It is made from distilled fermented plums and contains a range from 20% to 75% alcohol. It usually is made with local varieties of *P. domestica*. In Serbia and Montenegro, the second largest producer of plums, 80% of the fresh market plums go into the production of slivovitz (Buric, 2003). Sloe plums or *P. spinosa* are used to flavor gin. Plums are also used for jams and jelly as well as dried for candy and baked goods.

Plums have always been desired for their taste, colors, and ease of drying without fermenting or spoiling. Table 4 lists the nutrient contents of a Japanese-type plum as well as a dried European plum (USDA, 2006). Dried plums have been well known for health related value, i.e., as a laxative (Tinker *et al.*, 1994; Stacewicz-Sapuntzakis *et al.*, 2001). (The marketing name of prunes has been changed to dried plums to dissociate that name with any negative connotations.) But, in fact, both fresh plums (Japanese) and dried plums (European) have health benefits. The fruits are all among the highest in antioxidant containing foods. In a top 20 list of foods containing antioxidants, dried plums ranked ninth, black plums ranked 16th, and plums, in general, ranked 19th (Wu *et al.*, 2004). In a study on several cultivars of plum as well as peaches, there was variability in the levels of various antioxidant compounds (Gil *et al.*, 2002). Cultivars with the darkest flesh and skin contain the most as predicted. There have also been a series of reports on the beneficial effects of dried plums on preventing the decrease in bone density associated with menopause or gonadal hormone deficient males (Franklin *et al.*, 2006).

1.4 Traditional Breeding

A successful cultivar is one that makes a profit for the grower. There are different ways to achieve this. One way is to have the fruit ripen at a unique time so that there would be less competition in the market at that time. Ideally, breeders would have a fruit with the same desired characteristics that ripens each week in the season. Another is to improve fruit quality in order to attract

Table 3 Amount of land in cultivated plums

Country	Hectares (1000)	
	2004	2000
China	1503.64	1454.51
Serbia and Montenegro	135.00	125.00
Germany	64.00	61.00
Romania	97.00	95.66
USA	44.50	51.85
France	19.30	19.26
Chile	14.46	13.07
Spain	12.87	18.43
Turkey	18.40	18.40
Italy	14.01	12.34
Russian Federation	60.00	52.00
Ukraine	22.70	26.70
Islamic Republic of Iran	14.50	14.92
Argentina	14.87	10.50
Poland	25.37	31.72
Japan	18.00	17.40
Uzbekistan	15.00	13.50
India	14.00	14.00
South Africa	5.50	5.60
Republic of Korea	6.60	4.37

Source: Data based on FAOSTAT reports 2000, 2004

more buyers. These qualities are size, shape, skin color, attractiveness of ground color, color of flesh, firmness, freeness of pit, texture, and fruit quality (flavor, sugar, acid, etc.) (Okie and Weinberger, 1996). A third way to improve profit is to lower production costs. This could come about by increasing disease and insect resistance, which could minimize chemical use, increase yields, and decrease inputs. Yields could be increased through having cultivars that are better adapted to a specific region as well as more highly productive trees. Decreasing inputs could come from a less vigorous tree that does not have to be pruned as much or a drought-tolerant tree needing less water. The bottom line is to be able to grow a tree and market desirable fruit at a profit.

Breeding programs for European plums have different goals depending on the location and market targeted. Collectively, these goals include shortened juvenility period, resistance to pests and disease (bacterial canker, silver leaf, and fruit rot), resistance to sharka, tolerance to abiotic stresses, adaptability to regional conditions, cold hardiness, semicompact tree size, late bloom, cross-fertility, changes in ripening time, increased productivity, maximized fruit set, large fruit size, red, purple, and blue color, higher fruit quality,

better flavors, higher sugar content, freestone, and long shelf life (Okie and Weinberger, 1996).

Breeding programs for Japanese plums have goals similar to that for European plums, and include disease resistance, cold hardiness, low chilling requirements, compact trees, improved productivity through better pollination, self-fertility, late blooming, specific ripening times, increased fruit size, black skin color (hide bruises), yellow skinned and other colors, red-fleshed plums for warm climates, better eating quality, firmness for shipping, and improved storage ability (4 weeks to transport by ship) (Okie and Weinberger, 1996).

Selection and classical breeding have been amazingly successful in generating a number of high-quality cultivars, which have then been adapted in individual breeding programs for the local areas they are grown in (reviewed in Ramming and Cociu, 1991; Okie and Weinberger, 1996; Okie and Ramming, 1999). Most of the European plums are derived from high-quality types, such as “Reine-Claude”, “Prune d’Agen”, “Italian”, and “Pozegaca”, which were selected hundreds of years ago but still are in the background of many cultivars. More recent introductions from breeding programs such as “Stanley” (1926, New York Agricultural Experiment Station), which has “Italian” background, have also been successful. In Japanese plums, more breeding has occurred through cross-species hybridization to yield cultivars that are able to adapt to different conditions, yet still the commercial markets are based on a limited number of old cultivars that were bred by Luther Burbank such as “Santa Rosa”, or directly from Japan such as “Burbank” and derivatives of those. Local adaptations of these cultivars provide most of the world’s commercial germplasm.

Rootstock breeding has taken a somewhat different approach in the utilization of interspecific hybrids as well as different species. The major rootstocks are derived from *P. domestica*, *P. cerasifera*, Marianna (*P. cerasifera* × *P. munsoniana*), and Peach (*Prunus persica*) (Ramming and Cociu, 1991; Okie and Weinberger, 1996). Improvements have been in “vigor, longevity, hardiness, nematode and disease resistance, dwarfing ability, tolerance to drought or flooding, compatibility and ease of propagation” (Okie and Weinberger, 1996).

New tools for breeding are becoming available. The first approach has been to develop genetic linkage maps of markers that are associated with

Table 4 Nutrient value of 100 g of plum and dried prunes^(a)

Nutrient	Units	Plum	Dried prunes	Nutrient	Units	Plum	Dried prunes	Nutrient	Units	Plum	Dried prunes
Water	g	87.2	30.92	Vitamins				Amino acids			
Energy	kcal	46	240	Vitamin C	mg	9.5	0.6	Tryptophan	g	0.009	0.025
Energy	kJ	19	1006	Thiamin	mg	0.03	0.051	Threonine	g	0.01	0.049
Protein	g		2.18	Riboflavin	mg	0.03	0.186	Isoleucine	g	0.014	0.041
Total lipid	g	0.28	0.38	Niacin	mg	0.42	1.882	Leucine	g	0.015	0.066
				Pantothenic acid	mg	0.14	0.422	Lysine	g	0.016	0.05
Carbohydrate, by difference	g	11.4	63.88	Vitamin B-6	mg	0.03	0.205	Methionine	g	0.008	0.016
Fiber	g	1.4	7.1	Folate, total	µg	5	4	Cystine	g	0.002	0.011
Sugars, total	g	9.92	38.13	Choline, total	mg		10.1	Phenylalanine	g	0.014	0.104
Sucrose	g	1.57	0.15	Betaine	mg		0.4	Tyrosine	g	0.008	0.021
Glucose	g	5.07	25.46	Vitamin B-12	µg	0	0	Valine	g	0.016	0.056
Fructose	g	3.07	12.45	Vitamin A, IU	IU	345	781	Arginine	g	0.009	0.037
Lactose	g	0	0	Retinol	mcg	0	0	Histidine	g	0.009	0.027
Maltose	g	0.08	0.06	Vitamin E	mg	0.26	0.43	Alanine	g	0.028	0.066
Galactose	g	0.14	0	Tocopherol, β	mg	0	0	Aspartic acid	g	0.352	0.801
Starch	g	0	5.11	Tocopherol, γ	mg	0.08	0.02	Glutamic acid	g	0.035	0.114
Minerals				Tocopherol, δ	mg	0	0	Glycine	g	0.009	0.047
Calcium, Ca	mg	6	43	Vitamin K	µg	6.4	59.5	Proline	g	0.027	0.13
Iron, Fe	mg	0.17	0.93	Lutein + zeaxanthin	µg	73	148	Serine	g	0.023	0.059
Magnesium	mg	7	41	Lipids				Other			
Phosphorus	mg	16	69	Fatty acids				Alcohol, ethyl	g	0	0
Potassium	mg	157	732	Saturated	g	0.02	0.088	Caffeine	mg	0	0
Sodium	mg	0	2	Monounsaturated	g	0.13	0.053	Theobromine	mg	0	0
Zinc	mg	0.1	0.44	Polyunsaturated	g		0.062	Carotene, β	µg	190	394
Copper	mg	0.06	0.281	Cholesterol	mg	0	0	Carotene, α	µg	0	57
Manganese	mg	0.05	0.299	Phytosterols	mg	7		Cryptoxanthin, β	µg	35	93
Fluoride	µg	2	4					Lycopene	µg	0	0

^(a)Reproduced from USDA Nutritional Standards Plum

the traits of interest. These then can be used to screen seedlings for the traits of interest by their linkage to markers, or marker-assisted selection (MAS). In plum, these techniques have been used to identify molecular markers that are close to the *Ma* gene, which is responsible for conferring nematode resistance in *P. cerasifera* (Claverie *et al.*, 2004a; Dirlewanger *et al.*, 2004a). Again using these markers, the location of the *Ma* locus has been narrowed and the actual physical piece of DNA with the *Ma* locus has been isolated in a bacterial artificial chromosome clone (Claverie *et al.*, 2004b). This knowledge is being used in a breeding program to “stack”, that is, to combine several genes for nematode resistance in a *P. cerasifera* rootstock (Dirlewanger *et al.*, 2004c; Lecouls *et al.*, 2004). In general, this approach works well with the traits of interest that are clear in their scoring and segregation patterns. As general

Prunus molecular maps are further developed (Dirlewanger *et al.*, 2004b), their use in plum breeding will increase. Details on molecular mapping and breeding in plums can be found in a recent review by Esmenjaud and Dirlewanger (2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Plums are represented by a wide variety of sexually compatible species that have a great deal of desirable traits, as well as undesirable traits (Ramming and Cociu, 1991). The existing germplasm used for both the European plum breeding programs (hexaploid) and the Japanese plum breeding programs (diploid) have been derived from limited germplasm. With the advent of marker-assisted breeding and larger general

Prunus maps to aid in introgressing desired traits from diverse germplasm into existing commercial cultivars (Dirlewanger *et al.*, 2004c; Lecouls *et al.*, 2004; Esmenjaud and Dirlewanger, 2006), what would be the reason for using transgenic breeding? The stock answer would be that transgenic breeding is needed (1) if a trait is not present in sexually compatible germplasm, or (2) if a trait is too closely linked to some essential trait to be separated through hybridizations, or (3) if only a single (or small number) trait is desired to be modified, i.e., not reassemble the traits as in a sexual hybridization.

One trait that would meet the first and third criterion for plum would be resistance to sharka disease. The biggest threat to the existing plum industry as well as the stone fruit industry is the devastating virus plum pox potyvirus (PPV), the causal agent of sharka (Dunez and Sutic, 1988; Nemeth, 1994). PPV is carried by aphids in a nonpersistent manner on their stylus and transmitted from tree to tree when the aphids probe (Kunze and Krczal, 1971). Grafting with infected budwood also transmits PPV. The symptoms of the infection include chlorosis of the leaves and fruit, hallmark rings on fruit, leaves, and even seeds, deformed fruit, reduced quality of the fruit, premature fruit drop and in the presence of other viruses, tree decline. PPV was first reported in Bulgaria during the World War I (Atanassov, 1932). It then proceeded to spread throughout Europe. By the 1990s, PPV had infected over 100 million stone fruit trees (Nemeth, 1994). It has now been detected outside of Europe in countries as far ranging as India (Thakur *et al.*, 1994), Chile (Rosales *et al.*, 1998), the United States (Levy *et al.*, 2000), and Canada (Thompson *et al.*, 2001). New reports have detected PPV in China, Pakistan, and Argentina (Navratil *et al.*, 2005; Dal Zotto *et al.*, 2006; Kollerova *et al.*, 2006). The only cure is to destroy the trees. In the United States, where there was only a limited outbreak in Pennsylvania, over 1400 acres were destroyed at a cost of 40.5 million US dollars.

Resistance to PPV is needed because if a tree were tolerant it would still be a source of inoculum for other trees. In looking for such a gene in sexually compatible species, it has been found that commercial stone fruit are not the only trees susceptible to PPV. In

laboratory tests over 30 different *Prunus* including *P. mexicana*, *P. maritima*, *P. angustifolia*, and *P. americana* exhibited susceptibility (Damsteegt *et al.*, 2004). There are reports of some natural resistance/tolerance to plum pox virus, but the terms resistance, tolerance, and immunity are not always consistent with each study, nor the isolate of PPV, making comparisons between cultivars difficult (reviewed in Kegler *et al.*, 1998). There are breeding programs specifically with the goal of at least PPV tolerance that have reported good success (Hartmann, 1998, 2002; Rankovic and Ogasanovic, 2000). Resistance reported in *P. domestica* appears to be through a hypersensitive response to PPV (Kegler *et al.*, 2001). While there are breeding programs with some promises of resistance, the necessity for plum cultivars to be locally adapted as well as high standards for fruit quality would necessitate that resistance be introduced in many lines to recombine all the traits necessary for a resistant commercial cultivar. This difficulty is what makes PPV resistance meet the third criterion for a transgenic breeding approach, i.e., a single trait that needs to be modified.

Genes for resistance to other viruses are potential targets for transgenic breeding for similar reasons that PPV resistance, is a target, though currently no other virus is as destructive as PPV. Lastly, a good use for transgenic breeding would be to change a single-gene expression pattern without rearranging the genes in a typical hybridization. An example would be to manipulate the keeping quality in all plums. One of the problems in our worldwide marketing is that to ship plums economically, it has to be done by boat, which means fresh market plums need to have a keeping life of about four weeks.

2. DEVELOPMENT OF TRANSGENIC PLUMS

2.1 Developing a Transformation System

An initial transformation system for *P. domestica* was developed using two different constructs (Mante *et al.*, 1991), pCGN7001 and pCGN7314 (Comai *et al.*, 1990). These contained a selectable gene, neomycin phosphotransferase, *NPTII*, from *Escherichia coli* (Rothstein *et al.*, 1980), driven by

a 35S cauliflower mosaic virus (CaMV) promoter region (Odell *et al.*, 1985) in one construct and by a mannopine synthetase, MAS, from *Agrobacterium tumefaciens*, promoter (Comai *et al.*, 1990) in the other construct. The gene of interest was the β -glucuronidase, *uidA* gene (*GUS*), from *E. coli* (Jefferson, 1987), driven by either the MAS promoter or a double 35S promoter and terminated by the MAS 3' end. The goal of the project was to establish a transformation system and to determine what promoters would be needed to detect activity of the gene of interest as well as the selectable marker.

Similar systems have been developed for *P. salicina*. Prieto *et al.* (2005) applied the system described above to *P. salicina* varieties using NPTII as the selective marker and green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Davis and Vierstra, 1996) as a reporter gene.

Yancheva *et al.* (2002) reported the development of transformation system in *P. domestica* using leaf tissue. The vector used was based on pSM-GFP containing the GFP as a marker gene driven by 35S promoter and containing NPTII as a selectable marker.

Dolgov *et al.* (2005) and Mikhailov *et al.* (2007b) have also reported a transformation system using leaf tissue from *P. domestica*. Their vector was pNOV35S-GFP, having a redshifted *GFP* gene driven by the 35S promoter and a phosphomannose isomerase gene (*pmi*) (from *E. coli*) based selection (Joersbo *et al.*, 1998) driven by a promoter from cestrium yellow leaf curling virus (CMPS).

Transformation rates in plum have been low, ranging from less than 1% to approximately 4% at the highest. In one study, several different constructs and strains were tested to determine the most efficient parameters for transformation of *P. domestica* (Padilla *et al.*, 2003). Two different plasmid backbones, pBISNI (Narashimhulu *et al.*, 1996) and pGA482GG (An, 1986) were used, both containing NPTII as the selectable marker. Various genes of interest were used including the *PPV-CP*, *TomRSV-CP*, *PNRSV-CP*, *PDV-CP*, and peach antisense *ACO1*. Overall results suggested that the pGA482GG background results in more efficient transformation of *P. domestica*. Other studies that used leaf tissue changed the selection scheme as well as the vectors and *Agrobacterium* strain resulted in lower efficiencies (Yancheva *et al.*, 2002; Mikhailov and Dolgov, 2007).

2.2 Methods of Transformation

The method used for generating all the transgenic plums has been by transformation with *Agrobacterium tumefaciens*. Mante *et al.* (1991) first devised a method for transforming plum hypocotyls. Basically an overnight culture of *Agrobacterium* (EHA 101) was grown at 28 °C in Luria broth medium with both kanamycin (50 mg l⁻¹) and gentamycin (100 mg l⁻¹), and then was concentrated to 10⁵–10⁶ cells ml⁻¹ of murashige and skoog (MS) liquid medium. The freshly cut plum hypocotyls were co-cultivated with 10⁶ cells in 10 ml of liquid shoot regeneration media (SRM) for 48–72 h, then washed and cultured for 6–10 days with carbenicillin and cefotaxime. Variations of this procedure have been used in all the transformation protocols, with variations on *Agrobacterium* concentration, co-cultivation time, strain used (EHA101, EHA105, C58/Z707, LB4404), and antibiotics used to kill the *Agrobacterium*. Reported transformation rates range from less than 1% to 4% (Padilla *et al.*, 2003).

The method reported for transformation of leaf tissue began with adventitious shoots induced from plum leaf using 5 mg l⁻¹ BAP (6-benzylaminopurine) and 0.5 mg l⁻¹ IBA (indole-3-butyric acid), modified to 2 mg l⁻¹ BAP and 0.1 mg l⁻¹ IBA. The youngest leaves were wounded, then exposed to the bacteria for 30 min. Following a 3-day co-cultivation in the dark, the leaves were induced to form shoots with 5 mg l⁻¹ BAP, 0.5 mg l⁻¹ IBA in the presence of cefotaxime for 10 days in the dark. The shoots were then transferred to media with mannose or hygromycin to select for the transformed tissue with the *pmi* gene and *hpt* gene, respectively. After several months, shoots and cali that survived were placed on micropropagation media with selection and rooted. Transformation rates were approximately 1.5–2.2% (Mikhailov and Dolgov, 2007; Mikhailov *et al.*, 2007a, b).

2.3 Selection of Transformed Tissue

Most of the constructs used in plum transformation contain the *NPTII* gene for selection using kanamycin. One of the difficulties in *Prunus* transformation has been the low rate of regeneration (see Section 2.4), and one of the

effects of kanamycin is slow growth. Several of the successful transformation experiments have varied timing of kanamycin selection. In Mante *et al.* (1991) selection using kanamycin at 75 mg l^{-1} took place 6–10 days after co-cultivation. Levels of $10\text{--}75 \text{ mg l}^{-1}$ of kanamycin were reported to cause control tissue to remain white, not to expand and not to produce shoots. Yancheva *et al.* (2002), in their leaf system, used a low level of selection (25 mg l^{-1}) immediately following co-cultivation resulting in a large number of “escapes” or clones that were not transgenic. To remedy this but still have good growth, the first cycle of selection was at 25 mg l^{-1} and the second round was at 50 mg l^{-1} . The study of Padilla *et al.* (2003) used two different selection strategies. The first was to place the hypocotyls sections on 80 mg l^{-1} kanamycin immediately following co-cultivation. This was compared to delaying the addition of kanamycin for 2 weeks. The late selection resulted in approximately the same number of transformed lines as the early selection but with more “escapes” than the earlier selection.

Two other selection schemes have been used with success, a gene for phosphomannose isomerase, *pmi*, that allows for plant growth on mannose, and a hygromycin phosphotransferase gene, *hpt*, that allows for plant growth on hygromycin (Mikhailov and Dolgov, 2007; Mikhailov *et al.*, 2007a, b). The mannose selections were difficult because transgenic tissue allowed nearby escapes (Mikhailov *et al.*, 2007a), which could be avoided by passaging on selective media but overall transformation rates were low ($>0.1\%$, $n = 1$). In comparisons of transformation rates resulting from early (3 days) and late (13–14 days) selection on hygromycin, the late selection had significantly higher transformation rates, 2.2% versus 0.27% (Mikhailov and Dolgov, 2007).

2.4 Regeneration of Whole Plants

Regeneration efficiency appears to be the key to successful transformation and appears to be a stumbling block for transformation of plum. The first breakthrough came in the 1989 (Mante *et al.*, 1989) in which thidiazuron, N-phenyl-HN-1,2,3-thidiazol-5-ylurea (TDZ), was used to induce plant regeneration from mature cotyledons in *P. domestica*. The basic protocol

that evolved from these studies (Mante *et al.*, 1991) used seeds from cold storage rather than freshly harvested seeds. The hypocotyls were sliced from mature cotyledons that had their embryonic axes removed, and placed on MS medium with $2.5 \mu\text{M}$ IBA and $7.5 \mu\text{M}$ TDZ. Shoots developed by 4 weeks, and these were transferred by 10 weeks to rooting media with $2.5 \mu\text{M}$ IBA in the light. In the case of transformed hypocotyls, shoots were transferred every 4 weeks on selective media for a total of five transfers before transferring to rooting media. Acclimation to soil and the greenhouse was a 2-week process in a 1:1 mixture of peat moss:perlite mix. Overall rates were approximately 60–80% of cotyledons producing shoots, only 20–25% rooted and approximately 90% were successfully acclimatized in the greenhouse. Several improvements have been made with the initial IBA level reduced to $0.25 \mu\text{M}$, the multiplication step for transformed shoots has been modified by transferring them to media with $0.2 \mu\text{M}$ IBA and $5 \mu\text{M}$ BA, and the efficiency of rooting was improved to 50% by dipping the shoots in $2500 \mu\text{M}$ IBA before placing them on media with $0.01 \mu\text{M}$ kinetin and $0.01 \mu\text{M}$ naphthalene acetic acid (Scorza *et al.*, 1994). Several changes have since been made to simplify the protocol (Padilla *et al.*, 2003). This involved the composition of the regeneration media, the substitution of $1\text{--}3 \mu\text{M}$ benzyl-aminopurine for IBA in the proliferation media, a shorter time on the regeneration media, and an increased level of α -naphthaleneacetic acid (NAA) to $5 \mu\text{M}$ in the rooting media allowed the IBA dip to be eliminated (Padilla *et al.*, 2003). The transfer of plantlets to soil in the greenhouse was also modified by leaving the plantlets in soil in the culture room to acclimatize before moving to the greenhouse (Padilla *et al.*, 2003).

Mikhailov *et al.* (2007b) have reported obtaining up to 80% regeneration from one cultivar of *P. domestica*, “Startovaya.” This was using the protocol described for transformations from leaf tissue. Even with this high rate, the subsequent transformation rates were only 2% at the highest (Mikhailov and Dolgov, 2007; Mikhailov *et al.*, 2007b).

Other reports of plum regeneration have been published both in *P. domestica* and other species. Escalettes and Dosba (1993) reported success with adventitious shoot regeneration from *P. maritima*,

P. domestica and *P. insititia* as well as a hybrid plum clone using TDZ with or without NAA with specific concentrations of hormones being clone dependent. The addition of silver nitrate enhanced regeneration possibly by blocking negative effects of ethylene. Ochatt (1992) reported regeneration of plants from protoplasts of *P. cerasifera* and *P. spinosa*. Using either a combination of NAA and Zeatin, or NAA, BAP, and Zeatin for one line of *P. spinosa* and one line of *P. cerasifera*, respectively, protoplasts were able to form microcallus from which fast-growing callus was obtained. Shoots were formed after transfer to media with NAA, BAP, and Zeatin, and then rooted and transferred to soil. A third approach to regeneration was taken by Pascual and Marin (2005) using two rootstocks, Marianna 2624 (*P. cerasifera* × *P. munsoniana*) and myrobalan 605 (*P. cerasifera*) leaf cultures. Leaf explants from *in vitro* cultured shoots were subcultured for one month with BAP and IBA then exposed to a 90-min pulse of 2,4-D before being cultured with NAA and BAP and PPM (“Plant Preservative Mixture”, Plant Cell Technology, Washington, DC) or substituting 2,4-D for the NAA. The combination of the 2,4-D pulse and the regeneration media with NAA and BAP resulted in 5–59% shoots, consistently about two- to sixfold more than controls that had no 2,4-D treatment. There were differences in which leaf explants responded to which treatment with whole leaves or basal sections yielding the highest rates. The majority of the shoots were rooted and transferred to soil with no apparent differences from the “mother” plants (Pascual and Marin, 2005).

There are two reports of regeneration with *P. salicina*, the first as part of a successful transformation (Prieto *et al.*, 2005) system modeled after Mante *et al.* (1991) with modifications of growth regulator ratios. The second group has developed the first step in a transformation system (Tian *et al.*, 2004) using a protocol based on Mante *et al.* (1991) with good success.

Several other groups have reported success with shoot regeneration of *P. domestica*, but not yet rooting. Bassi and Cossio (1991) were able to obtain high levels of shoots from leaf tissue but these shoots were not able to be rooted. Nowak and Miczynski (2002) also reported shoots from leaves using TDZ and 2,4-D as growth regulators, but rooting was not the goal. Lauri *et al.* (2001) and

Gentile *et al.* (2003) were able to regenerate from shoot apices of *P. domestica* and a plum hybrid (*P. insititia* × *P. domestica*) by culturing in the dark on BA and NAA through developing callus and inducing shoots by transferring to an auxin-free media in the light.

2.5 Testing of Activity, Stability, and Inheritance of Transgenes; Adverse Effects on Growth, Yield, and Quality

2.5.1 Developing transformation systems

Several groups have developed and are in the process of developing effective transformation systems (Table 5). Once the regenerated plants are obtained in these systems, it remains to determine if the gene of interest or marker gene is expressed. In the first report of plum transformation (Mante *et al.*, 1991) the activity of two genes, *NPTII* and *uidA* were measured using two different promoters for each. The nopaline synthase (NOS) promoter had higher levels of expression of *NPTII* than did the MAS promoter and the double 35S promoter had higher levels of expression of *uidA* protein than did the MAS promoter. This demonstrated that all the three promoters work in plum and that the expression can be measured at the protein level in plum. The results of Padilla *et al.* (2003) also detected GUS expression. *GFP* expression has been detected from plums transformed with a pNOV35SGFP vector where the *GFP* was under a 35S promoter though expression varied by tissue and age of tissue (Mikhailov *et al.*, 2007b). Preliminary results from *GFP* expression in the *P. salicina* system (Prieto *et al.*, 2005) and the *P. domestica* (Yancheva *et al.*, 2002) have not been presented.

2.5.2 PPV resistance

2.5.2.1 Pathogen-mediated resistance

To incorporate resistance to PPV, the idea of pathogen-mediated resistance was tested. The initial hypothesis was that expression of a gene encoding a viral protein would prevent that virus from replicating and hence provide resistance (Beachy *et al.*, 1990).

Table 5 Summary of vectors used for plum transformation

Construct	Purpose	Select ^(a)	Prom/ term ^(b)	Marker ^(c)	Prom/ term	Gene of interest ^(d)	Prom/ term	Additional ^(e)	Agrobacterium	Reference
pCGN7001	System	Kan	35S/			uidA	MAS/MAS		EHA101	Mante <i>et al.</i> , 1991; Comai <i>et al.</i> , 1990
pCGN7314	System	Kan	MAS/Mas			uidA	35S-35S/ MAS		EHA101	Mante <i>et al.</i> , 1991; Comai 1990
pSM-GFP	System System	Kan Kan				GFP GFP	35S/ 35S/		LBA4404, EHA105	Prieto <i>et al.</i> , 2005 Davis and Vierstra, 1996; Yancheva <i>et al.</i> , 2002
pBISNI/GUS	System	Kan	NOS/NOS			uidA	MAS/NOS	OCX/MAS- AE	EHA105	Narashimulu <i>et al.</i> , 1996; Padilla <i>et al.</i> , 2003
pNOV35S-GFP	System	Man	CMPS/			GFP	35S/		CBE21	Mikhailov <i>et al.</i> , 2007b
pCamGFP	System	Hygro				GFP			CBE21	Mikhailov and Dolgov, 2007
pBin-mGFP5- ER	System	Kan				GFP			CBE21	Mikhailov and Dolgov, 2007
pGA482GG/ CPRV-4	PPV resistance	Kan	NOS/NOS	uidA	35S/NOS	PRV-CP	35S/	5'39bCMV- CP	C58/Z707	Scorza <i>et al.</i> , 1991, 1995; Fitch <i>et al.</i> , 1992
pGA482GG/ PPVCP-33	PPV resistance	Kan	NOS/NOS	uidA	35S/NOS			TMV-leader	EHA101, C58/Z707	Scorza <i>et al.</i> , 1994; Ravelonandro <i>et al.</i> , 1992 Jacquet, 1998a
pGA482GG- PPV-CP33	PPV resistance	Kan	NOS/NOS	uidA	35S/NOS			TMV-leader		Damiano <i>et al.</i> , 2005, 2007

(Continued)

Table 5 Summary of vectors used for plum transformation (*Continued*)

Construct	Purpose	Select ^(a)	Prom/ term ^(b)	Marker ^(c)	Prom/ term	Gene of interest ^(d)	Prom/ term	Additional ^(e)	Agrobacterium	Reference
ihpRNA-B14/ pHellsGate 8	PPV resistance PPV	Kan Hygro	NOS/NOS			Hairpin PPV-CP PPV-CP, hairpin	35S/OCS		LBA4404	Hily <i>et al.</i> , 2007
pBISNI/PDVcp	PDV resistance	Kan	NOS/NOS			PDV-CP	MAS/NOS	OCS/MAS- AE	EHA105, LBA4404	Mikhailov <i>et al.</i> , 2007a Padilla <i>et al.</i> , 2003
pBISNI/ PNRSV-cp	PNRSV resistance	Kan	NOS/NOS			PNRSV-CP	MAS/NOS	OCS/MAS- AE	EHA105, LBA4404	Padilla <i>et al.</i> , 2003
pGA482/GG- TomRSVcp	TomRSV resistance	Kan	NOS/NOS	uidA	35S/NOS	TomRSV-CP	35S/NOS		EHA105, LBA4404	Padilla <i>et al.</i> , 2003
pGA482G/GI/ antiACO1	Improve Keeping Quality	Kan	NOS/NOS	uidA	35S/NOS	Anti-ACO	35S/NOS		EHA105	Padilla <i>et al.</i> , 2003; Callahan and Scorza, 2007
pCambian vectors	PPV resistance	Kan/ Hygro	35S/NOS	uidA		Hairpin PPV-CP	tCUP/NOS			Tian, personal communication

^(a)Selection was Kan (kanamycin), Man (mannose) from the phosphomannose isomerase gene, and Hygro (hygromycin)

^(b)Promoter/terminator for selection was 35S from cauliflower mosaic virus, MAS from mannopine synthase, NOS from nopaline synthase, CMPS from cestrium yellow leaf curling virus

^(c)Marker gene was uidA for β -glucuronidase (GUS)

^(d)GFP, green fluorescent protein; PPV-CP, papaya ringspot virus coat protein; PPV-CP, plum pox virus coat protein; PDV, prune dwarf virus; PNRSV, *Prunus* necrotic ringspot virus; TomRSV, tomato ringspot virus; anti-ACO, antisense ACC oxidase

^(e)Additions are 5' sequences added to the gene of interest. OCS/MAS-AE is trimer OCS/MAS activating elements, 5'39bCMV-CP is the 5' untranslated region to the 1st 39 bases of the coat protein of cauliflower mosaic virus, and TMV-leader is the leader sequence for tobacco mosaic virus

2.5.2.2 *PRV transgene*

Initially, the construct used to transform papaya for resistance to papaya ringspot virus (PRV), (Fitch *et al.*, 1992), was used to transform *P. domestica* (Scorza *et al.*, 1991, 1995). The idea was that the similarity between the two potyviruses, PPV and PRV, would provide “cross-protection.” The PRV coat protein gene had a 35S promoter from CaMV, the 5′ untranslated region, translation initiation codon and the first 39 nucleotides of the CaMV coat protein gene upstream of the *PRV-CP* gene followed by the 35S polyadenylation terminator sequence. The selection gene was *NPTII* with a NOS promoter (Beven *et al.*, 1983; An, 1986) and NOS terminator. The marker gene was the *uidA* gene from *E. coli*, encoding for β -glucuronidase or GUS under a 35S promoter and NOS terminator from nopaline synthase from *A. tumefaciens* (Depicker *et al.*, 1982; Beven *et al.*, 1983).

Four independent lines were isolated that grew on kanamycin selection. Leaves were positive when assayed for GUS expression and DNA analyses indicated the presence of the marker genes as well as the *PRV-CP* gene. Three lines were propagated to test for resistance in a containment greenhouse (Scorza *et al.*, 1995). The plants were graft inoculated with PPV infected tissue and monitored for symptoms and by enzyme-linked immunosorbent assay (ELISA) for a period of up to 19 months. All lines eventually had positive ELISAs and symptoms. In a study where branches were examined above or below the graft inoculation, it appeared that one line had detectable virus by real time-polymerase chain reaction (RT-PCR) only in branches that were above the inoculation point and no detectable virus after 19 months below that graft point but did have detectable virus by 32 months (Scorza *et al.*, 1995). The overall conclusion was that at least in the three lines tested, the *PRV-CP* was not able to provide a mechanism for long-term resistance to PPV.

2.5.2.3 *PPV-CP transgene*

Ravelonandro used the coding sequence of the coat protein from PPV as the pathogen gene (Ravelonandro *et al.*, 1992). The leader sequence

from the tobacco mosaic virus (TMV) coat protein and an ATG start codon was placed in frame to the coding sequence of the *PPV-CP* gene and placed this between a 35S promoter sequence and a NOS terminator sequence. It was then inserted into the same backbone sequence as the PRV construct between the selection gene *NPTII* and the marker gene, *uidA* (Scorza *et al.*, 1994). Damiano *et al.* (2005, 2007) have repeated the transformation of *P. domestica* with the same construct and Mikhailov *et al.* (2007a) have made a construct with the coat protein gene driven by a double 35S promoter.

Jacquet *et al.* (1998a) designed the *PPV-CP* gene to minimize the potential for heteroencapsidation in transgenic plants by deleting the DAG amino acid triplet that is required for aphid transmission, and by deleting the first 420 bases of the coat protein gene. The two genes were then cloned into the pGA482GG plasmid between the 35S promoter and in front of the NOS terminator sequence resembling the above construct except for the manipulated coat protein gene (Jacquet *et al.*, 1998a, b; Ravelonandro *et al.*, 2000).

Transformation with the *PPV-CP* construct pGA482GG/PPV-CP-33 generated 22 independent lines of *P. domestica*, which tested positive for GUS activity, kanamycin resistance, and were PCR positive for *PPV-CP* (Scorza *et al.*, 1994). Five of the lines were further assayed for the integration of the transgenes, the level of *CP*-mRNA (messenger RNA) and presence of *CP*-protein. All five lines had stably integrated transgenes ranging from an apparent one copy to three or more copies in C5. One line, C6 had a deletion of the *uidA* gene. The levels of *CP*-mRNA ranged from undetectable in C6 to low in C5 and very high in C4. The levels of *CP*-protein were correlated to the level of detectable mRNA with none found in C6 and C5 and the most found in C4 (Scorza *et al.*, 1994).

After proliferation, the five lines from the initial transformations were bud grafted onto rootstocks in order to test for resistance to PPV. These plants were aphid inoculated with PPV strain D (Ravelonandro *et al.*, 1997) and graft inoculated with PPV strain M (Ravelonandro *et al.*, 1997; Scorza *et al.*, 2001). These plants were followed for three or four years in the greenhouse with several cold-induced-dormancy periods. In the aphid inoculated plants, by 3 weeks 8/8 of the C5 transgenic plants were immunoblot negative for

PPV-HC or helper component protein while 51 out of 57 of the control and other transgenic lines were positive. By three years, all of the 57 control and non-C5 transgenic lines were positive with most showing symptoms while none of the C5 lines were positive or exhibited symptoms.

Similar results were obtained in the two experiments that were inoculated by grafting on infected budwood. The C5 line never had detectable symptoms, nor did it have positive immunoblots or ELISAs during the three or four years while all the other lines at one time or another had symptoms, positive immunoblots and positive ELISAs. In one set of experiments, C5 did, however have virus detectable by IC-RT-PCR and uninfected buds grafted on the infected C5 lines did show virus symptoms within several months (Scorza *et al.*, 2001). In the other set of experiments, no virus could be detected in C5 using IC-RT-PCR and uninfected buds grafted onto C5 remained virus free (Ravelonandro *et al.*, 1997).

It was determined that the mechanism for the resistance of the C5 line was due to post-translational gene silencing (PTGS) (Scorza *et al.*, 2001; Hily *et al.*, 2005). In comparison to the other transgenic lines, C5 had high rates of transcription of the *CP*-transgene, very low levels of mRNA accumulation, specific methylation of the *CP*-transgene and very low levels of virus upon graft inoculation (Scorza *et al.*, 2001). It was postulated that the complex arrangement of the insertion of transgenes, specifically the arrangement of the coat protein gene, induced the silencing mechanism to trigger the destruction of sequences related to the *CP* such as the infecting PPV. In looking for small interfering RNAs (siRNAs), one of the hallmarks of silencing, two size classes of siRNAs were detected in uninfected C5 and neither was detected in another multicopy transgenic line, C3 nor in controls (Hily *et al.*, 2005). In infected lines the smaller class of siRNAs was detected in all while only the larger class was detected in the resistant C5 suggesting that that larger class of siRNAs was responsible for the resistance of C5.

One of the major questions was would this PTGS mechanism of resistance to PPV work in the field. Hily *et al.* (2004) analyzed the level of resistance in a field planting in Poland of C5 trees and other transgenic lines and control trees under both natural infection by aphids and by chip-bud inoculation. After four years, none of

the uninoculated C5 trees had become infected with PPV while 37 out of 39 other trees were infected, mostly in the first season. As was seen in one greenhouse experiment (Scorza *et al.*, 2001), when the C5 trees were inoculated through chip budding, PPV could be detected in a few leaves by IC-RT-PCR and by ELISAs. Symptoms were evident only in a few leaves after one year and those disappeared by four years. C5 in the field had high levels of specific methylation of the coat protein transgene relative to other transgenes and relative to other transgenic lines such as C3. C5 also had only low levels of detectable *CP*-mRNA, similar to the levels seen in the greenhouse studies (Scorza *et al.*, 1994). Results from field trials in Poland (8 years) and Spain (7 years) were similar in that no PPV was found in uninoculated C5 trees, while the majority of the other trees in the plantings were infected (Malinowski *et al.*, 2006). In the chip-bud-inoculated lines in Poland, all trees except C5 had detectable PPV by symptoms by the first year. C5 exhibited symptoms on less than five leaves per tree in year 2 and in the following years. Only in C5 was the detection of PPV by ELISA or IC-RT-PCR limited to those leaves that exhibited symptoms (Malinowski *et al.*, 2006). Overall, the field trials demonstrated that C5 was highly resistant to natural infection through aphids and was significantly resistant when chip-bud inoculated.

To determine if the resistance could be transferred, hybridizations were made with C5 (Ravelonandro *et al.*, 1998; Scorza *et al.*, 1998). C5 pollen was used to hybridize with a *P. spinosa* rootstock (apparently tetraploid) (Ravelonandro *et al.*, 1998) with the goal of transferring the PPV resistance to rootstocks. GUS expression determined that about half of the progeny (44/97) had received a transgene from C5 and PCR was used to confirm that those progeny had also received the *CP*-transgene from C5. After two cycles of growth and cold-induced dormancy (CID), the hybrid seedlings that had been chip-bud inoculated with PPV were symptomless, ELISA negative and negative on immunoblots indicating that they were resistant to PPV like the C5 controls (Ravelonandro *et al.*, 2001).

The *PRV-CP* transgenic lines were hybridized with C5 as well as selfed, yielding seedlings that contained only the *PPV-CP* insert, or only the *PRV-CP* insert, or both inserts or neither (Scorza

et al., 1998). These were graft inoculated with PPV and monitored. After eleven months following inoculation, only those seedlings that contained the *PPV-CP* had no symptoms and were 100% ELISA negative. All the rest of the plants had detectable symptoms. The pattern of multiple fragments that hybridized to the *PPV-CP* probe, seen in DNA blot analyses of C5 was seen in all the resistant seedlings, supporting the fact that the complex pattern of transgene insertion in C5 segregates as a single-linked loci with the resistance phenotype.

Based on earlier results that the resistance could be easily transferred, Ravelonandro *et al.* (2002) moved that resistance into hybrids with commercial *P. domestica* cultivars. Seedlings were screened for GUS expression and those that tested positive (56:59 and 16:24) were further analyzed using PCR assays to determine if the *PPV-CP* were present and to confirm the presence of the *uidA* gene. A number of the plants that contained the transgene *PPV-CP* as well as nontransgenic controls were replicated and then inoculated by either grafting on to an infected microrootstock or by chip budding. Infection was monitored by immunoblots or by IC-RT-PCR. All of the *PPV-CP* carrying seedlings were negative for virus through two cycles of growth and CID (Ravelonandro *et al.*, 2002), again confirming the transfer of the resistance as a single Mendelian gene.

The transfer and linkage of the multiple transgenes in C5 was confirmed in field studies over a number of years by looking at the genotypes of open pollinated C5 fruit in which there was an 1:1 segregation of GUS activity to no activity (118:100 and 120:92), and in 50 DNA blot analyses of GUS positive progeny, 49 had the expected pattern of *CP* fragments. The 50th had a more complex pattern suggesting hybridization with a nearby *PPV-CP* transgenic line other than C5 (APHIS, 2006, petition for deregulation).

Molecular characterization of the resistant progeny supported the inheritance of the PTGS mechanism of resistance (Scorza *et al.*, 2001; Hily *et al.*, 2004). Resistant seedlings had highly methylated *CP*-transgene DNA. They also had the two size classes of siRNA and low levels of *CP*-mRNA. This was apparent from an early stage of germination (2 weeks) as well as in ungerminated embryos (Hily *et al.*, 2004).

C5 has been patented as “HoneySweet” (Scorza *et al.*, 2004), and in all the testings, propagating sexual and asexual, and in grafting on many different types of rootstock, no unusual phenotypes have been detected. C5 is an upright tree form with vigorous growth, good productivity and yields high fruit quality with an average brix of 21.5°, medium- to large-sized fruit with blue skin and yellow flesh. A complete description of the tree and fruit are in the plant patent (Scorza *et al.*, 2004).

2.5.2.4 *PPV hairpin transgenes*

Based on the mechanism of PTGS to obtain the resistance in the C5 as well as many other reports in the literature, constructs were made that formed hairpin or double stranded RNAs from several of the *PPV* genes. The majority of these have been tested in tobacco but the constructs are ready for *Prunus* systems using not only a 35S promoter but a *rolC* promoter from *Agrobacterium rhizogenes* (Schmulling *et al.*, 1989; Pandolfini *et al.*, 2003; Di Nicola-Negri *et al.*, 2005). Hily *et al.* (2007) have taken a similar approach using both a short- and a full-length fragment of the *PPV-CP* gene as well as having them driven by a 35S promoter or a peach chlorophyll A/B binding protein minimal promoter. The hairpin constructs of the *PPV-CP* were placed in a pHellsgate 8 vector (Wesley *et al.*, 2001) with the sense and antisense orientations separated by a pyruvate orthophosphate dikinase intron and terminated with an octopine synthase terminating sequence. The selective gene was *NPTII*. After testing in *Nicotiana benthamiana*, *P. domestica* were transformed with the most effective construct that carried the full-length *CP* under the 35S promoter (Hily *et al.*, 2007).

Using knowledge from the studies that demonstrated that C5 resistance was based on a PTGS-silencing mechanism, many groups designed constructs that enhanced the generation of a PTGS response to obtain resistance to PPV. This was well demonstrated in tobacco using various *PPV* genes and several promoters (Pandolfini *et al.*, 2003; Di Nicola-Negri *et al.*, 2005; Hily *et al.*, 2007). In these studies, a large number of the transformants exhibited PTGS, 5/5 with the *rolC* promoter-*PPV-CP* (Pandolfini *et al.*, 2003), 41/41 with the 35S promoter and various pieces of the *PPV P1*,

HC-Pro and *P3* genes (Di Nicola-Negri *et al.*, 2005), and 37/48 using different lengths of the *PPV-CP* and the 35S and a Chlorophyll A/B binding protein promoter (Hily *et al.*, 2007). Only Hily *et al.* (2007) have introduced the hairpin construct into plum yielding lines that produce siRNAs. One line contained the 35S promoter and the full-length *CP* sequence and produced siRNAs in similar amounts to that of C5 and of the same two size classes seen in C5. The *CP*-transgene was highly methylated, which is consistent with the PTGS phenotype. Inoculation by aphids resulted in no detectable virus in the transgenic line tested while the susceptible controls were all positive. These results suggest that the hairpin constructs are an efficient way to generate resistance to PPV.

2.5.2.5 Other PPV resistance lines

A number of laboratories have reported transformation of plum with genes that could confer PPV resistance (da Camara Machado *et al.*, 1999; Damiano *et al.*, 2005, 2007; Ilardi *et al.*, 2007; Mikhailov *et al.*, 2007a). The results of resistance testing have not been reported.

2.5.3 Regulation of ethylene and effects on ripening

The complete cDNA (complementary DNA) sequence of *ACC Oxidase 1* gene from peach (Callahan *et al.*, 1992) was PCR amplified and digested with *EcoRI*, gel purified and ligated into an *EcoRI* digested pGA482GGI vector. The vector was constructed from the pGA482GG (Fitch *et al.*, 1992) vector by inserting a *uidA* gene with an intron (S. Gelvin, personal communication). The *uidA* was driven by MAS promoter and a 35S promoter and terminated by an octopine synthase terminator (pCNLS6). A 35S promoter and NOS terminator with a multiple cloning site in between was inserted between the NPTII selectable marker and the *uidA* marker. The antisense ACO *EcoRI* fragment was cloned into this multiple cloning site (Padilla *et al.*, 2003; Bill Farmire, personal communication).

Callahan and Scorza (2007) used *P. domestica* to investigate the effects of down-regulating ethylene

on the ripening characteristics. Fifteen antisense *ACO* lines were analyzed for the amount of ethylene evolved during ripening as well as the time of ripening and the brix of the fruit as it ripened. Results after three years of fruiting suggested that the antisense fruits were slower to ripen, had lower levels of ethylene, and had slightly lower levels of brix in comparison to 14 lines of nontransgenic seedling controls. This suggested that the antisense *ACO* gene was down-regulating the amount of ethylene produced, which was correlated with slower softening and a lower brix. Molecular studies to confirm the phenotyping are in progress.

2.6 Specific Regulatory Measures Adopted

All PPV experiments were performed with the required permits. As PPV is a foreign pest in the United States, all inoculation studies were done in a P3 quarantine greenhouse at Fort Detrick, MD. PPV was classified as a select agent and stricter controls as well as security measures were undertaken that restricted the possession of genes in addition to the virus. Again, work was done strictly in Fort Detrick by fully approved personnel.

APHIS field permits as well as greenhouse permits were obtained for all transgenic work in the United States. The field permits initially called for all transgenic propagatable material to be collected and burned including prunings. The trees were contained prior to and throughout bloom time so no release of pollen was possible. Since *P. domestica* is a hexaploid and not compatible with most *Prunus* species, when it was necessary to analyze fruit, restrictions were modified to allow open pollination as long as there were no cross-compatible trees in the area. All fruit and seed as well as clippings had to be collected and destroyed.

In order to further test and potentially release the PPV-resistant transgenic cultivar “HoneySweet” (C5) in the United States, it must be approved by three different federal agencies. “USDA-APHIS has jurisdiction over the planting of genetically engineered plants. EPA has jurisdiction over planting and food and feed uses of pesticides engineered into plants. These

Table 6 Time table for transgenic breeding. Development of a virus-resistant plum, “HoneySweet”

Years Pre-1	Isolation and characterization of gene of interest (Ravelonandro <i>et al.</i> , 1992) Isolation of appropriate promoter Construction and testing of appropriate vector with gene of interest and promoter of interest in model system (Ravelonandro <i>et al.</i> , 1992)
Year 1–2	Transformation/regeneration system for plum (Mante <i>et al.</i> , 1989, 1991) Transformation/regeneration of plum with gene of interest (Scorza <i>et al.</i> , 1994) Molecular analyses for gene expression (Scorza <i>et al.</i> , 1994) Proliferation and plant acclimatization (Scorza <i>et al.</i> , 1994) Plant propagation (Scorza <i>et al.</i> , 1994)
Years 2–5	Greenhouse tests for PPV resistance (Ravelonandro <i>et al.</i> , 1997; Scorza <i>et al.</i> , 2001) Gene expression studies (Callahan <i>et al.</i> , 2001) Determination of mechanism of resistance (PTGS) (Scorza <i>et al.</i> , 2001)
Years 5–12	Resistance trials conducted in the field, multi-environment, multi-PPV Isolates. (Malinowski <i>et al.</i> , 1998, 2006; Hily <i>et al.</i> , 2004) Fruit quality and yield tests (Scorza <i>et al.</i> , 2005/2007, personal communication) Transgene expression field tests (Hily <i>et al.</i> , 2004; Hughes Watson and Scorza, 2004) Hybridization tests to transfer resistance (Scorza <i>et al.</i> , 1998; Ravelonandro <i>et al.</i> , 1998, 2001, 2002)
Years 13+	Deregulation petition to APHIS (May 2006), Petition to FDA (October 2006), and EPA (2007, in progress)

are referred to as plant-incorporated protectants, or PIPs. Food and Drug Administration (FDA) has jurisdiction over food and feed uses of all foods from plants” (United States Regulatory Agencies Unified Biotechnology Web site, <http://usbiotechreg.nbii.gov/FAQRecord.asp?qryGUID>

=3). A petition for deregulation has been submitted to APHIS. This has been accepted and put up for public comment (APHIS, 2006, petition and environmental assessment). Table 6 presents a time course for the development of “HoneySweet” (Figure 1).

**Figure 1** Fruit from the PPV-resistant transgenic cultivar “HoneySweet” [Courtesy of Ralph Scorza]

3. FUTURE ROAD MAP

3.1 Expected Products

The majority of programs using transgenic plums are working to generate PPV resistance (Table 7), so the first predicted commercial product of transgenic breeding could be a PPV-resistant plum. It could come from the release of C5-“HoneySweet” directly or it may come from a cultivar resulting from breeding with “HoneySweet” to introduce the resistance into a plum type of choice (Ravelonandro *et al.*, 1998, 2002; Scorza *et al.*, 1998). In the United States, if “HoneySweet” is deregulated, the progeny of “HoneySweet” will automatically be deregulated in regard to the transgenes from “HoneySweet.” This would mean that the fastest PPV-resistant plums would come from hybridizations with “HoneySweet.” In this manner the PTGS method of resistance could be combined with nontransgenic germplasm with different modes of resistance resulting in a cultivar with stacked resistance mechanisms.

The next generation of transgenic plums will still be for resistance to PPV as well as other viruses using the same mechanism of resistance, PTGS (Padilla *et al.*, 2003; Dolgov *et al.*, 2005; Hily *et al.*, 2005). This generation will use the knowledge from “HoneySweet” to speed up the process of finding resistant lines, for example, by the introduction of hairpin constructs to induce the PTGS system (Mikhailov *et al.*, 2007a; Hily *et al.*, in press), and utilizing genes that will make the deregulation easier, such as using genes

that have been deregulated previously. This next generation of transgenic plums for release will be produced to minimize the use of existing intellectual property where possible to maximize the potential for commercial releases.

The third generation of potential products will be improvements in traits such as fruit quality aspects, productivity and disease resistance; traits that have been successfully approached through conventional breeding but will be better targeted or with a novel phenotype not easily obtained through conventional breeding (Prieto *et al.*, 2005; Callahan and Scorza, 2007). The identification of these improved traits will come through better knowledge of the molecular biology and physiology of those traits, as well as knowledge gained from genome sequencing of both peach and apple by the Joint Genome Institute and the Istituto Agrario San Michele all’Adige, respectively.

3.2 Addressing Risks and Concerns

3.2.1 Environmental challenges

APHIS considers eight potential environmental impacts for C5 plum including: (1) potential impacts from gene introgression, (2) potential impacts based on relative weediness, (3) potential impact on nontarget organisms, (4) potential impacts on biodiversity, (5) potential for viral interactions, (6) potential impacts on commercial use, (7) potential impacts on organic

Table 7 Status of transformed plum

Trait of interest	Group	Location	Gene of interest	Greenhouse results	Field trials	Releases	Reference
PPV resistance	Scorza/Ravelonandro	USA	PPV-CP	PPV resistance	Yes	Deregulation	Scorza <i>et al.</i> , 1994
PPV resistance	Scorza/Ravelonandro	USA	PRSV-CP	No long-term PPV resist	Yes		Scorza <i>et al.</i> , 1995
PPV resistance	Ravelonandro/Scorza	France	PPV-CP-DAG/RC	PPV recovery			Ravelonandro <i>et al.</i> , 2000
PPV resistance	Scorza/Ravelonandro	USA	PPV-CP-hairpin RNAi	PPV resistance			Hily <i>et al.</i> , 2005, 2007
Delayed softening	Callahan/Scorza	USA	peach-anti-ACO	Wound-induced ethylene	Yes		Callahan and Scorza, 2007
PPV resistance	Tian/Brown	Canada	PPV-P1/RNAi				Personal communication
PPV resistance	Mikhailov/Dolgov	Russia	PPV-CP/pmi/RNAi				Mikhailov <i>et al.</i> , 2007a
PPV resistance	Damiano	Italy	PPV-CP	PPV resistance			Damiano <i>et al.</i> , 2005, 2007

farming, and (8) potential impacts on raw or processed agricultural commodities (APHIS, 2006, environmental assessment). A number of these points have been addressed. *P. domestica* is hexaploid suggesting that its ability to outcross to wild relative is very low, since there are few wild hexaploid *Prunus* species. If it did outcross, the effect of the incorporation of C5 transgenes for kanamycin resistance, PPV resistance and *uidA* on wild relatives does not appear to confer any kind of advantage except to be resistant to PPV. On the point of weediness, C5 has a normal upright plum growth habit, hence there should be no impact on increased weediness. The impact on nontarget organisms was addressed in the petition for nonregulatory status (<http://www.aphis.usda.gov/brs/aphisdocs/04.26401p.pdf>), with a study on insects found on C5 plants. No differences were seen between transgenic and nontransgenic trees. In addition, NPTII and GUS proteins that C5 could produce (C5 has no detectable PPV coat protein), have been “classified as exempt from the requirement of a tolerance in or on all raw agricultural commodities when used as plant-pesticide inert ingredients” (59 Federal Register 49353, September 28, 1994 and 66 Federal Register 42957–42962, August 16, 2001 for NPTII and GUS, respectively). Biodiversity issues are a factor of the weediness or advantage/disadvantage conferred by the transgenes in C5 and as there appear to be none, there should be no effect on biodiversity.

The case for viral interactions with a consequent development of new viruses has been investigated. Virus interactions can have three major results. The first is that a viral genome may be “packaged” by a coat protein made by the transgenic tree that allows the “new” virus to be transmitted by aphids to a new host or heterologous encapsidation (Lecoq *et al.*, 1993; Tepfer, 2002). A study to prevent any transencapsidation of the transgene was done by Jacquet *et al.* (1998a, b). Constructs were made of the *PPV-CP* gene with either a deletion of the DAG, amino acid triplet responsible for aphid transmission, or a deletion of the start codon. These were tested in tobacco with good success both in generating resistance and in preventing transmission. The constructs were transferred to plum and are still in the process of being evaluated (Ravelonandro *et al.*, 2000). “HoneySweet” makes no detectable coat protein

(Scorza *et al.*, 1994), hence none is available for the potential encapsidation.

The second potential risk with virus interaction is for recombination between viral genomes. Since the mechanism of resistance in “HoneySweet” is PTGS, there are very low levels of RNA in the cytoplasm and similar RNAs are targeting for degradation (Scorza *et al.*, 2001), so presumably any recombinant virus would also be targeted for degradation. The risk of a synergy effect that would enhance the infection of another virus in the presence of the transgene was addressed when Ravelonandro *et al.* (2007) co-infected “HoneySweet” and controls with several different viruses mixed with PPV, including apple chlorotic leaf spot virus (ACLSV), prune dwarf virus (PDV), and *Prunus* necrotic ringspot virus (PNRSV). None of these viruses affected the “HoneySweet” response and resistance to PPV.

Approaches to minimize the spread of transgenes have been developed but have yet to be tested in plum. One approach to prevent the dissemination of transgenes through sexual material is to excise the transgene in pollen and fruit (Mlynárová *et al.*, 2006) or by restricting the transgenes to chloroplasts (Daniell, 2002). Transmission of transgenes to associated microorganisms is another possible mechanism for the release of transgenes. As of yet, no transmission has been documented (Kay *et al.*, 2002; Nielsen and Townsend, 2004).

3.2.2 Human health challenges

The worries about affects of transgenic food are the effect of foreign genes on human health as well as the transmission of genes to microflora in the human gut. The transgenic product consumed will be the fruit for transgenic plums. “HoneySweet” has had fruit analyzed (Scorza *et al.*, 2005, 2007; APHIS, 2006, petition) with no parameters outside the normal range of other nontransgenic plum cultivars. A study has also been done for FDA approval on predicting any new potentially antigenic proteins in C5, again with negative results. The potential new products of “HoneySweet”, NPTII protein and *uidA* protein, do not appear to be a problem (Gilissen *et al.*, 1998; Kuiper *et al.*, 2001). The potential for transfer of those genes to intestinal

organisms is under research from other groups as the marker genes as well as others are in a very large number of transgenic crops already released (Netherwood *et al.*, 2004). A recent European Food Safety Authority statement (<http://www.efsa.europa.eu/en/science/gmo/statements0/npt2.html>), concluded that the likelihood of transferring the *NPTII* gene from plants into the human gut was very low and the possible transfer than into a bacterium was very low with resulting stable incorporation and expression being a very low probability. This low probability accounts for the transfer and expression not being seen under natural conditions. They further conclude that the *NPTII* gene poses neither any risk to human health nor to the environment.

3.3 Expected Technologies

The most desired technology would be a reliable transformation system that is not cultivar specific. This will come as new groups are developing transgenic plum lines and more information is gained about plant hormone ratios in plum (Table 7). Along with a reliable transformation system should come improved selection schemes, like mannose or hygromycin (Dolgov *et al.*, 2005; Mikhailov *et al.*, 2007a) and novel promoters to help regulate the genes of interest and to possibly minimize expression of transgenes in edible parts. With the base knowledge from genomics and expression systems such as microarrays, genes can be identified that will have expression in desired tissues and developmental stages as well as in response to various stimuli. Appropriate promoter sequences can be selected from these. Systems that have been developed in other plants will be transferred to plum, such as systems that excise marker genes or marker-free transformation techniques (Yoder and Goldsbrough, 1994; Ow, 2002; Zhang *et al.*, 2003; Goldstein *et al.*, 2005; López-Noguera *et al.*, 2007). This is all expected technologies as they work in other plant systems and with time will be modified to work with plum.

As with the transformation systems being applied to plum, other current molecular tools are being applied to *Prunus* and can then be utilized for plum in order to identify traits of interest for manipulation and what processes those

traits interact with. There are continuing studies on deducing the *Prunus* genome (<http://www.bioinfo.wsu.edu/gdr>). Data from molecular mapping studies in various *Prunus* crops are being applied to a consensus map (Dirlewanger *et al.*, 2004b) as well as The Rosaceae Consortium of Mapping Populations (RosPOP) (<http://www.mainlab.clemson.edu/gdr/community/international/rospop.shtml>) for quantitative trait loci (QTL) and mapping resources. There will be more microarrays available (ESTree Consortium, 2005) to use to determine RNA expression patterns. With these expected technologies, more complex traits can be analyzed and may find themselves amenable to transgenic breeding.

3.4 Specific Details for Intellectual Property Rights (IPR), Public Perceptions, Industrial Perspectives, Political, and Economic Consequences

IPR issues as well as public acceptance, Industry support and acceptance and the resulting political and economic consequences are important issues to address in the commercial production of transgenic crops. Obviously, these issues have been dealt with in the releases and uses of corn and soybeans with various novel genes for herbicide resistance or insect resistance. The potential commercial releases of transgenic plums are many years behind as are the specific details. At present time, there are no commercial releases of transgenic plums. Only “HoneySweet”, the PPV-resistant transgenic plum has even been patented (Scorza *et al.*, 2004). Future releases will follow the established approval processes by APHIS, EPA, and FDA, and then the specific details for IPR and acceptance can be approached and in general will be no different than for those crops commercially developed prior to plums.

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Berry Crops

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1. INTRODUCTION

1.1 History, Origin, and Distribution

1.1.1 Blueberry and cranberry

Several species of *Vaccinium* are important commercially. Most production comes from species of section *Cyanococcus* including cultivars of *Vaccinium corymbosum* L. (highbush blueberry) and *Vaccinium ashei* (rabbiteye blueberry), and native stands of *Vaccinium angustifolium* (lowbush blueberry). Highbush cultivars are further separated into northern or southern types depending on their chilling requirements and winter hardiness. *Vaccinium macrocarpon* (large cranberry), a member of section *Oxycoccus*, is also an important domesticated species. *Vaccinium myrtillus* (bilberry, whortleberry) in section *Myrtillus* and *Vaccinium vitis-idaea* (lingonberry) in section *Vitis-idaea* are collected primarily from the wild.

All of the wild, edible *Vaccinium* species have been harvested for thousands of years by indigenous peoples. There are suggestions that the Indians of eastern North America intentionally burned native stands of lowbush blueberries to renew their vigor. The cultivation of *Vaccinium* by immigrant Europeans first began in the early 19th century when cranberry farmers in the Cape Cod area of Massachusetts started building dykes and ditches to control the water levels in native stands. Highbush and rabbiteye blueberries were first domesticated at the end of the 19th century. Plants

were initially dug from the wild and transplanted into New England and Florida fields.

Most of the commercial production of blueberry now comes from highbush and lowbush types, although rabbiteyes are important in the North American southeast and hybrids of highbush × lowbush (half-highs) have made a minor impact in the upper midwest of the United States. There is also growing interest in growing rabbiteye cultivars in the Pacific Northwest and Chile. Highbush blueberries are grown in 36 states in the United States, in six Canadian provinces, and in Australia, Chile, Argentina, New Zealand, and a number of countries in Europe. The largest acreages are in Michigan, New Jersey, North Carolina, Oregon, and Washington in the United States, and British Columbia in Canada. There are strong signs that an important highbush industry will be established in California. Commercial production of lowbush blueberries is restricted to Maine, Quebec, and Nova Scotia. Cranberries are grown primarily in Wisconsin, Massachusetts, New Jersey, Washington, and Oregon, with limited plantings in British Columbia, Michigan, Nova Scotia, and Quebec.

1.1.2 Cane fruit

The European red raspberry, *Rubus idaeus* subsp. *vulgaris* was first mentioned in the historical record by Pliny the Elder. He described it as “ida” fruit grown by the people of Troy at the base of

Mount Ida. However, it is likely that these plants originally came from the Ide Mountains of Turkey, as raspberries were not native to Greece (Jennings, 1988). Raspberries gradually grew in popularity over the centuries and by the 1500s, *R. idaeus* subsp. *vulgaris* was cultivated all over Europe. The North American subsp. *strigosus* was introduced into Europe in the early 19th century and natural hybrids with subsp. *vulgaris*, resulted in much advancement. In fact, most red raspberry cultivars dating from this period are hybrids of these two species (Daubeney, 1983; Dale *et al.*, 1993).

The black raspberry of North America, *Rubus occidentalis*, was not cultivated until the 19th century, probably because of its abundance in the wild and difficulties with its propagation (Jennings, 1988). It does not sucker like red raspberries and growers had to learn to propagate it by bending over the stem tips. Most early cultivars of black raspberry were wild selections, and in fact, purple raspberry hybrids of black and red raspberry may have been the first raspberry cultivars that contained black raspberry genes.

The most important European blackberry, *Rubus laciniatus* Willd., was probably domesticated in the late 17th century in England and was imported to the Northwestern coast of North America before 1860 (Jennings, 1988). *R. laciniatus* quickly became feral in the western United States and the widely grown "Thornless Evergreen" was selected from the wild in the early 1930s.

The earliest cultivars of eastern North American blackberries were selected in the 1800s, as forests were actively cleared and numerous wild species spread and hybridized. Three natural hybrids of *Rubus allegheniensis* × *Rubus frondosus*, "Lawton", "Dorchester", and "Texas Early" (Crandell), played a particularly key role in the domestication of the crop, although early cultivars were also selected from pure *R. allegheniensis* ("Snyder"), *Rubus argutus* ("Early Harvest"), and *Rubus ursinus* ("Aughinbaugh"), and interspecies crosses of *R. allegheniensis* × *R. argutus* ("Eldorado"), and *Rubus baileyanus* × *R. argutus* ("Lucretia" and "Austin Mayes").

1.1.3 Strawberries

Fragaria vesca, the alpine strawberry or fraise de bois, was the first strawberry domesticated. It was

originally brought into gardens by the ancient Romans and Greeks, and by the 1300s, was being grown all across Europe (Darrow, 1966). *F. vesca* had its widest popularity in the 1500s and 1600s in Europe before the introduction of strawberry species from the New World. The musky flavored *Fragaria moschata* (Hautbois or Hautboy) was also planted in gardens by the late 15th century, along with the green strawberry, *Fragaria viridis*. *F. viridis* was used solely as an ornamental all across Europe, while *F. moschata* was utilized for its fruit by the English, Germans, and Russians.

F. vesca dominated strawberry cultivation in Europe, until *Fragaria virginiana* from eastern Canada and Virginia began to replace it in the 1600s. All of the clones that arrived in Europe were wild in origin, as the aboriginal peoples of North America did little gardening with strawberries. A Chilean clone of *Fragaria chiloensis* found its way into Europe in the 1700s compliments of a French spy, Captain Amédée Frézier (Darrow, 1966; Wilhelm and Sagen, 1974). It was domesticated 1000 years ago by the indigenous Mapuches and was spread widely by the Spanish during the colonization period. Unfortunately, early reports on the Chilean strawberry were negative, as the plants were largely barren because Frézier had inadvertently brought back staminate plants. French horticulturalists solved the problem when they discovered that the "Chili" would produce fruit when pollinated by *F. moschata* or *F. virginiana*. The Chilean strawberry reached its highest acclaim in Brittany, and by the mid-1800s, there was probably more *F. chiloensis* cultivated in France than its native land.

Unusual seedlings began to appear in Brittany and other gardens with unique combinations of fruit and morphological characteristics. While the origin of these seedlings was initially clouded, the great French Botanist Antoine Nicholas Duchesne determined in 1766 that they were hybrids of *F. chiloensis* × *F. virginiana* and he named them *Fragaria* × *ananassa* to denote the perfume of the fruit that smelled like pineapple (*Ananas*). It is not clear where the first hybrids of the Pineapple or Pine strawberry appeared, but they must have arisen early in the commercial fields of Brittany, and in botanical gardens all across Europe.

Formal strawberry breeding was initiated in England in 1817 by Thomas A. Knight. He was

one of the first systematic breeders of any crop, and used clones of both *F. virginiana* and *F. chiloensis* in his crosses. Thomas Laxton of England was the most active breeder during the later part of the 18th century and released a number of important cultivars including “Noble” and “Royal Sovereign”. Charles Hovey, of Cambridge, Massachusetts, produced the first important North American cultivar, “Hovey.” It was the first cultivar of any fruit to come from an artificial cross in America and for a while made the strawberry the major pomological product in the country (Hedrick, 1925).

The dessert strawberry, *F. × ananassa*, now dominates strawberry cultivation and is grown in all arable regions of the world. *F. vesca* is generally restricted to home gardens where its small, aromatic fruits are considered a delicacy; most of the cultivars grown are everbearers. *F. chiloensis* is currently grown to a small extent in Chile, but has been largely replaced by *F. × ananassa*. Neither *F. viridis* nor *F. moschata* is of any current commercial importance.

Most of the strawberries of *F. × ananassa* grown across the world are bred to fruit under short-day conditions, although day-neutral types dominate in California and other Mediterranean climates (Hancock, 1999). Two major production systems are utilized in the world—hills and matted rows. The matted row system employs runners as the primary yield component. Both mother and daughter plants are allowed to runner freely, with periodic training into narrow rows. The hill or “plasticulture system” relies on crowns as the primary yield component, and any runners that form are removed. The hill system is used primarily in areas having warm winters and either hot or moderate summers such as California, Florida, Italy, and Spain. Matted rows are used to grow short-day cultivars in climates with short summers and cold winters, such as continental Europe and northern North America.

1.2 Botanical Description

1.2.1 Blueberry and cranberry

The genus *Vaccinium* is very widespread, with high densities of species being found in the Himalayas,

New Guinea, and the Andean region of South America. The origin of the group is thought to be South American. Estimates of species numbers vary from 150 to 450 in 30 sections (Luby *et al.*, 1991). The taxonomy of the commercially important sections *Cyanococcus*, *Oxycoccus*, *Vitis-idea*, and *Myrtillus* (Table 1) has been difficult to resolve due to complex polyploidy series ($x = 12$) and a general lack of chromosome differentiation and crossing barriers within sections. The primary mode of speciation has been through unreduced gametes.

All the polyploid *Cyanococcus* are likely to be of multiple origins and active introgression between species is ongoing. Most homoploids freely hybridize and interploid crosses are frequently successful, through unreduced gametes. The tetraploid highbush blueberry *V. corymbosum* has been shown to be an autopolyploid (Krebs and Hancock, 1989). Vander Kloet (1980) found the variation pattern in highbush and rabbiteye to be so complex that he placed all crown forming section taxa ($2x$, $4x$, and $6x$) under the name *V. corymbosum*. Most horticulturalists still distinguish the hexaploid *V. ashei* from tetraploid *V. corymbosum*, and continue to recognize at least one distinct diploid, *Vaccinium elliottii* (Luby *et al.*, 1991). The lowbush blueberry, *V. angustifolium* appears to be a direct descendant of *Vaccinium pallidum × Vaccinium boreale*, but introgression with *V. corymbosum* may have also influenced its subsequent development (Vander Kloet, 1977).

V. macrocarpon is an endemic of eastern North America and is thought to be the most primitive species in section *Oxycoccus* (Camp, 1945). Its closest relatives are diploid, tetraploid, and hexaploid races of *Vaccinium oxycoccus* that have circumboreal distribution. Gene exchange is now severely limited between the species due to a disjunct distribution and a flowering date difference of 3 weeks (Vander Kloet, 1988). *V. myrtillus* is very similar to *Vaccinium scoparium* and may have been derived in the Rocky Mountains of North America (Camp, 1945). There has been little speculation about the origin of *V. vitis-idea*, but it must be closely related to *V. myrtillus*, since hybrids have been discovered between these two species at numerous locations across northern Europe (Luby *et al.*, 1991).

Table 1 Species of blueberries and cranberries (*Vaccinium*) $2n = 2x = 24^{(a)}$

Section	Species	Ploidy	Location
<i>Cyanococcus</i>	<i>V. boreale</i> Hall and Aald.	2x	Northeastern North America
	<i>V. myrtilloides</i> Michx.	2x	Central North America
	<i>V. pallidum</i> Ait.	2x, 4x	Mid-Atlantic North America
	<i>V. tenellum</i> Ait.	2x	Southeastern North America
	<i>V. darrowii</i> Camp	2x	Southeastern North America
	<i>V. corymbosum</i> L.	2x	Southeastern North America
	<i>V. elliotii</i> Chapm.	2x	Southeastern North America
	<i>V. angustifolium</i> Ait.	4x	Northeastern North America
	<i>V. hirsutum</i> Buckley	4x	Southeastern North America
	<i>V. myrsinites</i> Lam	4x	Southeastern North America
	<i>V. corymbosum</i> L.	4x	Eastern North America
	<i>V. ashei</i> Reade	6x	Southeastern North America
	<i>V. macrocarpon</i> Ait.	2x	North America
	<i>V. oxycoccus</i> L.	2x, 4x, 6x	Circumboreal
<i>Vitis-Idaea</i>	<i>V. vitis-idaea</i> L.	2x	Circumboreal
<i>Myrtilus</i>	<i>V. myrtilus</i> L.	2x	Circumboreal
	<i>V. scoparium</i> Leiberg ex Coville	2x	Northwestern North America
	<i>V. caespitosum</i> Michx.	2x	North America
	<i>V. deliciosum</i> Piper	4x	Northwestern North America
	<i>V. membranaceum</i> Dougl. Ex Hook	4x	Western North America
	<i>V. parvifolium</i> Smith in Rees	2x	Northwestern North America
	<i>V. chamissonis</i> Bong.	2x	Circumboreal
	<i>V. ovalifolium</i>	4x	Northwestern North America
	<i>V. uliginosum</i> L.	2x, 4x, 6x	Circumboreal

^(a)Source: Luby *et al.*, 1991; Galletta and Ballington, 1996

1.2.2 Cane fruit

Raspberries and blackberries are in the genus *Rubus* of the Rosaceae. There are 12 subgenera recognized within *Rubus*, with the domesticated raspberries being found in the *Eubatus* and *Cylactis* (Table 2), while the domesticated blackberries are found in *Idaeobatus*, *Caesii*, *Suberecti*, and *Corylifolii* (Table 3). *Eubatus* are found in two geographically distinct sections, the *Moriferi* (Europe and Asia) and *Ursini* (western North America). *Cylactis* has an arctic and subarctic distribution. The *Corylifolii* species are located in eastern North America, being mostly concentrated in the southern United States. The *Caesii* contains only one species, *Rubus caesii*, which is found in Europe and Asia. Species of the *Suberecti* are located in the United Kingdom, Scandinavia, and North America. These species may be derivatives of *R. caesius* crossed with *R. idaeus*. Genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) have been utilized to

distinguish between raspberry and blackberry chromosomes, and identify translocations (Lim *et al.*, 1998).

The subgenus *Idaeobatus* contains about 200 wild species with nine sections. Almost all of the raspberry species in the *Idaeobatus* subgenera are diploid ($2n = 14$), with a few triploid and tetraploid types. *Idaeobatus* species are concentrated in northern Asia, but are also located in east and South Africa, Europe, and North America (Jennings, 1988). The greatest diversity is found in southwest China, which may be the center of origin of the subgenus.

The most important commercial taxa of *Idaeobatus* are: (1) the European red raspberry (*R. idaeus* ssp. *vulgatus* Arrhen.), (2) the North American red raspberry (*R. idaeus* ssp. *strigosus* Michx.), (3) the North American red raspberry (*R. strigosus* spp. *strigosus* Michx.), and (4) the black raspberry (*R. occidentalis* L.). Crosses between black and red raspberries have produced the purple raspberries (*R. neglectus* Peck). The

Table 2 Representative blackberry species. $2x = 2n = 14^{(a)}$

Subgenus	Species	Ploidy	Location
<i>Eubatus</i>	<i>R. bartoni</i> Newton		Europe
	<i>R. laciniatus</i> Willd.	4x	Europe
	<i>R. macropetalus</i>	12x	Western North America
	<i>R. nitidioides</i> Wats.	4x	Europe
	<i>R. procerus</i> Muell	4x	Europe
	<i>R. ruticanus</i> var. <i>inermis</i> E. Merc.	2x	Europe
	<i>R. thysiger</i> Banning and Focke	4x	Europe
	<i>R. ulmifolius</i> Schott.	2x	Europe
	<i>R. ursinis</i> Cham. et Schlecht	8x	Western North America
	<i>R. vitifolius</i> Cham. et Schlecht	8x	Western North America
<i>Caesii</i>	<i>R. caesius</i> L.	2x	Europe
<i>Corylifolii</i>	<i>R. canadensis</i> L.	3x	Eastern North America
	<i>R. rubrisetus</i> Rydb.	2x	North America
	<i>R. baileyanus</i> Britt.	4x	Eastern North America
<i>Idaeobatus</i>	<i>R. idaeus</i> ssp. <i>Vulgatus</i> Arrhen	2x	Europe
	<i>R. idaeus</i> ssp. <i>Strigosus</i> Michx.	2x	North America
	<i>R. glaucus</i> Benth.	4x	South America
<i>Orobatus</i>	<i>R. macrocarpus</i> Benth.	6x	South America
<i>Suberecti</i>	<i>R. allegheniensis</i> Porter	2x	Eastern North America
	<i>R. argutus</i> Link	2x, 4x	Eastern North America
	<i>R. cuneifolius</i> Pursh.	2x	Eastern North America
	<i>R. setosus</i> Bigel	2x	Eastern North America
	<i>R. frondosus</i> Bigel	4x	Eastern North America
	<i>R. triviales</i> Michx.	2x	Eastern North America

^(a)Source: Jennings, 1988; Daubeney, 1996

important arctic raspberries *R. arcticus* L. and *R. stellatus* Sm. are contained in *Cylactis*, which is closely related to the *Idaeobatus*. The Andean blackberry, *R. glaucus* Benth., is also classified in the *Idaeobatus*, even though its fruit adhere to the receptacle. It is likely a natural allotetraploid hybrid of a diploid black raspberry and a diploid South American blackberry, perhaps *R. bogotensis* (Jennings, 1988).

The major commercial taxa of raspberries share a considerable amount of interfertility. The red raspberries of Europe, *R. idaeus*, and the American *R. strigosa*, are completely interfertile and should probably be considered the same species. *Rubus arcticus* and *R. stellatus* are also highly interfertile and have been jointed as subspecies of *R. arcticus* (ssp. *arcticus* and ssp. *stellatus*). Hybrids between these two species are more commonly cultivated than pure species types (Jennings *et al.*, 1989). The cross of *R. idaeus* × *R. occidentalis* is only successful if *R. occidentalis* is used as the female parent, although bud pollination and heat treatment can help

overcome this unilateral incompatibility (Hellman *et al.*, 1982). At least 40 additional species in *Idaeobatus* have also been used in raspberry breeding, along with a few species in the *Cylactis*, *Anoplobatus*, *Chamaemorus*, and *Malachobatus* (Daubeney, 1996).

Blackberry cultivars generally have complex backgrounds and are often composed of species at several ploidy levels in four subgenera: *Eubatus*, *Caesii*, *Suberecti*, and *Corylifolii*. Four diverse groups of blackberries have been domesticated: (1) the European blackberries that were derived from a group of diploid and polyploidy species ($2n = 28, 42$, and 56). The background of the European cultivars is so complex that the designation *R. fruticosus* L. agg. is sometimes used (Daubeney, 1996), (2) erect blackberries and trailing dewberries domesticated from mostly diploid and tetraploid species ranging across eastern America, and (3) trailing black raspberries generated from only polyploid species from western America ($2n = 56, 84$). Blackberries and raspberries have also been hybridized to produce intersectional

Table 3 Representative raspberries species. $2x = 2n = 14^{(a)}$

Subgenus	Species	Ploidy	Location
<i>Idaeobatus</i>	<i>R. biflorus</i> Buch.	2x	China
	<i>R. cockburnianus</i> Hemel.	2x	China
	<i>R. corchorifolius</i> L.	2x	China
	<i>R. crataegifolius</i> Bunge	2x	China
	<i>R. coreanus</i> Mig.	2x	China
	<i>R. flosculosus</i>	2x	China
	<i>R. kuntzeanus</i>	2x	China
	<i>R. idaeus</i> subsp. <i>vulgaris</i> Arrhen	2x	Europe
	<i>R. idaeus</i> subsp. <i>strigosus</i> Michx.	2x	North America
	<i>R. innominatus</i> S. Moore	2x	China
	<i>R. glaucus</i> Benth.	2x	South America
	<i>R. lasiostylus</i> Focke	2x	China
	<i>R. macropetalus</i> Dough.	12x	Western North America
	<i>R. mesogaeus</i>		China
	<i>R. niveus</i> Thurb.	2x	India, Asia
	<i>R. occidentalis</i> L.	2x	Eastern North America
	<i>R. parvifolius</i> L.	2x, 4x	Japan, China
	<i>R. phoenicolasius</i> Maxim.	2x	Japan
	<i>R. pileatus</i> Folke	2x	Europe
	<i>R. pungens</i> Cambess.	2x	Indonesia
	<i>R. sachalinensis</i> Leveille	4x	East Asia
	<i>R. spectabilis</i> Pursh.	2x	Western North America
	<i>R. vulgatus bushii</i> Rosanova	4x	Caucasus
<i>Anoplobatus</i>	<i>R. odoratus</i> L.	2x	Eastern North America
<i>Chamaemorus</i>	<i>R. chamaemorus</i> L.	8x	Circumpolar/Subarctic
<i>Cylactis</i>	<i>R. acaulis</i> Michx.	2x	Alaska/Yukon
	<i>R. arcticus</i> L.	2x	Scandinavia
	<i>R. stellatus</i> Sm.	2x	Alaska/Yukon
<i>Orobatus</i>	<i>R. macrocarpus</i> Benth.	6x	South America

^(a)Source: Jennings, 1988; Daubeny, 1996; Finn *et al.*, 2002

hybrids such as Loganberries and tayberries ($2n = 42$), and Boysenberries and youngberries ($2n = 49$).

1.2.3 Strawberry

The strawberry belongs to genus *Fragaria* in the Rosaceae. Its closest relatives are *Duchesnea* and *Potentilla*. There are four basic fertility groups in *Fragaria* that are associated primarily with their ploidy level or chromosome number. The most common native species, *F. vesca*, has 14 chromosomes and is considered a diploid; its genome size is relatively small at 164 Mb (Akiyama *et al.*, 2001). The most important cultivated strawberry, *F. × ananassa*, is an octoploid with 56 chromosomes. It is an accidental hybrid of *F. chiloensis* and *F. virginiana* that arose in the mid-1700s when plants of *F. chiloensis* from Chile were

planted in France next to *F. virginiana* from the eastern seaboard of the United States.

An accurate taxonomy of the strawberry species is still emerging (Table 4). While the European and American species of *Fragaria* have been rigorously defined by Staudt (1959, 1989), the species situation in Asia is much more ambiguous (Hummer, 1995). There is no overall consensus of what species exist in China and published descriptions have sometimes relied on limited collections. Diploid, tetraploid, and hexaploid species are found in Europe and Asia, but octoploids are restricted to the New World and perhaps the Iturup Island northeast of Japan (Staudt, 1989). Only one diploid species, *F. vesca*, is located in North America. The genomic complement of the octoploids is likely AAA'A'BBB'B' (Bringhurst, 1990), with *F. vesca* likely being the A genome donor. The B genome donor has not been clearly elucidated, although

Table 4 Strawberry (*Fragaria*) species of the world. $2n = 2x = 14^{(a)}$

Species	Ploidy	Location
<i>F. vesca</i> L.	2x	Worldwide
<i>F. viridis</i> Duch.		Europe and Asia
<i>F. nilgerrensis</i> Schlect.		Southeastern Asia
<i>F. daltoniana</i> J. Gay		Himalayas
<i>F. nubicola</i> Lindl.		Himalayas
<i>F. innumae</i> Makino		Japan
<i>F. yesoensis</i> Hara. ^(a)		Japan
<i>F. mandshurica</i> Staudt		North China
<i>F. nipponica</i> Lindl. ^(a)		Japan
<i>F. gracilisa</i> A. Los.		North China
<i>F. pentaphylla</i> Lozinsk		North China
<i>F. corymbosa</i> Lozinsk		North China
<i>F. orientalis</i> Losinsk	4x	Russian Far East/China
<i>F. moupinensis</i> (French.) Card		North China
<i>F. × bringhurstii</i> Staudt	5x	California
<i>F. moschata</i> Duch.	6x	Euro-Siberia
<i>F. chiloensis</i> (L.) Miller	8x	Western North America and Chile
<i>F. virginiana</i> Miller		North America
<i>F. iturupensis</i> Staudt		Iturup Island
<i>F. × ananassa</i> Duchesne in Lamarck		Worldwide

^(a) According to Staudt, *F. nipponica* and *F. yesoensis* are the same species

molecular evidence is accumulating that Japanese *F. innumae* may be it (Davis and DiMeglio, 2004).

The most likely origin of the octoploids is that they originated in northeastern Asia when *F. vesca* combined with other unknown diploids, and the polyploid derivatives then migrated across the Bering Strait and dispersed across North America (Hancock, 1999). It is possible that *F. chiloensis* and *F. virginiana* are extreme forms of the same biological species, separated during the Pleistocene, which subsequently evolved differential adaptations to coastal and mountain habitats. The two species are completely interfertile, carry similar cpDNA restriction fragment mutations (Harrison *et al.*, 1997) and have very similar nuclear internal transcribed spacer (ITS) regions (Potter *et al.*, 1997).

Polyploidy in *Fragaria* probably arose through the unification of $2n$ gametes, as several investigators have noted that unreduced gametes are relatively common in *Fragaria* (Hancock, 1999). Staudt (1984) observed restitution in microsporogenesis of a F_1 hybrid of *F. virginiana* × *F. chiloensis*. In a study of native populations of *F. chiloensis* and *F. vesca*, Bringhurst and Senanayake (1966) found frequencies of giant pollen grains to be approximately 1% of the total.

Over 10% of the natural hybrids generated between these two species were the result of unreduced gametes.

The inheritance patterns of the octoploids are somewhat in dispute. Lerceteau-Köhler *et al.* (2003) concluded that *F. × ananassa* has mixed segregation ratios using amplified fragment length polymorphism (AFLP) markers, as they found the ratio of coupling versus repulsion markers fell between the fully disomic and polysomic expectations. However, two other studies evaluating isozyme, simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) segregation observed predominantly disomic ratios, indicating that the octoploid strawberry is completely diploidized (Arulsekaran and Bringhurst, 1981; Ashley *et al.*, 2003).

While there appear to be some barriers to interfertility among the diploid strawberries, they all can be crossed to some extent, and meiosis in the hybrids is regular, even in cases where the interspecific hybrids are sterile (Hancock, 1999). There are at least three overlapping groups of diploid species that are interfertile (Bors and Sullivan, 1998): (1) *F. vesca*, *F. viridis*, *F. nubicola*, and *F. pentaphylla*, (2) *F. vesca*, *F. nilgerrensis*, *F. daltoniana*, and *F. pentaphylla*, (3) *F. pentaphylla*,

F. gracilis, and *F. nipponica*. *Fragaria iinumae* may belong in group 3 or in an additional group, as no fertile seeds have been recovered when it was crossed with either *F. vesca*, *F. viridis*, or *F. nubicola*, but it has not been crossed with enough other species to accurately classify it. *Fragaria iinumae* does, however, have a glaucous leaf trait that is unique among the diploids, and its chloroplast RFLPs cluster it with *F. nilgerrensis* in a group that is isolated from the rest (Harrison *et al.*, 1997).

The incorporation of traits from a number of lower ploid species has been accomplished through pollinations with native unreduced gametes or by artificially doubling chromosome numbers. The utility of this approach has been shown for a wide range of species in *Fragaria* and in the related genus *Potentilla* (Hancock, 1999). Particular success in incorporating lower ploidies into the background of *F. × ananassa* has come through combining lower ploidy species and then doubling to the octoploid level (Bors and Sullivan, 1998).

1.3 Economic Importance

1.3.1 Blueberry and cranberry

The highbush blueberry is by far the most important commercial crop in *Vaccinium*, with over 85 000 tons of highbush fruits being produced annually on over 20 000 ha (USDA Agricultural Statistics). The estimated area of rabbiteye production is currently about 3000 ha, with half the acreage in Georgia. The total annual production is over 5500 tons. Half-high production is restricted to a few hundred hectares in Minnesota and Michigan. Annual production of lowbush blueberries ranges from 25 000 to 40 000 tons on about 40 000 ha in primarily Maine and the Maritime provinces of eastern Canada. Cranberry production is about 170 000 tons annually on 9000 ha in Massachusetts, Oregon, and Michigan.

Blueberries and bilberries are eaten both as dessert fruits and in processed forms. About 50% of the highbush crop is marketed fresh and the remainder is processed. Blueberries are used primarily in pie fillings, yogurts, ice cream, and prepared muffin and pancake mixes. Syrups, jams, and preserves are also produced, but in limited quantities. The juice of blueberries is rarely

consumed directly as it has a very strong flavor and dark color. The fruits of cranberries are very tart and for this reason are mostly processed into juices or processed in baked goods. Blueberries and cranberries are sometimes added to dried products after dehydration using an explosion-puffing process.

An average blueberry fruit is composed of approximately 83% water, 0.7% protein, 0.5% fat, 1.5% fiber, and 15.3% carbohydrate (Hancock *et al.*, 2003). Cranberries contain 88% moisture, 0.2% protein, 0.4% fat, 1.6% fiber, and 7.8% carbohydrate. Blueberries have 3.5% cellulose and 0.7% soluble pectin, while cranberries contain 1.2% pectin. The total sugars of blueberries amount to more than 10% of the fresh weight, and the predominant reducing sugars in blueberries are glucose and fructose, which represent 2.4%. The edible portion of the cranberry is composed of 2.66% glucose, 0.74% fructose, and 0.14% sucrose. Its pulp contains measurable amounts of lignin, glucose, arabinose, and xylose.

The overall acid content of *Vaccinium* fruit is relatively high. Ripe cranberries range from 2% to 3%, while blueberries fall in the range of 1–2%. The primary organic acid in blueberries is citric acid (1.2%). They also contain significant amounts of ellagic acid, a compound thought to reduce the risk of cancer (Maas *et al.*, 1991). The cranberry contains high levels of several organic acids, including quinic (1.3%), citric (1.1%), malic (0.9%), and benzoic (0.6%). Ingestion of cranberries leads to increased acidity of the urine through conversion of its high quinic and benzoic acid contents to hippuric acid by the body. The high acidity and possible antibacterial effects of hippuric acid may relieve urinary tract infections and reduce some types of kidney stones.

Compared to other fruits and vegetables, blueberries and cranberries have intermediate to low levels of vitamins, amino acids, and minerals (Hancock *et al.*, 2003). Blueberries contain 22.1 mg of vitamin C per 100 g of fresh weight and cranberries contain 7.5–10.5 mg. Blueberries are unusual in that arginine is their most prominent amino acid.

In general, blueberries are one of the richest sources of antioxidant phytonutrients among the fresh fruits, with total antioxidant capacity ranging from 13.9 to 45.9 μmol Trolox equivalents per gram fresh berry (Ehlenfeldt and Prior,

2001; Connor *et al.*, 2002a, b). Berries from the various *Vaccinium* species contain relatively high levels of polyphenolic compounds, with chlorogenic acid predominating. Total anthocyanins (ACYs) in blueberry fruit range from 85 to 270 mg per 100 g, and species in the subgenus *Cyanococcus* carry the same predominant ACYs, aglycones, and aglycone-sugars, although the relative proportions vary (Ballington *et al.*, 1988). The predominant ACYs were delphinidin-monogalactoside, cyanidin-monogalactoside, petunidin-monogalactoside, malvidin-monogalactoside, and malvidin-monoarabinoside.

Among the other *Vaccinium*, cranberries have total ACYs varying from 25 to 100 mg per 100 g fruit, with the most important ACYs being cyanidin-3-monogalactoside, peonidin-3-monogalactoside, cyanidin-3-monoarabinoside, and peonidin-3-monoarabinoside. The various *Ericaceae* species also contain appreciable amounts of several carotenoids.

The major volatiles contributing to the characteristic aroma of blueberry fruit are *trans*-2-hexanol, *trans*-2-hexanal, and linalool (Hancock *et al.*, 2003). The predominant volatiles in the bilberry are *trans*-2-hexanal, ethyl-3-methyl butyrate, and ethyl-2-methyl butyrate. In the cranberry, 2-methyl butyrate is rare, but α -terpineol predominates. Benzaldehyde also contributes to the aroma of the cranberry.

1.3.2 Cane fruit

Raspberries are grown in 37 countries on over 70 000 ha; worldwide production is over 414 000 metric tons, with a 38% increase over the last 10 years (FAO, 2002). Russia and Serbia now account for almost 50% of the worldwide raspberry production, with the United States having about 13% and Poland about 11%. Over 95% of the raspberry hectareage is under red cultivars. The major production areas in North America are the Pacific Northwest (Oregon, Washington, and British Columbia), California, and the eastern United States (New York, Michigan, Pennsylvania, and Ohio). Mexico and Chile export significant amounts of raspberries to North America from November to May.

The highest production of blackberries and hybrid berries is in North America (42%) and Europe (30%) (Strik *et al.*, 2006). Serbia, Oregon, Hungary, and California are the leading production regions, with significant hectareage also being found in China, Guatemala, Spain, Canada, and Australia. Worldwide production of blackberries and hybrid berries is over 140 000 metric tons on about 20 000 ha. Worldwide blackberry production has almost doubled over the last 10 years.

Raspberries are commonly eaten fresh, individually quick frozen, and processed into juice, yogurt, jam, and flavorings. Raspberry juice is generally blended with apple, pear, or grape juice, as the flavor is too intense for direct consumption. Most blackberries and hybrid berries are processed into preserves, jams, jellies, and pastry fillings. Quick-frozen blackberries are also gaining in popularity.

Raspberry fruits are composed primarily of water (87%), with pectins making up 0.1–1.0% of the remaining soluble fraction (Green, 1971). A typical raspberry is composed of 5–6% sugar, with the main sugars being glucose, fructose, and to a lesser degree sucrose. One hundred gram of fresh raspberries have 0.42–1.40 g of protein, 0.20–0.55 g of fat, 5.8–11.6 g of carbohydrate, 3.0–7.4 g of fiber, 0.40–0.51 g of ash, and contain 31–49 kcal of food energy.

Raspberry fruits contain only small amounts of most vitamins, although vitamin C is found in significant levels. The predominant amino acids include alanine, asparagine, aspartic acid, glutamic acid, γ -aminobutyric acid, leucine, serine, and valine. Raspberry juice contains from 0.10% to 0.14% polyphenols; most of these being catechin, chlorogenic, ferulic, and neochlorogenic acids. The color of raspberries is imparted primarily by ACYs, with cyanidin and pelargonidin glucosides predominating (Jennings and Carmichael, 1980). Up to six sugars may be attached at the 3-position-one monoglycoside, three diglycosides and two triglycosides. The cyanidin glucosides predominate in raspberries and blackberries.

A considerable amount of recent research has documented variation patterns in the antioxidant capacity of *Rubus* species and crosses. Deighton *et al.* (2000) measured the antioxidant properties of domesticated and wild species, along with total phenol, ACY, and ascorbic acid contents. Antioxidant capacities ranged from 0 to 25.3 μ mol

Trolox equivalents per gram, with *R. caucasicus* having the highest values. Ascorbic acid and ACYs had only a minor influence on antioxidant capacity. Moyer *et al.* (2002) found that the western blackberry species *R. ursinus* and its derivatives were higher in antioxidant capacity than the eastern blackberry cultivars and most European *Rubus* species. In a comparison of antioxidant capacity in cultivars of thornless blackberry, red raspberry, and black raspberry, Wang and Lin (2000) found black raspberries to have the highest antioxidant capacity, followed by blackberries and red raspberries.

A large number of volatile compounds are found in raspberry fruit (Shamaila *et al.*, 1993; Moore *et al.*, 2002). One of the most abundant ketones, 1-(p-hydroxyphenyl)-3 butanone produces an odor very characteristic of raspberry (Larsen and Poll, 1990). Other important volatile compounds include alcohols, acids, esters, carbonyls, and naphthalene. Numerous aromas have also been identified in blackberry, although no single volatile has been described as characteristically blackberry (Klesk and Qian, 2003).

1.3.3 Strawberry

Strawberries are the most important soft fruits grown worldwide. The cultivated strawberry, *F. × ananassa*, is a regular part of the diets of millions of people and is cultivated in all arable regions of the globe from the arctic to the tropics. Annual world production of strawberries has steadily grown through the ages, with quantities doubling in the last 20 years to over 3.2 million metric tons. Most of the production is located in the northern hemisphere (98%), but there are no genetic or climatic barriers preventing greater expansion into the southern hemisphere.

The United States is the leading producing nation with approximately 25% of the world's crop, followed by Spain, Japan, Poland, Italy, and the Korean Republic. California dominates the strawberry industry in the United States with over 80% of the total production. The industries in Spain, the Korean Republic, and the United States have grown steadily over the last two decades, while production in Japan, Italy, and Poland have declined in the last decade, after dramatic increases in the 1970s and 1980s.

The strawberry is widely appreciated for its delicate flavor, aroma, and nutritional value. Ripe strawberries are composed of approximately 90% water and 10% total soluble solids (Hemphill and Martin, 1992), and contain numerous important dietary components. They are extremely high in vitamin C and a standard serving of strawberries (10 fruit) supplies 95% of the recommended dietary requirements (National Academy of Sciences, 1989; Maas *et al.*, 1996). The main soluble sugar components in strawberries are glucose and fructose, which are over 80% of the total sugars and 40% of the total dry weight (Wrolstad and Shallenberger, 1981). The primary organic acid is citric acid, which composed 88% of the total acids (Green, 1971). The strawberry also contains significant levels of ellagic acid, which is thought to be an anticarcinogenic (Maas *et al.*, 1991).

Red color develops through the production of ACYs, primarily pelargonidin-3-glucosidase (Wrolstad *et al.*, 1970; Kalt *et al.*, 1993), although at least eight pelargonidin- and two cyanidin-based ACYs have been detected in strawberry juice (Bakker *et al.*, 1994). Cyanidin 3-glucoside is the second most common ACY. The total concentration of ACYs varies 16-fold across cultivars, and there is some variation in ACY composition, although no clear associations between individual ACYs and color have been observed (Bakker *et al.*, 1994).

Glucose, fructose, and sucrose are the major soluble sugars found in the fruit of strawberries during all stages of ripening. Glucose and fructose are found in almost equal concentrations (Maas *et al.*, 1996), and they rise continuously during fruit development from 5% in small green fruit to 6–9% in red berries (Kader, 1991). Sucrose levels are generally much lower, and show little accumulation until about the middle of fruit development (Forney and Breen, 1986). Invertases probably play an important role in determining sweetness, by regulating hexose and sucrose levels (Ranwala *et al.*, 1992; Manning, 1998).

Strawberry flavor is a complex combination of sweetness, acidity, and aroma. The most intensely flavored fruits generally have high levels of both titratable acidity (TA) and soluble solids, while the blandest fruit are low in both these components (Kader, 1991). The primary components of flavor have not been completely elucidated, but strawberry aroma is thought to originate from a

complex mixture of esters, alcohols, aldehydes, and sulfur compounds (Schreier, 1980; Dirinck *et al.*, 1981; Pérez *et al.*, 1996). Hundreds of volatile esters have been correlated with strawberry ripening and aroma development, with methyl- and ethyl-esters of butanoic and hexanoic acids being among the most prevalent (Larsen and Poll, 1992; Pérez *et al.*, 1992, 1996). Other components in high concentration are trans-2-hexenyl acetate, trans-2-hexenal, trans-2-hexenol, and 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furanol). Concentrations of these volatiles vary widely among cultivars and produce large variations in aroma quality (Hirvi, 1983; Shamaila *et al.*, 1992; Pérez *et al.*, 1996, 1997).

Aroma and fragrance content also varies across species. Several researchers consider 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furanol) and 2,5-dimethyl-4-methoxy-3(2H) furanone (mesifurane) as being particularly important aroma contributors (Pyysalo *et al.*, 1979; Larsen and Poll, 1992; Sanz *et al.*, 1994; Pérez *et al.*, 1996). The wild species *F. vesca* and *F. virginiana* have much stronger aroma than the cultivated types (Hirvi and Honkanen, 1982). *F. vesca* contains high amounts of ethyl-acetate, but low amounts of methyl butyrate, ethyl butyrate, and furanone. *Fragaria nilgerrensis* contains high levels of ethyl acetate and furanone, but low levels of methyl butyrate and ethyl butyrate. Hybrids between *F. vesca* and *F. × ananassa* have intermediate levels of fragrance and aroma, while crosses between *F. nilgerrensis* and *F. × ananassa* more closely resemble *F. nilgerrensis*.

1.4 Traditional Breeding

1.4.1 Blueberry and cranberry

Blueberry breeding is a very recent development. Highbush breeding began in the early 1900s in New Jersey, with the first hybrid being released in 1908 by F. Coville of the United States Department of Agriculture (USDA). He conducted the fundamental life history studies of the blueberry that served as the basis of cultivation such as soil pH requirements, cold and day-length control of development, pruning strategies, and modes of propagation. Working with Elizabeth White and others, he collected several outstanding wild clones of *V. corymbosum* and *V. angustifolium*,

which he subsequently used in breeding improved types. Over 75% of the current blueberry acreage is still composed of his hybrids, most notably “Bluecrop”, “Jersey”, “Weymouth”, “Croatan”, “Blueray”, “Rubel”, and “Berkeley.”

G. Darrow took over the USDA program and made important contributions on the crossability and speciation of the native species, and began working on the development of southern highbush types with R. Sharp in Florida. Out of that work came the important cultivar Sharpblue, which was grown commercially until very recently. P. Lyrene took over the breeding work when R. Sharp retired and has released a number of important cultivars including “Misty” and “Star.” These cultivars are known for their high fruit quality and plant vigor.

A. Draper eventually took over the breeding work at the USDA and focused on mixing the genes of most wild *Vaccinium* species into the cultivated highbush background. He released a prodigious number of southern and northern highbush cultivars, with improved fruit color and firmness, smaller pedicle scars, and higher productivity (Hancock and Galletta, 1995). His “Duke” and “Elliott” have been major successes, and several of his other cultivars show great promise including “Nelson” and “Legacy.”

Numerous other highbush breeding programs have made substantial contributions over the last 50 years. S. Johnson at Michigan State University was one of the first highbush breeders outside the USDA and worked hard to improve the cold tolerance of highbush by crossing it with *V. angustifolium*. The strategy did not work, but he and his replacement, J. Moulton, produced two important cultivars, “Bluejay” and “Northland.” J. Hancock is now at MSU and has released three new northern highbush cultivars that show high promise, “Aurora”, “Draper” and “Liberty.” J. Moore and now J. Clark in the University of Arkansas have maintained a healthy interest in southern wild species as gene donors, and recently released “Ozarkblue”, a very high quality late type. In North Carolina, J. Ballington has worked at the climatic interface between northern and southern highbush types, and has generated a number of exciting cultivars including the Southern highbush type “O’Neal”, and the likely intermediate types “Reveille”, “Arlen” and “Summit.”

Several other highbush breeding projects have produced attractive cultivars. The ones making

the biggest impact are “Brigitta Blue” selected by D. Jones and R. Bell in Australia from open-pollinated seeds collected by Johnston, and “Reka” and “Nui” released by N. Patel in New Zealand.

Rabbiteye breeding was initiated in the 1940s by G. Darrow in collaboration with O. Woodward at the Georgia Agricultural Experiment Station and E.B. Morrow at the North Carolina Experiment Station. This work was continued by M. Austin and S. Nesmith in Georgia, G. Galletta and Ballington in North Carolina, and R. Sharp and P. Lyrene in Florida. These breeding programs have resulted in significant improvements in fruit color, size, texture, and attractiveness over the original wild selections. The most important cultivars have been “Tifblue” (1955) and “Climax” (1975) from Georgia and “Powderblue” and “Primier” (1978) from North Carolina.

Cranberries have been bred sporadically since the mid-1900s, although most of the crop is still produced from the wild selections “Early Black”, “Howes”, “McFarlin” and “Searles”. In 1929, the USDA began a cooperative cranberry breeding project between the New Jersey, Massachusetts, and Wisconsin Departments of Agriculture. Out of this program came “Pilgrim” with improved size, color (purplish red), keeping quality, productivity, and resistance to the blunt-nosed leafhopper and “Stevens” with improved color (deep red), firmness, and resistance to softening. “Crowley” was introduced from the Washington Agriculture Station in the 1960s as a better pigmented replacement for “McFarlin.” Cranberry breeding programs are currently being conducted at Rutgers University (N. Vorsa) and the University of Wisconsin (E. Zeldon and B. McCown).

Little breeding work has been conducted on lowbush blueberries, although the lowbush types have been hybridized to produce “half-highs”. The major cultivars of this type are “Northland” developed by Johnston in Michigan in the 1960s and “North Country” developed more recently by J. Luby in Minnesota. The “half-highs” have much higher yields and larger fruit than lowbush, but have low enough stature to be protected by snow in areas with extreme winter cold.

Blueberries are all primarily outcrossing with varying levels of self-fertility, depending on species and genotype. In general, northern highbush blueberries have the highest levels of self-fertility,

followed by southern highbush and rabbiteye. Northern highbush are generally planted in solid blocks, although having a pollinator would be beneficial for most cultivars. Southern highbush and rabbiteye need pollinators, and alternate row plantings are recommended. Lowbush fruit is harvested from highly variable native stands, and as such considerable natural cross-pollination occurs. Cranberries are self-fertile, but cross-pollination can enhance seed production (Galletta and Ballington, 1996).

Blueberries and cranberries are asexually propagated through cuttings and tissue culture. All breeding programs have relied primarily on pedigree breeding where elite parents are selected each generation for intercrossing. Interspecific hybridization has played a major role in the development of highbush blueberries. Probably the most important success was the transfer of a low chilling requirement from the diploid *V. darrowii* to the northern tetraploid highbush *V. corymbosum* via unreduced gametes. One hybrid produced by Draper, US 75 (Fla 4B × Bluecrop), was completely fertile and is the source of the low chilling requirement in many of the southern highbush cultivars.

Several other strategies have been used to incorporate the genes of native species into the cultivated highbush background. *V. corymbosum* has been crossed with *V. angustifolium* to produce “half-high” types with greater winter tolerance than highbush. Draper produced interspecific tetraploid hybrids of *V. myrsinites* × *V. angustifolium* that could be directly crossed with highbush. He colchicine-doubled diploid hybrids of *V. myrtilloides* × *V. corymbosum* and then crossed this hybrid with *V. corymbosum*. He also generated numerous hybrids of diploid and polyploid species and the most fertile were ones were used as breeding parents. Interspecific crosses have been attempted among the other genera of *Vaccinium*, but with only limited utility (Luby *et al.*, 1991).

Most of the highbush types now being released are complex hybrids. Some of the most dramatic examples are “O’Neal” that contains genes from the four species (*V. corymbosum*, *V. darrowii*, *V. ashei*, and *V. angustifolium*) and “Sierra” that possesses the genes of five species (*V. corymbosum*, *V. darrowii*, *V. ashei*, *V. constablaei*, and *V. angustifolium*). “Biloxi” contains the genes from

five species (*V. corymbosum*, *V. darrowii*, *V. ashei*, *V. atrococcum*, and *V. angustifolium*), and has fewer *V. corymbosum* than non-*V. corymbosum* genes in its genome.

The current goals of southern highbush breeders are to obtain early ripening types with high plant vigor, disease resistance, and a later bloom (particularly in Florida). Established breeding lines are being used for this purpose, along with hybrids derived from native *V. ashei*, *V. elliottii*, and *V. darrowi*. Rabbit-eye breeders hope to expand harvest dates, improve flavor, and improve storage life. The northern highbush breeders are concentrating on flavor, longer fruit storage, expanded harvest dates, disease and pest resistance, and machine harvestability. Established breeding lines are being used by northern highbush breeders, along with complex hybrids made up of *V. darrowi*, *V. angustifolium*, *V. constablei*, and most of the other wild species. Even though it has limited winter hardiness, *V. darrowii* has proven to be an interesting parent in colder climates, because it passes on a powderblue color, firmness, high flavor, heat tolerance, and potential upland adaptations. Cranberry breeders are focusing on early maturing fruit, uniform large size, intense color, keeping quality, high productivity, and plant vigor.

1.4.2 Cane fruit

The first formal breeding work on red raspberries was begun in North America. The most enduring cultivar from this early period was “Latham”, which was introduced in 1914 by the Minnesota Fruit Breeding Farm (Jennings, 1988). It was the leading cultivar in eastern North America until 1930 when “Newburgh” (Geneva Experiment Station) and “Chief” (Minnesota Fruit Breeding Farm) were released. “Latham” and “Chief” ultimately became important in eastern Europe where hardiness was at a premium.

Another important early cultivar was “Cuthbert”, found in New York as an accidental hybrid of “Hudson River Antwerp” (*R. idaeus*) and a native (*R. strigosus*). It was a dominant cultivar from the 1940s to the 1980s. Two other early cultivars that had a major impact were “Lloyd George” found in the wild in 1919 by J. J. Kettle in Kent, England and “Pyne’s Royal” released in 1913 by T.B. Pyne of Topsham Nurseries. Later

cultivars that became dominant were “Willamette” and “Canby”, which were developed by George Waldo and released in the 1940s, “Malling Jewel” introduced in 1950 by Grubb at East Malling Research Station, “Meeker”, which was introduced by C.D. Swartz from Washington in 1967, and “Glen Clova” released by D. Jennings in 1969 from the Scottish Horticulture Research Institute.

“Lloyd George” has been a particularly important parent, being in the direct ancestry of 32% of the North American and European cultivars in 1970 (Oydin, 1970). This cultivar contributed several important traits including primocane fruiting, large fruit size, and resistance to the North American aphid. Jennings (1988) speculated that the success of “Lloyd George” hybrids “was possibly achieved because they combined the long conical shape of “Lloyd George” receptacle with the more rounded shape of the American raspberries”. A key example of such a hybrid is “Willamette”, which is a cross of “Newburg” × “Lloyd George” and has dominated the industry in western North America for over a half century. Other especially important cultivars have been the florican fruiting types “Malling Jewel” from the United Kingdom, “Glen Moy” and “Glen Ample” from Scotland, “Chilcotin”, “Skeena”, “Chilliwack”, “Tulameen”, and “Malahat” from British Columbia and the primocane fruiting “Heritage” from New York and “Autumn Bliss” and “Autumn Britten” from the United Kingdom.

The most active black raspberry breeding was conducted at Geneva, New York by R. Wellington. A number of important cultivars were released from there in the 1920s and 1930s including “Dundee”, “Naples”, “Rachel”, “Bristol” and “Evans” (Jennings, 1988). Bristol is still an important cultivar, known for its vigor and fruit size. George Slate followed Wellington and selected “Allen”, “Huron” and “Jewel” in the 1950s and 1960s, all noted for their large fruit. Another important cultivar, Munger, was released from Oregon in 1897.

Most of the early cultivars of blackberries were selected from the wild. The earliest systematic breeding of blackberries was done by Judge Logan in California in the late 1880s (Jennings, 1988). He selected the hybrid “Loganberry” from the natural cross of “Aughinbaugh” × “Red Antwerp” and “Mammoth” (“Black Logan”)

from “Aughinbaugh” × “Texas Early”. Darrow and Waldo of the USDA developed a number of popular blackberry cultivars for the Pacific Northwest in the middle of the 20th century including “Pacific”, “Cascade”, “Chehalem”, “Olallie”, and “Marion.” “Marion” is still widely grown, known for its rich flavor and high productivity. The cultivar “Silvan” was selected in 1984 in Victoria, Australia from seed generated in Oregon between “Marion” and a Bosenberry hybrid (McGregor and Kroon, 1984). It has much higher yields than “Marion” and large, excellent flavored fruit.

The most successful, early breeding work on blackberries in the eastern United States was done at Geneva, New York by Slate in the 1950s who introduced the cultivars “Hedrick” and “Darrow.” A significant later advancement was made when Scott and Ink (1966) at the USDA at Beltsville, Maryland transferred the recessive gene for spinelessness from the British cultivar “Merton Thornless” to generate “Thornfree” and “Darrow.” Later releases from Maryland were “Dirkson Thornless”, “Black Satin”, “Hull Thornless”, and “Chester Thornless.” Some of the newer blackberry releases that have made a major impact in colder climates are “Loch Ness” from Scotland and “Ćaćanska Bestrna” from Serbia. The most important blackberry cultivars for hot climates are currently “Brazos” from Texas, “Cherokee”, “Navaho”, and “Arapaho” from Arkansas, and “Tupy” from Brazil (Strik *et al.*, 2006).

Today, the major objectives of raspberry and blackberry breeding programs are high quality, good yields, suitability for shipping if fresh market, machine harvestability, and suitability for processing for the industrial market, adaptation to local environments, and improved pest and disease resistance (Finn and Knight, 2002). Resistance to *Phytophthora fragariae* is a widespread goal in all raspberry breeding programs. In Europe, breeders are most concerned with cane *Botrytis*, spur blight, and cane spot. Raspberry bushy dwarf virus (RBDV) is a major concern in the Pacific Northwest where RBDV has reached epidemic proportions. The development of spineless erect and trailing types is of paramount importance in raspberries. Efforts are also underway to develop raspberries that can tolerate hotter and more humid conditions and blackberries

that can flourish in colder conditions. High recent interest also revolves around developing primocane fruiting blackberries.

Wide hybridization has played a particularly important role in raspberry breeding. In fact, some of the most important breeding progress was made in the 19th century by crossing North American *R. strigosus* with European *R. idaeus*, and genes from at least six other species in *Idaeobatus* have been incorporated into red raspberry cultivars including *R. occidentalis*, *R. cockburnianus*, *R. biflorus*, *R. kunzeanus*, *R. parvifolius*, and *R. pungens oldhamii*, along with two *Cylactis* species (*R. articus* and *R. stellatus*) and an *Anoplobatus* species (*R. odoratus*) (Daubeny, 1996). Finn and Knight (2002) report that over 70 *Rubus* species are now being evaluated as possible parents.

Blackberry breeding also has had a rich tradition of interspecific hybridization, with different ploidy levels often being crossed. Most blackberries are tetraploid, but ploidy levels range from diploid to $2n = 14x = 98$ (Thompson, 1995a, b). The “Loganberry” is an allohexaploid derived from a reduced gamete of octoploid *R. ursinus* and an unreduced gamete of diploid *R. idaeus* (Crane, 1940; Thomas, 1940). Other interspecific polyploidy hybrids were selected in the late 1800s and early 1900s including the “Laxtonberry” (septaploid of “Loganberry” × the raspberry “Superlative”), “Phenomenal Berry” (hexaploid of “Aughinbaugh” × the raspberry “Cuthbert”), “Youngberry” (septaploid of “Phenomenal Berry” × a dewberry hybrid of *R. baileyanus* × *R. argutus*), and Boysenberry (septaploid of “Loganberry” × a trailing blackberry).

Raspberries are generally outcrossing, but some blackberries are apomictic, which complicates breeding. Darrow and Waldo (1933) reported that crosses with “Thornless Evergreen” produced 87% apomictic progeny, while Hall *et al.* (1986) found 94% apomicts in their crosses of “Thornless Evergreen”. The hybrid offspring are very difficult to identify without biochemical or molecular markers.

1.4.3 Strawberries

The dessert strawberry is an outcrossed crop that is relatively sensitive to inbreeding (Morrow and Darrow, 1952; Melville *et al.*, 1980), and it

can be asexually propagated by runners, so most modern varietal improvement programs have been based on pedigree breeding where elite parents are selected each generation for intercrossing. The germplasm base of strawberry is considered to be relatively narrow (Sjulin and Dale, 1987), but if adequate population sizes are maintained, changes in levels of homozygosity across generations appear to be minimal (Shaw, 1995). Since highly heterozygous genotypes can be propagated as runners, few breeding programs have developed hybrid cultivars using inbred lines, although some cultivars have been generated in this manner.

Selfing has been successfully employed in a number of instances to concentrate genes of interest (Hancock *et al.*, 1996) and backcrossing has been used occasionally to incorporate specific traits. Barritt and Shanks (1980) moved resistance to the strawberry aphid from native *F. chiloensis* to *F. × ananassa*. Bringham and Voth (1978, 1984) transferred the day neutrality trait from native *F. virginiana* spp. *glauca* to *F. × ananassa*. Approximately three generations were necessary to restore fruit size and yield to commercial levels.

Formal strawberry breeding was initiated in England in 1817 by Thomas A. Knight (Darrow, 1966; Wilhelm and Sagen, 1974). He was one of the first systematic breeders of any crop, and used clones of both *F. virginiana* and *F. chiloensis* in his crosses. He produced the famous “Downton” and “Elton” cultivars, noted for their large fruit, vigor, and hardiness. Michael Keen, a market gardener near London, also became interested in strawberry improvement about this time and developed “Keen’s Imperial” whose offspring, “Keen’s Seedling” is in the background of many modern cultivars. This cultivar dominated strawberry acreage for close to a century.

Thomas Laxton of England was the most active breeder during the later part of the 18th century and released a number of important cultivars including “Noble” and “Royal Sovereign.” These two cultivars were grown on both sides of the Atlantic Ocean, and were popular until the middle of the 20th century. “Nobel” was known for its earliness, cold hardiness, and disease resistance. “Royal Sovereign” was popular because of its earliness, productivity, flavor, attractiveness, and hardiness.

Charles Hovey, of Cambridge, Massachusetts, produced the first important North American

cultivar, “Hovey”, by crossing the European pine strawberry, “Mulberry” with a native clone of *F. virginiana* in 1836. It was the first cultivar of any fruit to come from an artificial cross in America and for a while made the strawberry the major pomological product in the country (Hedrick, 1925).

Albert Etter of California developed dozens of cultivars around the turn of the century utilizing native *F. chiloensis* clones (Fishman, 1987). His most successful cultivar was Ettersburg 80 (1910), which was widely grown in California, Europe, New Zealand, and Australia. Renamed as “Huxley”, it was still popular in England as late as 1953. Ettersburg 80 was extremely drought resistant, of outstanding dessert and jam quality due to its solid bright red color, and was unusually hardy for a California type. Other outstanding Etter cultivars were “Ettersburg 121”, “Fendalcino” and “Rose Ettersburg.” While these releases were very successful as cultivars, they may have had their greatest impact as breeding parents. Most California cultivars (and many others) have an Ettersburg cultivar in their background (Darrow, 1966; Sjulin and Dale, 1987).

In the middle of the 20th century, a number of particularly successful breeding programs emerged in Scotland, England, Germany, and Holland. In Scotland, Robert Reid developed a series of red stele resistant cultivars utilizing American “Aberdeen” as a source of resistance. His cultivar “Auchincruive Climax” dominated acreage in Great Britain and northern Europe until its demise due to June yellows in the mid-1950s. In England, D. Boyle produced a large series of cultivars with the prefix “Cambridge”. “Cambridge Favorite” (1953) became the most important of the group and dominated the acreage in Great Britain by the 1960s. In Germany, R. von Sengbusch’s produced a “Senga” series, of which “Senga Sengana” (1954) became paramount. “Senga Sengana” was widely planted for its processing quality and is still important in Poland and other eastern European countries. In the Netherlands, H. Kronenberg and L. Wassenaar’s released several cultivars, of which “Gorella” (1960) made the greatest impact. It was noted for its size, bright red glossy skin, and red flesh. B. Meelenbroek who followed in this program released “Elsanta” (1981), considered the ideal fresh market cultivar for its bright color, flavor, and regular size.

Most of the breeding advances in the eastern United States have come from the USDA (Hancock, 1999). George Darrow at Beltsville, Maryland developed “Blakemore”, which became the major southern US cultivar in the mid-1930s and “Fairfax” was widely planted in the middle of this century from southern New England to Maryland and westward to Kansas. These two cultivars were used extensively in breeding, finding their way into the ancestry of a diverse array of cultivars grown in all parts of the United States. Other important releases from Darrow were “Pocahontas”, “Albritton”, “Surecrop” and “Sunrise”. D.H. Scott, A.D. Draper, and G.J. Galletta followed Darrow and released “Redchief” (1968), “Earliglow” (1975), “Allstar” (1981), and “Tribute” and “Tristar” (1981). All of these cultivars are still grown today, and Tribute and Tristar were the first day neutrals widely grown in the eastern United States. An active USDA breeding program has also been conducted at Corvallis, Oregon initially by Darrow, G.F. Waldo, and F.J. Lawrence, and now C. Finn. Some of the more important cultivars emerging from this program were “Siletz” (1955) and “Hood” (1965). “Hood” is considered the premier berry for processing.

Several other state and federal supported programs have released important cultivars in the United States and Canada. Some of the most significant ones from the United States were “Honeoye” and “Jewel” (New York), “Raritan” (New Jersey) and “Sweet Charlie” (Florida). From Nova Scotia came “Bounty”, “Glooscap”, and “Kent.”

H. Thomas and E. Goldsmith’s of the University of California released the important cultivars “Lassen” and “Shasta” in 1945. “Shasta” was widely grown in the central coast of California in the 1950s and 1960s because of its large size, firmness, and long season. “Lassen” was grown extensively in southern California about the same period, prized for its short rest period, and high productivity. R. Bringham and V. Voth took over the California-Davis program in the 1950s and generated a succession of internationally important, Mediterranean adapted cultivars including “Tioga” (1964), “Tufts” (1972), “Aiko” (1975), “Pajaro” (1979), “Chandler” (1983), “Selva” (1983), “Camarosa” (1992) and “Seascape” (1991). Most recently,

Doug Shaw has released “Diamonte” from this program.

The greatest concentration of breeding activity outside of Europe and the United States has been in Japan. Two very important cultivars were produced there: H. Fukuba’s “Fukuba” (1899), noted for its large size and high flavor (Darrow, 1966), and K. Tamari’s “Kogyoku” (1940), respected for its vigor, earliness, and fruit size (Mochizuki, 1995). “Fukuba” was the most important cultivar in forcing culture until the early 1970s. “Kogyoku” was one of the leading field grown cultivars after World War II, until it lost importance to the American import “Donner” in the 1950s.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Since blueberries, cane fruits, cranberries, and strawberries are all asexually propagated, transgenic breeding could be used to rapidly introduce individual genes of interest without changing the inherent desirable characteristics of existing cultivars. To date, however, transformation technologies have been little utilized in these crops.

Resistance has been found for many of the important pest and disease problems in the small fruit crops, but in many cases the resistance is quantitative. This makes it difficult to incorporate multiple resistances into a new cultivar via hybridization and at the same time maintain high fruit quality and yield. The incorporation of transgenics that produce high levels of simply inherited resistance to these diseases would be highly beneficial, particularly if the transgenes are effective against multiple pests and/or diseases. There are also a few instances where no known resistance to a major disease or pest exists. In these cases, transgenic approaches might prove to be the only route to provide needed variability.

Small fruits are grown across a wide environmental zone, but several abiotic factors commonly limit their productivity, such as salinity, frost, and cold. As with disease and pest resistance, there are often sources of resistance available to these abiotic stresses, but in many cases the amount of variability among improved genotypes is low and the inheritance is quantitative. More variability can often be found in wild populations,

but at least three generations of backcrossing are necessary to approach commercial quality fruit. These factors have made it very difficult to improve resistance to salinity, frost, and cold without negatively impacting on fruit quality and yield. The incorporation of single transgenes improving these traits would greatly speed the development of genotypes with expanded abiotic tolerances.

1.5.1 Blueberry and cranberry

Blueberries are subject to a wide array of diseases. Probably the most widespread problems in highbush blueberry are mummy berry (*Monilinia vaccinii-corymbi* (Reade), blueberry stunt, blueberry shoestring virus, stem blight (*Botryosphaeria dothidea* (Mouq. Ex Fr.) Ces and de Not.), stem canker (*Botryosphaeria cortices* Demaree and Wilcox), *Phytophthora* root rot (*Phytophthora cinnamomi* Rands), *Phomopsis* canker (*Phomopsis lokoyae*), botrytis fruit rot (*Botrytis cinerea* Ters. Ex Fr.), and anthracnose fruit rot (*Colletotrichum gloeosporioides* (Penz.) Penz and Sacc.). Rabbiteye blueberries are much more resistant to diseases than highbush types, with only botrytis blossom and twig blight, stem blight and mummy berry being major problems. Lowbush is most negatively impacted by botrytis stem and twig blight and red leaf disease caused by *Exobasidium vaccinii* (Fckl.) Wor. The most serious disease problems of cranberry are fruit rots caused by *Guignardia vaccinii* Shear, *Godronia cassandrae* Peck, and *Ceuthospora lunata* Shear. Resistant or tolerant genotypes have been described for most of the above diseases, but the genetics of resistance has only been determined for *Phytophthora* root rot, *Phomopsis* canker, stem canker, and stem blight in blueberry (Luby *et al.*, 1991; Galletta and Ballington, 1996). Genetic resistance to these diseases is quantitative.

Several insects and arthropods do significant damage to highbush blueberries including blueberry maggot (*Rhagoletis pomonella* Walsh), sharp-nosed leafhopper (stunt vector) *Staphytopius magdalenensis* Prov., and blueberry aphid (shoestring virus vector) (*Illinoia pepperi* Mac. G.), cranberry fruit worm (*Acrobasis vaccinii* Riley), cherry fruitworm (*Graphditha packardi* Zell), and the plum curculio (*Conotrachelus nenuphar* Herbst). Lowbush and rabbiteye blueberries have

far fewer major pests than highbush types; however, significant damage is caused by cranberry fruitworm and stunt in rabbiteye blueberry, and maggot in lowbush. The most serious pests of cranberries are the leafhopper *Sclerorachus vaccinii*, which vectors false blossom disease, black root weevil *Brachyrhynchus sulcatus* Fabr., cranberry tip worm *Dasyneura vaccinii* Smith, black-headed fireworm *Rhopobota vacciniana* Pock, and the cranberry fruit worm. Very little variation in resistance has been reported to most of these pests except the sharp-nosed leafhopper, which was quantitatively inherited in *V. ashei*, but recessive in *V. elliotii* (Ballington *et al.*, 1993).

Among the abiotic factors limiting blueberries, high pH, temperature extremes, and insufficient soil drainage are the most important problems. The *Vaccinium* are “acid loving” and as such require soils below pH 5.8 for high vigor; they also do not tolerate flooded roots for more than a few days. Among the cultivars, there is little variation for these traits, although a number of wild species may have greater tolerance to soil pH (Luby *et al.*, 1991; Galletta and Ballington, 1996). Frost damage during the spring when blossoms begin to open is very prevalent across most of the highbush production regions and in the northern highbush production zones, winter cold often causes severe damage to flower buds and young shoots. There is little variation in frost tolerance among rabbiteye and highbush cultivars, although there is a considerable amount of quantitative variability in cold tolerance, with northern cultivars being hardier than southern ones. High temperature during harvest in the northern production areas often causes fruit softening and negatively impacts on storage life, but no formal screens have been made to search for variability in this characteristic. Cold hardiness is a complex interaction between rate of acclimation and deacclimation and deep winter tolerance.

In blueberries, the fruit characteristics most sought after are flavor, large size, light blue color (a heavy coating of wax), a small scar where the pedicel detaches, firmness, and a long storage life. Fruit firmness is probably paramount in determining the shelf life of blueberries, along with sugar to acid ratio. Considerable quantitative variability exists for all these traits, and some native species like *V. darrowi* have provided especially useful variability. High antioxidant capacity has

also become an important fruit quality parameter in blueberries. Considerable amounts of variability have been observed in this characteristic that is quantitatively inherited (Connor *et al.*, 2002a, b). ACY concentration is one of the major fruit quality factors in cranberries. Other important characteristics are uniform color, high aroma, firmness, glossiness, and ability to retain texture in storage. All of these characteristics appear quantitative in nature.

1.5.2 Cane fruit

The primary factor associated with low yields and degeneration of raspberry cultivars are viruses. The most important virus diseases are RBDV, raspberry yellow net (RYNV), black raspberry necrosis (BRNV), raspberry leaf mottle (RLMV), and raspberry leaf spot (RLSV). These four viruses are vectored by the aphids *Amphorophora idaei* Börner in Europe and *A. agathonica* Hottes in North America. Most cultivars are tolerant to raspberry yellow net and black raspberry necrosis (Jennings, 1988), but there are no published sources of resistance to RBDV, raspberry leaf mottle, and raspberry leaf spot.

Other important viruses are arabis mosaic virus and strawberry latent ringspot virus (SLRV) in England and southern Europe. These two viruses are vectored by *Xiphinema diversicaudatum* Micoletsky. The tomato ringspot virus (TRSV) is one of the most damaging viruses in North America, where it causes “crumbly fruit” and is vectored principally by the nematode *Xiphinema americanum* Cobb. Resistance and/or tolerance has been identified for strawberry latent ringspot, arabis mosaic, raspberry yellow net, black raspberry necrosis, and the two aphids (Daubeney, 1996), but no sources of resistance have been reported to raspberry leaf mottle, raspberry leaf spot or *X. americanum*. The resistance to arabis mosaic virus is regulated by a single gene (Jennings, 1964).

A number of fungal diseases cause serious damage to raspberries. The most important disease of fruit is gray mold, *Botrytis cinerea*. Most cultivars are susceptible to this disease, although quantitatively inherited sources of resistance are available (Jennings *et al.*, 1989). The most prevalent cane diseases of raspberries are midge

blight and cane blight. Midge blight is a disease complex instigated by damage from the raspberry midge, *Resseliella theobaldi* Barnes. Cane blight is caused by *L. coniothyrium*, which generally enters through wounds caused by mechanical harvesting. Spur blight (*Didymella applanata* (Niessl) Sacc) is a very important disease in the Pacific Northwest and eastern Europe. Only partially resistant genotypes have been identified to midge and cane blight (Jennings *et al.*, 1989; Williamson and Jennings, 1992). Hairy canes with gene *H* tend to be less affected by spur blight, with most sources of resistance being additive (Jennings, 1988; Williamson and Jennings, 1992).

Several other fungal problems commonly damage raspberries including cane spot or anthracnose (*Elsinoë veneta* (Burkh.) Jenkins), leaf spot (*Sphaerulina rubi* Demaree and Wilcox), yellow rust (*Phragmidium rubi-ideae* (DC.) Karst), and late yellow rust (*Pucciniastrum americanum* (Farl.) Arth.). Major resistance genes exist for these two fungal pathogens (Anthony *et al.*, 1986; Williamson and Jennings, 1992). Root rots are also often problems in both North America and Europe. The primary causal organism is *Phytophthora megasperma* Dreschsler in Europe and *Phytophthora erythroseptica* Pethyby in North America. Only quantitative resistance is known to these pathogens.

Blackberries are generally much more resistant to fungal diseases than raspberries. However, the following diseases can be quite important in North America: orange rust (*Gymnoconia pecking* (Howe) Trott.), anthracnose, rosette (double blossom) (*Cercospora rubra* (Wint.) Plakidas), leaf spot, and *Verticillium* wilt (*Verticillium albo-atrum* Reinke and Berth.). Purple blotch (*Septocytia ruborum* (Lib.) Petrak) is the most serious disease in Europe, while downy mildew (*Peronospora sparsa* Berk.) is very important in New Zealand. Quantitatively inherited resistance to all these diseases has been reported (Jennings, 1988; Williamson and Jennings, 1992).

The raspberry midge, mentioned above, and the raspberry beetle (*Byturus tomentosus* Degeer) are the most important insect pests of raspberry in Europe. The western raspberry fruitworm (*B. bakeri* Barber) and the eastern raspberry fruitworm (*B. rubi* Barber) are major problems in North America. Other serious pests across the world are the clay colored weevil (*Otiiorhynchus*

singularis L.), the black vine weevil (*Otiorhynchus sulcatus* Fab), and the two-spotted spider mite (*Tetranychus urticae* Koch) in the Pacific Northwest. Quantitative resistance has been described to the raspberry beetle (Jennings *et al.*, 1989), but there are no published reports on resistance to raspberry fruit worms, clay colored weevil, black vine weevil, and two-spotted spider mite.

Lack of winter cold tolerance limits the range of successful raspberry and blackberry cultivation in the continental climates of central and eastern Europe, and eastern and central North America. Blackberries are generally more sensitive to cold than raspberries, and black raspberries are less hardy than red raspberries. The inheritance of winter hardiness is under complex genetic control. Winter hardy raspberries have four key characteristics: (1) rapid hardening in the fall before severe temperatures occur, (2) long rest or deep dormancy making them resistant to temperature fluctuations in the spring, (3) the ability to re-harden if initial cold tolerance is lost, and (4) late bud break (Daubeney, 1996). It has been proven difficult to combine cold hardiness with early flowering and fruiting (Jennings *et al.*, 1989).

While blackberries are less winter hardy than raspberries, there is still a wide range in the hardiness of cultivars and species (Hall, 1990). The European cultivar "Merton Thornless" is one of the least tolerant ones, and its use as a source of spinelessness led to reductions in hardiness in Europe and North America (Jennings *et al.*, 1989). The largest gain in hardiness has come from breeding cultivars with trailing canes that are covered by snow.

Raspberries grown in the colder regions can also be limited by fluctuating spring and fall temperatures, and low temperatures during fruiting. There is considerable variability among raspberry genotypes in their ability to set high proportions of their drupelets under cool conditions. In general, the raspberry cultivars developed in the United Kingdom are better adapted to cool temperatures than those developed in the Pacific Northwest, with some exceptions. The northwestern cultivar "Meeker" has been shown to have good drupelet set at both locations (Dale and Daubeney, 1985).

Heat and drought are limiting in southern Europe, southeastern North America, and much of the southern hemisphere. In many of these

areas, cultivars with a low chilling requirement are also needed, as hot summer temperatures are often associated with limited chilling hours. Considerable variability has also been found among raspberry and blackberry cultivars for adaptation to high summer temperatures. In most cases, those cultivars with high temperature tolerance also have a reduced chilling requirement, and most of the heat tolerant genotypes are also drought tolerant. The wild Asiatic raspberry species have been most widely used as sources of high temperature tolerance and a low chilling requirement, although genes regulating a low chilling requirement have been found in cool weather adapted *R. ideaus* (Rodriquez and Avitia, 1989).

The most desired cultivars are erect and sparsely spined, with adequate but not excessive cane numbers and cane heights (Jennings *et al.*, 1989). Spines are more prominent in blackberries than raspberries, and thus have received a higher priority in their breeding. Most raspberry cultivars are not completely spine free, but spineless ones do exist and many at least have reduced numbers on the basal portions of canes. There is an apparent association between spinelessness and susceptibility to winter injury that requires large population sizes to identify recombinants (Moore, 1984). Dominant genes for spinelessness have been found, but to date most of the genes used to produce spineless cultivars are recessive (Daubeney, 1996).

The critical traits associated with high fruit quality in raspberry and blackberry are flavor, soluble solids, acidity, color, texture, skin strength, fruit size, seed size, ability to maintain integrity, ease of removal, nutritional content, and ripening season. Raspberries with an intense red color without any blue are considered best for the fresh market, yogurt, and pie fillings (Jennings *et al.*, 1989). Blackberries must retain their black color and not turn red in storage and after freezing. Seeds are more of a problem in blackberries than raspberries, but in general, low numbers of small seeds are best. Considerable genetic variability exists for all of these fruit characteristics among cultivars and species, although few studies have been performed to determine their genetics. Barritt (1982) found very high heritability for fruit firmness in a diverse breeding population of raspberries containing genotypes from the Pacific

Northwest and the United Kingdom. Single genes have been identified for fruit color, fruit size, and crumbly fruit (Daubeney, 1996).

Connor *et al.* (2005a) estimated narrow-sense heritabilities for antioxidant capacity (AA), total phenolic content (TPH), and fruit weight from progeny of a factorial mating design of seven female and six male red raspberry genotypes. Heritability estimates were all high, at 0.54, 0.48, and 0.77 for AA, TPH, and fruit weight, respectively. In further work evaluating individual ACY content with total ACY content and antioxidant capacity in the same families, Connor *et al.* (2005b) found high values of h^2 for individual ACYs (0.54–0.90), but ACY content and profile information were “inefficient proxies and predictors of AA in red raspberry fruit”. The inclusion of a pigment-deficient *R. parvifolius* × *R. ideaus* hybrid resulted in significant female and male contributions to variation, but its removal from the analysis made female × male interaction negligible.

1.5.3 Strawberries

Several soil pathogens damage strawberry roots, resulting in vigor declines and death in severe cases. Two very common problems across the world are red stele or red core caused by *Phytophthora fragariae* Hickman and verticillium wilt caused by *Verticillium albo-atrum* Reinke & Berth. and *V. dahlia*. Black root rot is also widespread and is caused by a complex of organisms including *Pythium*, *Rhizoctonia*, and the root lesion nematode (*Pratylenchus penetrans* Cobb). *Fusarium* wilt or *Fusarium* yellows (*Fusarium oxysporium* Schl. f. sp. *fragariae* Winks and Williams) is of major importance in Japan, Korea, and Australia. Resistant and/or tolerant genotypes have been found for all four of these major soil pathogens, but for all of them, the genetics are complex (Hancock, 1999).

Fumigation has been widely employed to control these soil pathogens, but the impending ban on methyl bromide (MB) fumigation has stimulated increased interest in developing resistant cultivars. The identification of a transgene that conferred broad resistance to a number of soil pathogens would be particularly desirable. Without fumigation, cultivars yield 50% less fruit on average. Screens of the California breeding

population on fumigated and nonfumigated soil have uncovered little general resistance to the total array of soil pathogens normally found in strawberry soils (Larson and Shaw, 1995a, b), although a screen of eastern breeding material did uncover some tolerant individuals (Patrica and Hancock, 2005).

Three foliar diseases are very widespread and routinely cause serious damage including, leaf blight (*Phomopsis obscurans* (Ell. and Ev.) Sutton), Ramularia leaf spot (*Mycosphaerella fragariae* (Tul.) Lindau), and leaf scorch (*Diplocarpon earliana* (Ell. & Everh.) Wolf). *Alternaria* leaf spot or black leaf spot (*Alternaria alternaria* (FR.) Keissler) causes serious damage in Europe, New Zealand, and Korea. Powdery mildew (*Sphaerotheca macularis* (Wallr. Ex Fr.) Jaesz) is also found across most of the strawberry range, although it rarely does economic damage. Angular leaf spot, *Xanthomonas fragariae* Kennedy and King, is a rapidly growing problem in strawberries all across the world (Maas *et al.*, 1995). For most of these diseases, only quantitative resistance has been found with moderate to high levels of heritability. Only black leaf spot resistance has been reported to be controlled at a single locus (Yamamoto *et al.*, 1985).

Anthrachnose is a common problem in strawberries, causing a wide array of symptoms including fruit rot, crown rot, and lesions of the stolons, petioles, and leaves. Anthracnose diseases of strawberry are caused by *Colletotrichum fragariae* A.N. Brooks, *C. acutatum* J.H. Simmonds, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. *Colletotrichum acutatum* is the primary pathogen causing crown rot in Europe (Denoyes-Rothan and Baudry, 1995), while *C. fragariae* is the most common cause of crown rot in the southeastern United States. Sources of resistance to anthracnose fruit and crown rots exist in strawberry; however, the genetic factors conditioning host resistance to crown and fruit infection differ and only a few genotypes are resistant to both fruit and plant. Strong environmental × genotype interactions affect the expression of resistance, and multiple isolates of *C. acutatum*, *C. gloeosporioides*, and *C. fragariae*, vary in pathogenicity to *Fragaria* genotypes (Smith and Black, 1987).

Phytophthora cactorum (Leb. & Cohn) Schroet also causes widespread incidences of severe crown (Cactorum crown rot) and fruit rots (leather rot),

particularly in warm climates. Other important fungal fruit rots are as follows: (1) botrytis fruit rot or gray mold (*Botrytis cinerea* Pers. ex. Fr.), which is a worldwide problem, (2) mucor fruit rot (*Mucor mucedo* L. ex Fries), sometimes important in the United States and United Kingdom, (3) rhizopus leak (*Rhizopus* spp.) a particular problem in the United Kingdom but worldwide in scope, (4) tan-brown rot, (*Discohainesia oenotherae* (Cook & Ellis)), a major problem in humid strawberry regions, (5) *Phomopsis obscurans* (Ellis & Everh.) Sutton in Florida, and (6) septoria hard rot (*Septoria fragariae*) rarely a problem in the United States but common in Europe and Australia. Moderate to high levels of heritability have been found to gray mold and leather rot.

Strawberries across their range are hosts to numerous viruses and phytoplasma. Among the most important are the aphid-borne viruses involved in the yellows complex (mottle, mild yellow-edge, crinkle, and vein banding viruses) and the nematode-borne viruses (raspberry ringspot virus, tomato black ring, strawberry latent ringspot virus, and arabis mosaic virus) (Maas, 1998). There are also two important phytoplasma diseases spread by leaf hoppers, Aster yellows that are caused by a cultivar of species and green petal or clover phyllody. Resistance has been identified to the regional virus complexes found in the Pacific Northwest, but no formal genetic analysis has been performed.

The nematodes causing the most widespread problems include the northern root-knot nematode (*Meloidogyne hapla* Chitwood), root lesion nematode, and the needle nematode (*Longidorus elongatus* Dorylaimida). Resistance has been described for all these pests, although no genetic studies have been conducted.

Two aphids are widespread that damage strawberries, the strawberry root aphid, *Aphis forbesi*, and the strawberry aphid *Chactosiphon fragaefolii* (Corherell). The strawberry aphid is found all across the range of cultivation, while the strawberry root aphid is restricted to east of the Rockies in the United States. The latter is most important as a vector of virus disease. Resistant genotypes have been identified for the strawberry aphid, and resistance has been shown to be regulated by more than one locus with partial dominance and additive action (Hancock, 1999). Other important strawberry pests are plant

bugs (*Lygus* spp.), root weevils (*Otiorhynchus* spp.), strawberry weevil (*Anthonomus signatus* Say), two-spotted spider mite (*Tetranychus urticae* Koch.), and the cyclamen mite (*Steneotarsonemus pallidus* (Banks)). Moderate levels of resistance have been identified to two-spotted spider mite, strawberry aphid, and black vine weevil.

Excess salt from irrigation water is a major production problem in many arid agricultural regions. Water containing more than 100 ppm sodium or chloride ions results in enough salt accumulation to cause yield loss without visible plant injury (Brown and Voth, 1956). Some cultivars have been shown to be more "salt tolerant" than others, but no formal genetic studies have been made.

Strawberries generally bloom in early spring, when the chance of frost is relatively high in many strawberry production regions. Flower buds, open flowers, and young fruit are all injured by frost. Pistillate parts are most sensitive to damage; however, some damage is likely to all parts if temperatures fall to -2°C (Darrow, 1966). Differences in bloom tolerance to frost have been described in the older literature (Ourecky and Reich, 1976); however, all of our modern cultivars have flowers that are highly sensitive to frost.

Winter freezing injury to the strawberry crown and inflorescence buds is a serious limitation to strawberry production throughout the upper half of the Northern Hemisphere. Nonacclimated strawberry plants are usually killed when the crown temperature remains at -3°C for more than 1 or 2 h (Scott and Lawrence, 1975). Acclimated strawberry plants can survive crown temperatures of -12°C to -15°C , although injury such as decreased vigor is visible at higher temperatures. The greatest amount of damage occurs in the late winter to early spring when temperatures rapidly rise and then drop again. Early breeders dramatically improved the winter hardiness of northern cultivars using quantitative variability, but little improvement in this trait has been made in the last few decades.

Several factors can restrict consumer acceptance of strawberry fruit including size, firmness, flavor, nutrition, and color. There is considerable variability for these traits available in most breeding populations, although inheritance is generally polysomic. Size of fruit is inherited quantitatively, with 6 to 8 allelic pairs controlling

fruit expansion (Hancock, 1999). Flesh firmness and skin toughness are often correlated positively and are generally inherited quantitatively. Skin and flesh color have been shown to be largely under the control of additive variation, although a few genes appear to have much larger effects than others. Internal and external colors are probably regulated by separate sets of genes as correlations between these two parameters are small.

Soluble solids and acidity are controlled with varying levels of additive and dominance control (Shaw *et al.*, 1987; Shaw, 1988). Shaw (1988) found little difference in the soluble solids and total sugars in his breeding population, although he did observe significant genotypic variation in sucrose, glucose, fructose, and acidity levels. Wenzel (1980) found a negative association between soluble solids concentration and yield. Vitamin C content has also been shown to be polygenic, with some parents displaying partial dominance for high levels and some progeny having higher levels than their parents (Hancock *et al.*, 1996). Several studies have described variation in the flavor of progeny families suggesting additive quantitative control (Darrow, 1966), although few formal genetic studies on this character have been conducted. Zubov and Stankevich (1982) found significant seedling variation in fruit consistency, ACY content and vitamin C, but not flavor.

2. DEVELOPMENT OF TRANSGENIC BERRY CROPS

2.1 Donor Genes

2.1.1 Blueberry and cranberry

Two groups have reported on the transformation of blueberry (Graham *et al.*, 1996; Song and Sink, 2004). The screenable reporter *gusA* driven by either the cauliflower mosaic virus (CaMV) 35S or a chimeric super promoter (Aocs)₃AmasPmas (Ni *et al.*, 1995) and terminated by T-nos was transformed into blueberry cultivars using *nptII* as the selectable marker. The *bar*, driven by P-nos and terminated by the gene 7 poly(A) signals (Becker *et al.*, 1992) was also transformed into blueberry plants after selection with glufosinate ammonium. High-level tolerance to glufosinate-herbicide Rely, 750–3000 mg l⁻¹ glufosinate ammonium, was

observed in the *bar*-expressing, greenhouse plants (Song *et al.*, 2007). Field trials showed little or no damage to plants from four independent transgenic events after application of a workable concentration of glufosinate ammonium, 750 mg l⁻¹, for weed control (Song *et al.*, 2007).

Successful transformation of cranberry has only been reported by a group from the University of Wisconsin-Madison. In their initial work, a vector containing the *gusA*, *nptII*, and *Bt* (*Bacillus thuringiensis* Subsp. *Kurstaki* crystal protein) was used to optimize transformation protocols for the cultivar Stevens (Serres *et al.*, 1992; Serres *et al.*, 1997). Both *gusA* and *Bt* were controlled by the CaMV 35S promoter, while *nptII* had a nopaline synthase promoter (P-nos). All three genes were terminated by the nopaline synthase polyadenylation region. In later work, they transformed two genes, *nptII* and *bar*, into “Pilgrim” to obtain herbicide-resistant plants (Zeldin *et al.*, 2002). The *bar* gene was driven by the CaMV 35S promoter and terminated by the soybean small subunit gene polyadenylation signal region (T-nos).

The genes encoding the cold-responsive proteins known as dehydrins have been cloned in blueberry. Muthalif and Rowland (1994a, b) originally examined changes in protein levels in the floral buds of the cold-tolerant, northern highbush “Bluecrop” and the cold-sensitive rabbiteye “Tifblue.” They found that three proteins of 65, 60, and 14 kDa increased in both cultivars in response to cold and became the predominant proteins. The highest levels of the dehydrins were found in “Bluecrop.” This correlation has held up across a number of other cultivars with varying levels of cold tolerance (Arora *et al.*, 1997; Panta *et al.*, 2001). A 2.0 kb blueberry complementary DNA (cDNA) was identified that encodes the 60 kDa dehydrin (*bbdhn1*) (Levi *et al.*, 1999). This clone was used to probe cold-hardened floral buds of “Bluecrop” and another five dehydrins were identified (*bbdhn2*–*bbdhn5*) (Rowland *et al.*, 2004).

A large expressed sequence tag (EST) library of highbush blueberry has been generated in the laboratory of L. J. Rowland at the USDA-ARS Fruit Laboratory at Beltsville, Maryland. Through traditional molecular genetic and genomic approaches, she and her collaborators have identified and isolated several genes associated

with cold hardiness including several members of the dehydrin gene family (Muthalif and Rowland, 1994a, b; Dhanaraj *et al.*, 2003, 2005). They have also conducted gene expression studies under field and cold room conditions using cDNA microarrays (Dhanaraj *et al.*, 2006a, b).

2.1.2 Cane fruit

Regeneration and transformation systems have been developed for blackberries and raspberries utilizing leaves, cotyledons, and internodal stem segments (Hassam *et al.*, 1993; Mathews *et al.*, 1995a; Graham *et al.*, 1997a; Kokko and Karenlampi, 1998; Meng *et al.*, 2004). Most of the *Rubus* transformations have been with marker genes *GUS*, *NPTII*, and *CSR* (chlorsulfuron herbicide resistance).

Mathews *et al.* (1995b) transformed “Canby”, “Chilliwick”, and “Meeker” red raspberries with the gene for S-adenosylmethionine (SAMase), as a potential strategy to delay fruit decay. Leaf and petiole explants were inoculated with *Agrobacterium* strain EHA 105 carrying the binary vectors pAG1452 or pAG1552 encoding SAMase under control of the wound and fruit-specific E4 promoter. Petiole explants produced the highest rates of transformation, and more transformants were recovered using hygromycin phosphotransferase (HPT) as the selective agent rather than neomycin phosphotransferase (NPTII). They reported on establishment of the transformants in soil, but have not published information on levels of resistance to decay.

There have been two attempts to generate resistance to RBDV using *Agrobacterium*-mediated transformation. Jones *et al.* (1998) isolated the coat protein gene (*cp*) from a resistance-breaking strain of RBDV and transformed plants with it in the sense and antisense orientation. Some of their transformants were partially resistant. Taylor and Martin (1999) sequenced the *cp* gene, mutations of the movement protein and nontranslatable RNA of RBDV, and transformed “Meeker” red raspberry with each of these constructs (Martin and Mathews, 2001). A small number of bramble genes have been identified and sequenced. Twenty genes associated with fruit ripening were identified by Jones *et al.* (1999) in the red raspberry “Glen Clova.” Most

of these genes were associated with cell wall hydrolysis and ethylene biosynthesis. Iannetta *et al.* (2000) cloned two putative endo- β -1,4 glucanase genes (*RI-EGL1* and 2) from ripe receptacle messenger RNA (mRNA). The expression of these genes were limited to ripe fruit receptacles, and the application of 1-methylcyclopropene (1-MCP) to green fruit indicated that ethylene accelerates raspberry abscission and increases EGase activity.

Ramanathan *et al.* (1997) cloned two genes for polygalacturonase-inhibiting protein (*PGIP1* and *PGIP2*) that could play a role in gray mold resistance. Plant polygalacturonase-inhibiting proteins (PGIPs) inhibit fungal endopolygalacturonases, which are released by fungi to degrade plant cell walls. Activity levels of *PGIP* were found to decline during floral and fruit development, but expression of *PGIP* was stable throughout development from closed flower to ripe fruit.

2.1.3 Strawberry

Two types of herbicide resistance have been engineered into strawberry through *Agrobacterium*-mediated transformation. The phosphinothricin acetyl transferase gene (*PAT*) was incorporated into the “Selekta” strawberry using *Agrobacterium*-mediated gene transfer (Duplessis *et al.*, 1995, 1997). Putatively transformed shoots were rooted and established in the greenhouse and most transgenic plants were found to be resistant to the herbicide glufosinate-ammonium (Ignite). The *CP4.EPSP* synthase gene, which confers resistance to glyphosate (Roundup®) was introduced into “Camarosa” (Morgan *et al.*, 2002), and when 73 independent transformations were sprayed with Roundup® in the nursery, a range of responses were noted from complete resistance to death. Expression levels of the *CP4.EPSPS* gene was strongly correlated with phenotype. The best lines were subsequently tested in the field and appeared to produce good quality fruit.

To provide insect resistance, the cowpea protease trypsin inhibitor gene (*CpTi*) (Agricultural Genetics Company, Cambridge, England) was incorporated into strawberry via *Agrobacterium*-mediated transformation using the *NPTII* marker (Graham *et al.*, 1995, 1997b, 2002). The insertion of *CpTi* into strawberry cultivars “Melody”

and “Symphony” was found to reduce vine weevil (*Otiorhynchus sulcatus*) damage in both greenhouse and field trials. The transgenic lines showed increased root growth, less larval feeding, and fewer pupae. In other work, strawberries transformed with the lectin *Galanthus nivalis* agglutinin (GNA), did not show any significant reduction in weevil feeding (Graham, 2005).

Enhanced resistance to *Verticillium dahliae* was observed in transgenic “Joliette” strawberry plants expressing a *Lycopersicon chilense* chitinase gene (*pcht28*) under the control of the CaMV 35S promoter (Chalavi *et al.*, 2003). A stipule regeneration system was used with *Agrobacterium*-mediated gene transfer. Constitutive expression of the chitinase gene was demonstrated by Northern analysis, and in growth chamber studies, transgenic strawberry plants had significantly higher resistance than controls, based on rates of crown infection and leaf wilting symptoms.

A gene encoding a PGIP has been cloned that shows developmental regulation and pathogen-induced expression in strawberry and likely plays a role in defense against fruit rots (Mehli *et al.*, 2004, 2005; Schaart *et al.*, 2005). After inoculation with *Botrytis cinerea*, fruit of five cultivars (“Elsanta”, “Korona”, “Polka”, “Senga Sengana”, and “Tenira”) showed a significant induction in PGIP expression and the most resistant one, “Polka”, had the highest constitutive expression. Work is ongoing to produce transgenic strawberries that overexpress PGIP sequences and screen them for resistance to *B. cinerea*.

The antisense of strawberry pectate lyase was incorporated into “Chandler” strawberry under control of the 35S promoter to increase fruit firmness (Jiménez-Bermúdez *et al.*, 2002). At full ripening, no differences in color, shape, and weight were noted between the transgenic and control plants, but the transgenics were significantly firmer. Pectase lyase activity was 30% lower in ripe transgenic fruit than the control. In another study, Jiménez-Bermúdez *et al.* (2002) found that expression of an antisense sequence of a strawberry pectate lyase gene reduced ascorbic acid content, presumably through reduced pectin solubilization in cell walls of transgenic plants.

Two endo- β -1,4-glucanase (EG) genes, *cell*, and *cel2*, have been isolated from strawberry that are closely related to tomato genes influencing softening. *Cell* is expressed specifically in ripening

fruit (Manning, 1998), while *cel2* mRNA is found primarily in young vegetative tissues and early green fruit (Trainotti *et al.*, 1999). *Cell* has been cloned into strawberry in the antisense orientation via *Agrobacterium*-mediated transformation using the plant binary vector pBINPLUS (Woolley *et al.*, 2001). In the transgenic strawberries, mRNA was strongly suppressed in ripe fruit; however, EG activity and firmness were not affected. The incorporation of *cell* had no effect on the transcription of *cel2*.

The S-adenosylmethionine hydrolase gene (*SAMase*) has been incorporated into strawberry, which controls ethylene biosynthesis and presumably effect fruit softening (Mathews *et al.*, 1995b). Strawberries are not climacteric fruit, but do have a limited response to ethylene and it is possible that reductions in ethylene biosynthesis during the post harvest period could slow down softening.

De la Fuente *et al.* (2006) has cloned *FaGAST* from strawberry, which encodes a small protein with 12 cysteine residues conserved in the C-terminal region that is similar to a group of proteins in other plant species that regulate cell division and elongation. Expression of *FaGAST* in transgenic *F. vesca* under the control of CaMV 35S resulted in delayed fruit growth, reduced fruit size, late flowering, and low sensitivity to gibberelin. Apparently, *FaGAST* plays a role in arresting fruit elongation during strawberry fruit ripening.

A number of recent molecular studies have searched for the genes in strawberry that are involved in cell wall modification during ripening and, therefore, influence fruit firmness. Harrison *et al.* (2001) identified and characterized a number of expansin genes (*FaExp2* to *FaExp7*), which likely induce cell wall extension *in vitro*. Messenger RNAs from most of these were present in leaves, roots, and fruit, except for *FaExp5*, which showed fruit-specific expression. Castillejo *et al.* (2004) isolated four pectin esterases from strawberry (*FaPE1* to *FaPE4*). *FaPE1* was specifically expressed in fruit and was upregulated by auxin treatment in green fruit and downregulated by exogenous applied ethylene in ripe and senescing fruits. The repression of *FaPE1* may be involved in textural changes during fruit senescence. Blando-Portales *et al.* (2004) identified a fruit-specific gene encoding for a HyPRP protein involved in the anchoring of polyphenols to cell membranes. Salentijn *et al.* (2003) found the expression of

two genes associated with lignin metabolism (cinnamoyl CoA reductase and cinnamyl alcohol dehydrogenase) to vary dramatically between soft fruited ("Gorella") and firm-fruited ("Holiday") cultivars.

Three full-length cDNAs encoding β -galactosidases (*Fa β gal1*, *Fa β gal2*, and *Fa β gal3*) were isolated from a library representing red fruit transcripts by Trainotti *et al.* (2001). Two of the genes had a C-terminus domain that was structurally related to known animal peptides with sugar-binding ability. Galactose is released during the dismantling of cell walls and the galactosidases are thought to play an important role in the mobilization of galactose. In a study of salt-extractable proteins from the cell walls of immature and ripe strawberry, Iannetta *et al.* (2004) identified seven abundant polypeptides, two of which were thought to be important determinants in the regulation of the sugar:acid balance (mitochondrial malate dehydrogenase and mitochondrial citrate synthase).

DNA microarrays have been utilized to identify and clone genes associated with strawberry flavor and aroma. Aharoni *et al.* (2000) identified a novel strawberry alcohol acyltransferase (SAAT) in *F. \times ananassa* cultivar Elsanta that is critical in flavor biogenesis in ripening fruit. This gene combines acyl-CoA and alcohol to generate the esters, the most important class of volatile compounds in fruit. Aharoni *et al.* (2004) also cloned the gene, *F. ananassa Nerolidol Synthase 1* (*FaNES1*), which was found in all three octoploid species, but not in *F. vesca* and *F. moschata*. It generates linalool and nerolidol when supplied by geranyl diphosphate or farnesyl diphosphate. They also found *F. vesca* to carry an insertion mutation in a terpene synthase gene that differs from the one in the cultivated strawberry (*F. ananassa Pinene Synthase*). This insertion limited its expression and further altered aroma by reducing quantities of pinene and myrcene.

A number of other genes have been characterized that are highly expressed during fruit ripening and maturation. Manning (1998) generated a cDNA library from messenger RNA isolated from ripe fruit, and identified a number of genes encoding enzymes of phenylpropanoid metabolism, and genes for cellulase, expansins, cysteine proteinase, and acyl carrier protein. Three mRNAs with fruit-specific, ripening-enhanced

expression have also been identified in ripening fruit using polymerase chain reaction (PCR) differential display (Wilkinson *et al.*, 1995). When sequenced, they had high homology with known proteins including: (1) an annexin, which may play a role in membrane function and cell wall structure, (2) chalcone synthase (CHS), which is a key enzymatic step in flavonoid biosynthesis, and (3) a ribosomal protein, most likely a 40S subunit. In addition, a gene (*njjs4*) has been identified, which is associated with the process of seed maturation and fruit ripening, and is related to the class-I LMW heat-shock-protein-like genes (Medina-Escobar *et al.*, 1998). Two auxin-induced and one auxin-repressed mRNAs from unknown genes have been cloned from receptacles of immature green fruit (Reddy *et al.*, 1990; Reddy and Poovaiah, 1990). Yubero-Serrano *et al.* (2003) identified a gene encoding a lipid transfer protein (*Fxaltp*) in strawberry fruit that responds to ABA, wounding, and cold stress. Aharoni *et al.* (2001) cloned the transcription factor *FaMYB1* from ripening fruit, which plays a key role in the biosynthesis of ACYs and flavonols.

Hoffman *et al.* (2006) used RNA interference (RNAi)-induced silencing to reduce activity of CHS in strawberry fruits (Figure 1). They used a construct (*ihpRNA*) containing the partial sense and corresponding antisense sequences of CHS separated by an intron (from a strawberry quinone oxidoreductase gene). An *Agrobacterium* suspension containing the gene was injected into 14-day-old fruit still attached to the plant. Almost white fruit were produced when the injection was repeated 3 days in a row.

DNA microarrays have also been used to profile cosmic patterns of gene expression during ripening. Aharoni and O'Connell (2002) found 441 transcripts to differ significantly between the achene and receptacle tissues. The most common transcripts found in achenes were those for signal and regulation cascades associated with achene maturation and stress tolerance. Representatives included phosphatases, protein kinases, 14-3-3 proteins, and transcription factors. Several genes were identified in the receptacle that encode proteins related to stress, the cell wall, DNA/RNA protein, and primary metabolism.

An NADPH-dependent D-galacturonic acid reductase gene (*GalUR*) was isolated and characterized from strawberry to determine its role in

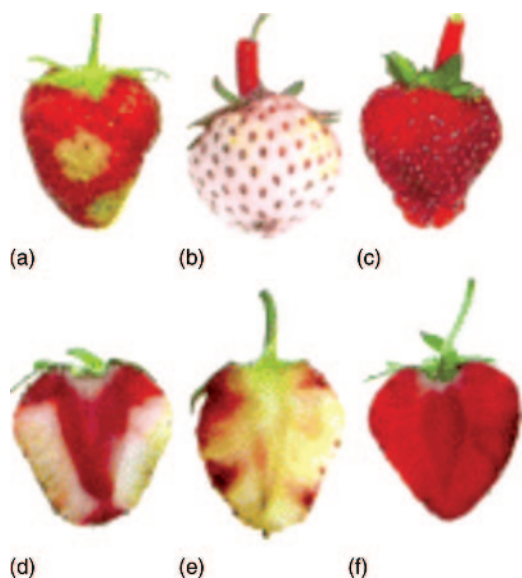


Figure 1 Silenced phenotypes of the *FaCHS-ihpRNA* transformed strawberries. (a) A chimeric phenotype where a solution of *Agrobacterium* containing the *ihpRNA* was injected. (b) An almost white fruit produced when the injection was repeated three times. (c) Control plants. (d, e, and f) cross sections of two chimeric fruit and a control [Reproduced from Hoffman *et al.*, 2006. © Blackwell Publishing]

vitamin C content (Angius *et al.*, 2003). Expression of *GalUR* correlated closely with ascorbic acid levels during strawberry fruit ripening and *GalUR* protein levels were found to be associated with ascorbic acid content in four species of *Fragaria* (*F. × ananassa*, *F. chiloensis*, *F. virginiana*, and *F. moschata*). The gene was not engineered into strawberry, but overexpression of *GalUR* in *Arabidopsis* enhanced vitamin C content two to threefold.

The acidic dehydrin WCOR410 from wheat was transferred to strawberry in an attempt to improve freezing tolerance (Houde *et al.*, 2004). The WCOR410 protein has been associated with the plasma membrane in wheat and its levels have been correlated with freezing tolerance. After acclimation, transgenic strawberry leaves had a 5°C improvement in freezing tolerance compared to controls. However, there was not a difference in the freezing tolerance of nonacclimated transgenics and controls, suggesting that another factor induces its expression during cold acclimation. Transgenic strawberries have also been developed that expressed antifreeze protein

gene (*AFP*) isolated from white flounder (Firsov and Dolgov, 1998), but no freezing trials were conducted.

In an earlier attempt to increase the freezing tolerance of strawberries, the transcription factor *CBF1* from *Arabidopsis* was overexpressed in the strawberry “Honeoye” (Owens *et al.*, 2002). The *CBF* genes are part of a family of cold- and drought-inducible transcription factors that bind to promoters containing a C-repeat/dehydration responsive element (CRT/DRE). This element is found in many cold-induced plant genes. *Agrobacterium*-mediated transformation with a CaMV 35S-*CBF1* construct was utilized to produce transgenic strawberries. The freezing tolerance of strawberry leaf discs from nonacclimated plants was significantly increased by 3–5°C. However, the freezing tolerance of floral tissue was not, despite expression of the *CBF1* transgene in receptacles at levels similar to developing leaves. A putative ortholog (*F. × ananassa CBF1* (*FaCBF1*)) was identified with 48% amino acid identity to *CBF1* from *Arabidopsis*.

To elucidate the molecular basis of cold acclimation in strawberry, Ndong *et al.* (1997) used differential screening to identify genes associated with low temperature acclimation. They identified three transcripts, *Fcor1-3* (*Fragaria cold-regulated 1-3*), whose levels changed dramatically after cold acclimation. Transcript accumulation for *Fcor3* was the most closely correlated with freezing tolerance, suggesting it may be a useful marker for this trait. *Fcor3* encodes a polypeptide that shows high identity with PSI polypeptides from spinach and barley.

The late embryogenesis abundant protein gene (*LEA3*) from barley (*Hordeum vulgare*) was used to transform the “Toyonaka” strawberry (Wang *et al.*, 2004) in hope of increasing the resistance of strawberry to salt stress. Calli from anthers were transformed by particle bombardment with plasmid pBY520. *In vitro* plants of transgenic strawberry had significantly less wilting than controls under 50 mmol l⁻¹ (19% vs. 62%) and 100 mmol l⁻¹ NaCl (43% vs. 96%).

Mezzetti *et al.* (2004) developed transgenic strawberries and raspberries carrying the *defH9-iaaM* auxin-synthesizing construct, composed of the regulatory region of the *DefH9* gene from snapdragon and the *iaaM* coding region from *Pseudomonas syringae*. The *defH9-iaaM*

gene was found to promote parthenocarpy in emasculated flowers of both strawberry and raspberry, and to increase fruit size, weight, and yield.

The FBP7 promoter (floral binding protein7) from petunia was found to be active in floral and fruit tissues of strawberry, using the β -glucuronidase gene as a reporter (Schaart *et al.*, 2002). β -glucuronidase (GUS) activity was found in floral and fruit tissues, but not vegetative ones, although *gus*-derived mRNAs were found in roots and petioles. The 35S promoter was found to be sixfold stronger than the FBP7 promoter.

Agius *et al.* (2005) used a transient expression system to conduct a functional analysis of homologous and heterologous promoters in strawberry fruit. The CaMV 35S promoter was fused to the *LUC* gene to optimize the transient assay. The GalUR promoter from strawberry was found to be active in fruit and under light regulation. Slight activity in fruit was found for the pepper fibrillin promoter, but not for the tomato polygalacturonase promoter.

A MADS box gene from strawberry, *STAG1*, has been cloned and characterized in transgenic plants (Rosin *et al.*, 2003). *STAG1* shares 68–91% sequence homology with *AGAMOUS* from numerous plant species. Analysis of the expression patterns of a *GUS* marker gene driven by the *STAG1* promoter revealed that *STAG1* was active in stamens, receptacles, petals, central pith, and vascular cells during floral development and achenes, pith, and cortical cells during fruit ripening.

Three major EST libraries of have been generated as genomic resources in strawberry. A cDNA library of over 1800 ESTs has been produced from whole plants treated with salicylic acid by Folta *et al.* (2005). A cDNA library of *F. vesca* with over 2500 ESTs has also been being constructed by Davis (2005). These efforts are part of a major Rosaceae Genomics Project centered at the Clemson University (http://www.mainlab.clemson.edu/gdr/about_gdr.shtml). Batley *et al.* (2005) have generated over 23 600 ESTs from a range of tissues, developmental stages, and experimental conditions, and identified 11 690 single nucleotide polymorphisms (SNPs) and 4200 SSRs for mapping. Their focus is on traits such as day neutrality and the fruit characteristics of firmness, flavor, taste, aroma, and color.

2.2 Transformation Methods Employed

2.2.1 Blueberry and cranberry transformation

2.2.1.1 Blueberry

Agrobacterium tumefaciens-mediated transformation is the only published approach that has yielded transgenic blueberry plants. Graham *et al.* (1996) described the transformation and regeneration of the “half high” blueberry “North Country” (*V. corymbosum* \times *V. angustifolium*), using *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) with a binary vector carrying an intron-containing *GUS* marker gene (Vancanneyt *et al.*, 1990). They found “North Country” to be hypersensitive to kanamycin and ticarcillin, and as a result, they did not use antibiotic selection. The regenerates were shown to be GUS-positive, but a Southern analysis was not performed to confirm transformation.

Song and Sink (2004) generated Southern blot-confirmed transgenic plants of highbush blueberry of four cultivars: “Aurora”, “Bluecrop”, “Brigitta”, and “Legacy” (Figure 2). Two selectable marker genes, *nptII* and *bar*, were used to produce the transgenic plants (Song and Sink, 2004; Song *et al.*, 2007). Their optimized transformation and selection protocols yielded transgenic plants at frequencies of 15.3% for “Aurora”, 5.0% for “Bluecrop”, 10.0% for “Brigitta”, and 5.6% for “Legacy”. Residual bacteria were successfully controlled using cefotaxime. The effect of glufosinate-ammonium (GS) on regeneration was also investigated and the combination of 0.1 mg l⁻¹ GS with 250 mg l⁻¹ timentin yielded effective shoot inhibition without leading to rapid death of explants. The protocol was then used to produce herbicide-resistant transgenic plants of “Legacy” (Song *et al.*, 2007). A detailed step-by-step description of the transformation procedure can be found in Song and Sink (2006).

Overall, *in vitro*-derived leaves have been found to be the most useful explant source in blueberry regeneration (Billings *et al.*, 1988; Callow *et al.*, 1989; Rowland and Ogden, 1992). Nickerson (1978) first reported low-bush blueberry regeneration from hypocotyl or cotyledon sections; however, shoot regeneration from seedling explants is not preferable since

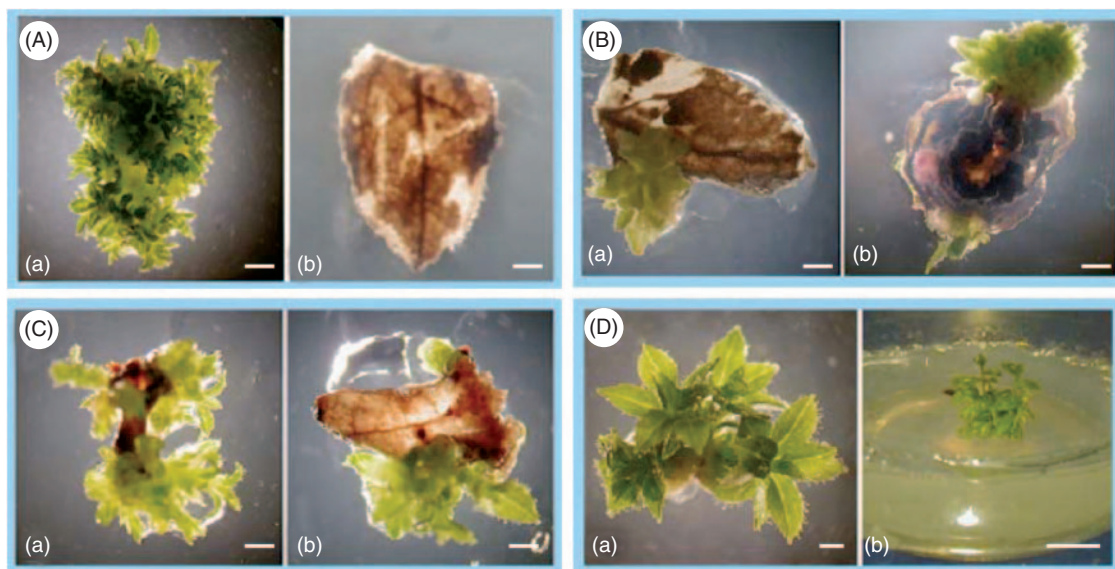


Figure 2 Transformation, selection, and regeneration of transgenic blueberry plants of the cv. Aurora. (A) Nontransformed leaf explants (a) on RM, (b) on RM containing 10 mg l^{-1} Km and 250 mg l^{-1} Cx, *Bar* = 1 mm. (B) Production of Km-resistant buds and shoots from surface (a) or wounded positions (b) of leaf explants, *Bar* = 1 mm. (C) Formation of Km-resistant shoot clusters from different positions of leaf explants, *Bar* = 1 mm. (D) Elongation of GUS-positive shoots on RM (a) or stock culture medium (b) containing 10 mg l^{-1} Km and 250 mg l^{-1} Cx, *Bar* = 1 mm (D-a), 1 cm (D-b) [Reproduced from Song and Sink, 2004]

blueberries are asexually propagated. Several medium and hormone combinations have been used to regenerate shoots. The cytokinin 6-(γ,γ -dimethylallylamino)-purine (2ip) was first examined to promote shoot regeneration from leaf explants, since it was effective for blueberry micropropagation. On woody plant medium (Lloyd and McCown, 1980) supplemented with $15 \mu\text{M}$ 2ip, 70–100% of leaf explants regenerated shoots from leaf explants of “Berkeley” and “Bluehaven”, and the mean number of shoots per regenerating leaf was about 7 (Billings *et al.*, 1988). On half strength MS medium +5 to $25 \mu\text{M}$ 2ip, adventitious shoots were produced on leaf explants of “Bluecrop”, “Bluejay”, and “Jersey”. Rowland and Ogden (1992) found that zeatin riboside was more effective than either of the cytokinins, 2ip and zeatin, in promoting shoot regeneration for leaf explants of “Sunrise”; whereas, “Bluecrop” and “Duke” produced no shoots on any of the media tested.

Hammerschlag’s group improved adventitious shoot organogenesis from leaf explants of five highbush cvs. using either thidiazuron (TDZ) at 1 or $5 \mu\text{M}$, or zeatin riboside at $20 \mu\text{M}$

(Cao and Hammerschlag, 2000; Cao *et al.*, 2002); a two-step pretreatment in liquid medium, including 4 days using $5 \mu\text{M}$ TDZ + $2.6 \mu\text{M}$ naphthaleneacetic acid (NAA) and 3 days using $7 \mu\text{M}$ zeatin riboside + $2.6 \mu\text{M}$ NAA, enhanced shoot regeneration of “Bluecrop” (Cao *et al.*, 2002). Shoot regeneration of six cultivars was evaluated after seven treatments on different media.

Song and Sink (2004) found that adventitious shoot regeneration occurred for all cultivars at high frequencies (86.7–100%), but the optimum treatment(s) varied among cultivars. TDZ and NAA were effective in promoting adventitious shoot formation for all of the cultivars in terms of the number of shoots per explant. Those cultivars that readily produced shoots by elongation of the buds on stem pieces placed horizontal on proliferation medium also regenerated shoots readily from leaf explants (Song and Sink, 2004).

Two studies have examined the factors influencing the early stages of transformation. Cao *et al.* (1998) used transient *gusA* expression to test the effects of *Agrobacterium tumefaciens*

strain, cocultivation, explant age, genotype, and sucrose concentration in the medium. They used 10 highbush cultivars and strains LBA4404 and EHA105, containing the binary vector p35SGUSint. LBA4404 was found to be much less virulent than EHA105, and 4 days of cocultivation with EHA105 yielded 50-fold more GUS-expressing zones than 2 days, with genotype and explant age also being important factors. Song and Sink (2004) found leaf explants to be susceptible to three *A. tumefaciens* strains EHA105, LBA4404, and GV3101 (Koncz and Schell, 1986), although EHA105 yielded higher frequencies of transient GUS expression than the other two. Six days cocultivation on filter paper overlaid medium yielded the highest level of transient GUS expression and did not lead to necrosis of the leaf explants. Transient GUS expression was also improved by using acetosyringone (100 μ M) and suitable medium. The blue staining cells, that were apparently susceptible to *A. tumefaciens*, were the ones active at the sites of tissue regeneration.

2.2.1.2 Cranberry

The first transgenic cranberry ‘Stevens’ was obtained using the particle bombardment method (Serres *et al.*, 1992), to which a patent (United State Patent 5240839) was issued in 1993 (<http://www.freepatentsonline.com/5240839.html>). This method was used later to produce transgenic ‘Pilgrim’ (Zeldin *et al.*, 2002). An outline of this transformation protocol is as follows. *In vitro*-derived, one-node stem sections, 5 mm, were pretreated for up to 20 days on BIM: Woody Plant Medium (Lloyd and McCown, 1980) + 0.13% D-gluconic acid + 2% sucrose + 0.3% agar + 0.1% gelrite + 10 μ M 2ip (N_6 -(2-isopentenyl)adenine) + 1 μ M TDZ (Serres *et al.*, 1992). One milligram gold particles were coated by 0.5 μ g plasmid DNA; 75 μ g DNA/bombardment was used. Stage I (2 days after bombardment): on BIM + 300 mg l^{-1} kanamycin (Km) (added before autoclaving); Stage II (3 weeks after bombardment): 5 ml overlay of 300 mg l^{-1} Km of sterile water on previous medium; Stage III (11 weeks after bombardment): on CMM + 300 mg l^{-1} for 9 days followed by a 200 mg l^{-1} Km of sterile water;

Stage IV (22 weeks after bombardment): on Km-free CMM.

Although successful, the bombardment method yielded a low frequency of transformation (0.15%). Additional efforts are therefore required to simplify the transformation protocol and improve transformation frequency. A higher shoot regeneration system now exists for five genotypes (‘Early Black’, ‘Pilgrim’, ‘Ben Lear’, ‘Stevens’, and ‘No. 35’) with 6.5–18.2 shoots/leaf (Polashock and Vorsa, 2002). Bombarded leaf explants of these genotypes could yield an improved transformation system. Particle bombardment-mediated transient transformation studies using leaf explants of these cultivars have yielded positive results, although stable transformation has not been reported (Polashock and Vorsa, 2002).

While *Agrobacterium*-mediated transformation has not been employed on cranberry, it has potential. Preliminary studies on *A. tumefaciens*-mediated transient transformation conducted by Vorsa’s group have proved that cranberry leaf explants are susceptible to *A. tumefaciens* strain EHA105 (Polashock and Vorsa, 2002). Also, leaf explants of cranberry are amenable to regeneration. Additional efforts should focus on optimizing *A. tumefaciens* infection conditions and developing effective antibiotic- or herbicide-selection systems for selective regeneration of transformed cells.

2.2.2 Cane fruit

A number of factors have been shown to play critical roles in determining regeneration and transformation rates of cane fruits including environmental conditions (Palonen and Buszard, 1998; Meng *et al.*, 2004), leaf orientation (McNicol and Graham, 1990), type of hormone (Fiola *et al.*, 1990; Millan-Mendoza and Graham, 1999), and most importantly genotype (Reed, 1990; Owens *et al.*, 1992; Graham *et al.*, 1997a). Both blackberries and raspberries have been regenerated from leaves, stem segments, and cotyledons (Hall *et al.*, 1986). Regenerate shoots of blackberry most readily form at the leaf–petiole junction from phloem parenchyma cells or cells surrounding the vascular bundles (Ke *et al.*, 1988). Protoplasts have also been isolated from various *Rubus* species, but no regenerates have been produced beyond callus

(Mezzetti *et al.*, 1999; Dunland *et al.*, 2000; Nita-Lazar *et al.*, 2000).

MS medium that is enriched with cytokinin and auxin is usually utilized for organogenesis (Swartz and Stover, 1996; Norton and Skirvin, 1997). Indole-3-butyric acid (IBA), NAA, or 2,4-dichloro-phenoxyacetic acid have been used successfully as auxins, and TDZ is the most effective cytokinin (Fiola *et al.*, 1990; McNicol and Graham, 1990). Explants are most commonly cultured in the dark for varying periods from 1 day to several weeks before being transferred into the light (Fiola *et al.*, 1990; McNicol and Graham, 1990; Meng *et al.*, 2004), and regeneration has been found to occur more readily at 20 °C than 25 °C (Turk *et al.*, 1993).

2.2.3 Strawberry

Strawberries were one of the first crops to be routinely proliferated through micropropagation (Zimmerman, 1991), and regeneration systems for *F. × ananassa* have been developed with disarmed strains of *A. tumefaciens* using anthers, callus, flower buds, leaf discs, protoplasts, petioles, stems, stipules, roots, and runners (Hokanson and Maas, 2001; Passey *et al.*, 2003). Callus, petiole sections, and leaf discs have also been used in combination with *A. tumefaciens* to regenerate *F. vesca* (Haymes and Davis, 1998; Alsheikh *et al.*, 2002). Genotypes vary widely in the success of the various techniques, and some are quite recalcitrant to all techniques. A genetic line of *F. × ananassa*, LF9, has been developed that produces transformed shoots in as few as 15 days (Figure 3; Folta *et al.*, 2006).

Most of the transgenic strawberries to date have been generated using *Agrobacterium*-mediated transformation systems. *Agrobacterium* strains LBA4404 and EHA105 have been most commonly employed, with pBIN19 derivatives as the binary vector (Graham, 2005). In most cases, leaf- or stem-based systems have been utilized in MS medium. The hormones have been BA plus 2,4-D or IAA. Regenerates have generally been selected using 25 mg l⁻¹ kanamycin, and contamination after inoculation has been limited by using cefotaxime, carbenicillin, and ticarcillin.

Biolistics have been employed in three instances with strawberry. De Mesa *et al.* (2000)

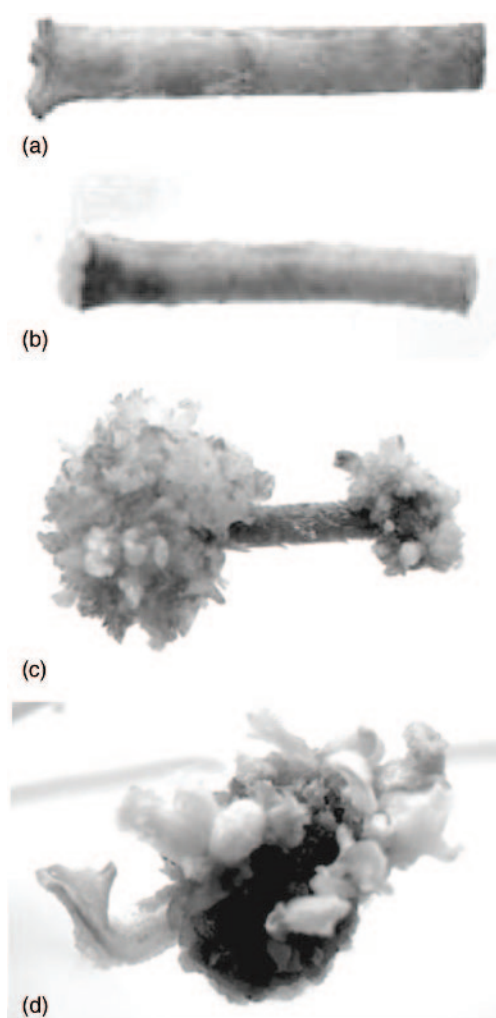


Figure 3 The progression of shoot regeneration in LF9 petiole cultured on medium supplemented with 0.05 μM 2,4-D in combination with 0.10–0.50 μM BA and 4.54 μM TDZ. (a) 0 days; (b) after 5 days; (c) after approximately 40 days; (d) a comparable explant grown in complete darkness with occasional inspection under light [Reproduced from Folta *et al.*, 2006. © Springer]

bombarded leaf discs with *Agrobacterium*-coated gold microprojectiles as a means to enhance stable transformation of GUS. Wang *et al.* (2004) bombarded strawberry calli with tungsten particles coated with the pBY520 plasmid using PDS-1000/He. Agius *et al.* (2005) used particle bombardment to effect transient transformation of strawberry fruit.

An effective marker-free transformation process has recently been successfully tested in strawberry (Schaart *et al.*, 2004). In it, a vector was constructed in which site-specific recombination left only the 35S promoter of cauliflower mosaic virus and a GUS encoding sequence. The system incorporated an inducible site-specific recombinase to eliminate the selectable marker. Fully marker-free transgenic “Calypso” plants were obtained through this procedure.

2.3 Testing

There have not been many reports on field trials of transgenic plants of *Vaccinium* species. Polashock and Vorsa (2002) mentioned in their review that field tests were conducted to evaluate transgenic “Stevens” and “Pilgrim” cranberries, both with *Bacillus thuringiensis* (*Bt*). Four transclones showed a significant increase in blackheaded fireworm (BHFw, *Rhopobota naevana*) mortality rate, although none of the 64 *Bt*-expressing transclones yielded significant BHFw larval mortality in *in vitro* feeding assays. Herbicide-resistant cranberry with *bar* was also evaluated under greenhouse and coldframe conditions; one transclone with *bar* showed moderate tolerance, 500 mg l⁻¹ glufosinate ammonium, to glufosinate-herbicide Liberty (Zeldin *et al.*, 2002). Similarly, *bar*-expressing blueberry “Legacy” showed high herbicide resistance under controlled conditions (Song *et al.*, 2007). Most recently, field trials are being conducted in Michigan to evaluate the *bar*-expressing “Legacy”; several transgenics were identified with strong herbicide resistance (Song *et al.*, 2007).

In an effort to engineer resistance to RBDV, Martin *et al.* (2004) produced 197 transgenic lines of “Meeker” red raspberry with either the coat protein gene, mutated forms of the movement protein gene, or nontranslatable RNA of RBDV. These lines were planted in a field with extreme disease pressure, and after 4 years, five lines were found to be resistant. Fruits from these lines had a similar ACY profile to those of wild type fruit.

To develop strawberry cultivars resistant to glyphosate, the *CP4.EPSP* synthase gene was introduced into “Camarosa” through *Agrobacterium*-mediated transformation. When 73 independent transformants were sprayed with

Roundup Ultra, they showed a wide range in symptoms, but 30 of them were highly tolerant. Southern analysis of the most tolerant lines showed that all but two of them had only a single copy of the *CP4.EPSP* synthase gene. The transformed, glyphosate-sprayed lines produced typical, good quality fruit.

Graham *et al.* (2002) trialed transgenic strawberries constitutively expressing the cowpea trypsin inhibitor (*CpTi*) to determine if they offered protection against feeding of the vine weevil (*Otiorhynchus sulcatus*). Over 3 years, two transgenic “Melody” and three transgenic “Symphony” lines showed significantly improved plant growth in comparison to controls and had a significant reduction in the number of pupae feeding on their roots. Yield was not assessed due to severe fruit rot, but no effects on the numbers of Carabid and other nontarget arthropods were noted.

Mezzetti *et al.* (2004) field trialed transgenic strawberries and raspberries carrying the *defH9-iaaM* auxin-synthesizing gene. The transgenic plants showed significant increases in fruit number, size, and yield. The increase in fruit yield was about 180% in cultivated strawberry, 140% in wild strawberry, and 100% in raspberry.

2.4 Specific Regulatory Measures Adapted

To date, no requests have been made for the unrestricted release of transgenic small fruit crops, and only a very limited number of field trials have been conducted. These have been conducted under the auspices of local Laboratory Biosafety Committees, the Animal and Plant Health Inspection Service (APHIS) of USDA, Food and Drug Administration (FDA), Environmental Protection Agency (EPA), State regulation, and International agreements.

3. FUTURE ROAD MAP

3.1 Expected Products

Weed infestation in fields is one of the major problems in all small fruit crops, particularly strawberry. To control weeds in many crops, nonselective and broad-spectrum herbicides, such

as glyphosate and phosphinothricin, are usually used although they can only be applied as a directed spray under the bushes and avoiding any contact with the green tissues. Herbicide-resistant plants can be expected to broaden the application of nonselective herbicides and to provide a simple, inexpensive, potent, and environmentally friendly management for weed control. Currently, two transgenic herbicide-resistant cropping systems are common for soybean, maize, rapeseed, and cotton: RoundupReady (active agent: glyphosate) and Liberty Link (active agent: glufosinate).

With the impending ban on the use of MB fumigation in strawberry production, growers have lost their most effective means to control weeds and soil pathogens. Yields in most of the cultivars now grown are reduced by 50% in nonfumigated soil, and only limited genetic variability for resistance has been found to the broad range of pathogens controlled by MB fumigation (Larson and Shaw, 1995a, b; Particka and Hancock, 2005). Herbicide-resistant strawberries with the *CP4-EPSP* synthase gene or *PAT* would allow for the effective control of weeds, and a transgene like *pcht28* that provided resistance to a broad range of fungal pathogens could restore considerable yield potential. Engineered resistance to *Phytophthora fragariae* root rot would also be extremely valuable in raspberry, as it is a major objective of all breeding programs. To minimize marketers concerns about the acceptance of transgenic fruit, targeted expression of these genes to just vegetative tissue would likely be required. The use of marker-free selection systems might also prove beneficial.

The incorporation of transgenes for fruit rot resistance and increased firmness in strawberries and raspberries, such as the antisense of genes for pectate lyase and polygalacturonase-inhibiting protein, would be highly beneficial if the public is prepared to accept them. Strawberries and brambles are a highly perishable fruit and even with controlled atmosphere storage and refrigeration, a high proportion of the harvested fruit are lost due to softening and fungal disease. In particular, *Botrytis* and *Phytophthora* infections are common sources of substantial crop losses in all of the small fruit crops.

Transgenic breeding could also provide small fruit crops with resistance to major virus diseases.

Of particular importance are raspberry bushy dwarf, blueberry stunt, blueberry shoestring, and strawberry crinkle and others in the yellows complex. In addition to expression of coat protein for protection against these diseases, application of RNAi technology could be exploited to obtain virus-resistant plants (Tenllado *et al.*, 2004; Hoffman *et al.*, 2006). Resistance to RBDV has already been engineered in raspberry through transgenic approaches, but the resistant lines have not gone through the regulatory system.

The distribution of small fruit cultivation is often restricted by low temperature stress. Frost tolerance at bloom is particularly important in all the small fruits and winter cold hardiness is often an issue, particularly in raspberries. While some genetic variability exists for these characteristics, very little improvement has been made by plant breeders. The incorporation of genes such as *CBF1* could provide the necessary genetic variability to improve this trait, if their phenotypic effects could be limited by targeting their expression to floral tissues or periods of cold (Kasuga *et al.*, 2004).

Cool temperatures during flowering also commonly limit small fruit yields. When temperatures are low during bloom, pollinator activity is greatly reduced leading to poor seed set, reduced fruit size, irregularly shaped fruits, and low yield. The incorporation of a gene that induces parthenocarpic fruiting like the *defH9-iaaM* auxin-synthesizing gene would be very beneficial in areas where cool conditions prevail during pollination.

Transgenic approaches might also be employed to modify the secondary metabolism of the small fruit crops to improve their nutritional quality and flavor (Scalzo *et al.*, 2005). Successful modification of the nutritional value of tomatoes through metabolic engineering and transformation has provided a novel example of the use of organ-specific gene silencing to enhance the nutritional value of fruits (Davuluri *et al.*, 2005). Biochemical pathways might be altered through transgenic approaches to enhance ACY production and strengthen aroma. Numerous genes have been identified in strawberries that are associated with aroma (Aharoni *et al.*, 2000, 2004) and flavonoid metabolism (Wilkinson *et al.*, 1995; Manning, 1998).

3.2 Addressing Risks and Concerns

Several obstacles are working against the acceptance of transgenic blueberries, cane fruit, cranberries, and strawberries. The economic value of these fruit crops is limited compared to many of the agronomic crops and as a result there is only modest private stimulus to develop new biotechnological products. A second issue is that they are all outcrossed crops with widespread, native relatives in close proximity to cultivated fields. Most transgenic crop releases to date have been with species that do not have nearby congeners, eliminating the risk of the movement of the transgene into wild species populations. The release of transgenic small fruit crops will require more scrutiny and in-depth ecological studies than has been necessary with those trans crops previously released (Hancock, 2003). The third issue associated with the commercial release of transgenic blueberries, cane berries, cranberries, and strawberries is reluctance by the fruit industry to introduce transgenic products, for fear that there will be a negative backlash from people leery of consuming genetically modified (GM) crops.

A strong influx of federal and state funds is needed to stimulate strawberry biotechnology research, along with a careful analysis of what people's real perceptions are about transgenic fruit. Until this happens, transgenic small fruit crops will remain an important research tool, but not a commercial entity. Utilizing marker-free transformation systems and targeted expression of transgenes will help minimize public concern, but the fear of the technology in general must be reduced before transgenic small fruit products will find their way into homes.

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Cherries

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Central Asia is the likely origin of the earliest diploid *Prunus* species (Watkins, 1976). Species in the section *Cerasus*, which includes sweet (*P. avium* L.), sour (*P. cerasus* L.), and ground (*P. fruticosa* Pallas) cherry, are thought to be derived from this ancestral *Prunus*. The *Cerasus* cherries developed to the west of the central Asian subgenus *Cerasus*' center of origin, while most other *Prunus* species evolved in the east.

Hedrick (1915) described the geographic range of wild sweet cherry as all of mainland Europe well into the southern part of erstwhile USSR and as far east as northern India, with the greatest prevalence between the Caspian Sea and the Black Sea. In contrast, the ground cherry's center of origin is western and central Asia (Watkins, 1976).

It is reported that sweet cherries were brought by the Roman General Lucullus from Cerasunt on the Black Sea to Rome in 74 BC, and they spread from there to Germany and Britain. Sweet cherries (mazzard) have been grown from southern Russia, north of the Caucasian mountains, to the north of France for centuries. The ground cherry has a wider area of distribution, which overlaps with the center of wild sweet cherry, thus giving rise to hybrids as sour cherries. The sour cherry is a native of the Carpathian Basin. Domestication

and cultivation have resulted in some ecotypes of sweet, sour, and ground cherries arising in the various areas of Europe and partly in Asia. Several sweet and sour types are adapted to harsh winter conditions (Iezzoni *et al.*, 1991; Faust and Surányi, 1997).

There is good evidence suggesting that *P. cerasus*, a tetraploid, arose from a natural cross between *P. avium* and *P. fruticosa*. The geographic ranges of the two species overlap in northern Iran and Turkmenistan, which is the center of origin of sour cherry. From there, sour cherry followed a similar course to Europe as sweet cherry, and ultimately came to North America with English settlers. It is more tolerant of the humid, rainy eastern conditions, and therefore proliferated more than sweets, where it is still cultivated today in greatest numbers. Michigan, the leading producer in North America, grows sour cherries along the eastern shore of Lake Michigan, where the moderating influence of the lake on winter and spring temperatures is beneficial to production.

Although, cultivated cherries are ubiquitous in the temperate zone, there has been little effort to take them further south into subtropical regions. Among cherry genotypes, there are cultivars that require low levels of winter chilling, although the existing high-quality cherry cultivars all have high chilling requirements. Commercial production of sweet cherries is constrained by rainfall during the ripening period, which causes cracking of

Bigarreau-type (firm-fleshed) sweet cherries as well as subsequent brown rot infection, which destroys the value of the fruit.

1.2 Botanical Features

The “true” cherries are now classified by botanists within the genus *Prunus*, subgenus *Cerasus*, family Rosaceae, rather than by the older system within the genus *Cerasus* (Webster, 1996). The subgenus *Cerasus* is divided into eight sections, and section 1, *Eucerasus*, has *P. avium*, *P. cerasus*, and *P. fruticosa*. Altogether, there are over 150 *Prunus* species.

The mature sour cherry is a medium-size tree with a rounder, more spreading habit than the erect sweet cherry. Tree height is maintained at 3 m or less in cultivation. Leaves are smaller than that of sweet cherry, elliptic with acute tips, mildly serrate margins, with long petioles. Individual flowers are the same as in sweet cherry. Sour cherry inflorescence buds usually produce 2–4 flowers, with long pedicels, as in sweet cherry. However, a higher proportion is borne laterally on 1-year wood rather than on spurs, as compared to sweets. Spurs are shorter lived on sour than sweet, gradually declining in productivity over 3–5 years. Sour cherries are the latest blooming of the stone fruits. Most sour cherries are self-fertile, and require no pollenizers. The fruit is a drupe, 20–25 mm in diameter, round or heart-shaped, glabrous, with a long pedicel attached. The pit is generally smooth, and encloses a single seed. Sour cherries generally have lower sugar and higher organic acid content than sweet cherries, giving them their distinct flavor. They are generally bright red in color, and exhibit less color variation than sweets, which are usually dark red to purple. “Montmorency” produces about two-third of its fruit laterally on longer wood, and one-third on spurs. Both sweet and sour cherries require only about 2–3 months for fruit development. No thinning is required for sour cherries, whereas sweet cherries often are pruned annually to reduce the crop load and increase fruit size.

Sour cherry is naturalized widely and its distribution area covers almost all European countries and Southwest Asia. However, it is cultivated in many additional parts of the world, mainly in North America. Both sweet and sour

cherries develop extensive root systems in deep, well-drained, gravelly to sandy-loam soils, which are optimum. Thus, aeration is more important than water-holding capacity, and flooding or heavy soils limit growth and production (Webster and Looney, 1996). Sweet cherry performance is best in cool, dry climates with limited exposure to spring frosts and diseases, and lack of rainfall during harvest. Rainfall and/or high humidity, especially at bloom or harvest, promote fungal and bacterial diseases that kill flowers and shoots or incite fruit rots. During harvest, rain causes the fruit to crack. Thus again, dry climates are preferred (Webster and Looney, 1996). These favorable environmental conditions explain why, in the United States, Washington, Oregon, and California are the leading producers. Conversely, sour cherries prefer cool, humid conditions; although they can be grown in the western US, fruit size is diminished by the arid climate. Sweet and sour cherries both require 1000–1500 h of chilling temperatures in winter; thus, they cannot be grown in warmer climates. Dormant buds are killed at about -29°C (-20°F), and the pistil is particularly sensitive to spring frosts.

The 2C value for diploid “Van” sweet cherry was reported as 0.67 pg (picogram), which is about one-half that of the tetraploid *Prunus* \times sp. “4x” of 1.36 pg, while tetraploid “Montmorency” sour cherry had a 2C of 1.42 pg (Dickson *et al.*, 1992). Twenty-six isozyme loci and one morphological trait (Tobutt and Nicoll, 1992), and 89 markers falling into 10 linkage groups (Stockinger *et al.*, 1996), have been accomplished for diploid $2n = 2x = 16$ sweet cherry. For sour cherry, $2n = 4x = 32$, restriction fragment length polymorphism maps of the cvs. Rheinische Schattenmorelle (RS) and Erdi Botermo (EB) were constructed using 86 progeny from the RS \times EB cross (Wang *et al.*, 1998). The RS map has 126 markers while EB has 95 markers in 19 and 16 linkage groups, respectively. Bridges between RS and EB were identified by 53 common markers to 13 homologous linkage groups. From a cytological perspective, sour cherry has disomic inheritance but occasional intergenomic pairing with pre- or postzygotic selection (abundant 2:1 ratios) (Beaver and Iezzoni, 1993). Mapping and expressed sequence tag (EST) data are both included in the Clemson Genome Database for Rosaceae (<http://mainlab.clemson.edu/gdr/genus/prunus/>

cherry/index.shtm). Details of genome mapping and molecular breeding have been reviewed thoroughly by Dirlewanger *et al.* (2006).

1.3 Economic Importance

Sweet cherries are grown in more than 45 countries of the world, and sour cherries in at least 35 countries (Webster and Looney, 1996). Based on the top 20 countries of production for each, world production of sweet cherries (1.86 million metric tons) is about 53% higher than that for sour cherries (1.2 million metric tons), and the worldwide sweet cherry crop value of US\$1.44 billion is about 134% higher than that for sour cherry (FAOSTAT, 2005). No single country accounts for more than 14% of world sweet cherry production (O'Rourke, 2006). The relatively short seasonality, high perishability, and delicate handling required for high-quality fresh sweet cherries likely contribute to this egalitarian worldwide production picture.

The market for fresh sweet cherries has remained very strong for more than the past decade (1995–2005), an otherwise turbulent period for most tree fruit markets (e.g., apple, peach, pear). Consequently, world harvested area of sweet cherries has been increasing dramatically, from 289 000 ha (1985) to 327 000 ha (1995) to 401 000 ha (2005), and the area actually planted to sweet cherries (but not yet bearing) is likely to be significantly higher yet (O'Rourke, 2006). The increase has been especially dramatic in North America, with a 48% increase in production area from 1995 to 2005. In terms of sweet cherry area harvested, the top countries are Spain, Germany, the United States, Italy, the Russian Federation, Turkey, and Iran. On a percentage increase basis, the most rapid increases in production area have been in the southern hemisphere regions of Chile, Argentina, Australia, New Zealand, and South Africa.

Although world production area has been increasing dramatically, actual world tonnage hit a record level (1.9 million metric tons) in 2000 but has been at a plateau between 1.8 and 1.9 million metric tons since then (O'Rourke, 2006). The top three countries of production are Turkey (260 000 t), the United States (250 000 t), and Iran (223 000 t), followed by Germany, Italy, and the

Russian Federation at about half the level of the top three. From a sweet cherry breeding point of view, this discrepancy between area planted and production suggests that significant production gains have yet to be made through improved adaptive traits associated with its many abiotic and biotic limitations.

Worldwide sour cherry production is led by the Russian Federation at a 2005 harvest of 235 000 t, followed by Ukraine (178 000 t), Turkey and Poland (about 140 000 t each), Serbia and Montenegro (116 000 t), the United States (98 000 t), and Hungary and Germany at about 81 000 t (FAOSTAT, 2005). A significant portion of the production in the Russian Federation, Ukraine, and Serbia and Montenegro is probably from “backyard” trees rather than commercially managed orchards (Webster and Looney, 1996).

The primary market value of sweet cherries is as a premium fresh product eaten out of hand, but industrial uses of processed sweet cherries are also important. The primary market value of sour cherries comes from industrial uses, though some are eaten fresh. Processed uses of sweet and sour cherries include frozen, canned, and dried forms for use in baking (e.g., pastries, pie filling, and dessert toppings); ice cream and yogurt; additives to snacks, breakfast cereals, confections, salads, etc.; juices, liqueurs, wines, and flavorings; preserves, jellies, and jams; as well as brined for maraschino cherry products.

During the past decade, both sweet and tart cherry fruits have become recognized for potential benefits to human health. Both are rich in vitamin C and potassium (Iezzoni *et al.*, 1991; Looney *et al.*, 1996) as well as multiple forms of phytochemical compounds, such as bioflavonoids and anthocyanins (Brown *et al.*, 1996). Certain forms of both of these compound groups have been shown to exhibit antioxidant and anti-inflammatory properties, including cyclooxygenase (COX-I and COX-II) inhibition comparable to over-the-counter drugs such as ibuprofen (Wang *et al.*, 1999, 2000; Seeram *et al.*, 2001; Tall *et al.*, 2004). Kang *et al.* (2003) also demonstrated inhibitory activities of tart cherry anthocyanins against colon cancer. Cherries may become a valuable nutritional supplement for human diets to help reduce the risk of, and alleviate the pain from, degenerative diseases such as arthritis (Seeram

et al., 2001). Concentrated sour cherry juice and powdered cherry extracts (in tablet form) for health consumption are additional industrial uses that are likely to increase future fruit utilization.

1.4 Traditional Cherry Breeding

The most important sweet cherry variety in North America is “Bing”, selected from a chance seedling in the late 1800s (Hedrick, 1915) for its outstanding flavor, firmness, size, and adaptation to the Pacific Northwest United States. Similarly, “Montmorency”, a seedling selected in Europe prior to the 1600s (Hedrick, 1915), has been the most important sour cherry variety in North America for more than a century, and it remains the industry standard today. Selections within commercial orchards, often by growers, of more productive “strains” of standards such as “Bing” or “Montmorency” historically have been a common tool used for genetic improvement from ancient cultures (Fogle, 1975) even to the present day. For example, in the late 1990s, commercial producers of “Lapins” sweet cherry have selected later ripening sports that subsequently have been named (“Sweet ValentineTM” and “Sweet Georgia”) and promoted commercially to extend the ripening season. The majority of the cherry rootstocks used worldwide are relatively unimproved seedlings of *P. avium*, *P. cerasus*, or *P. mahaleb*. Clearly, there is great potential for genetic improvement of sweet and sour cherries as well as rootstocks via traditional and modern breeding efforts.

1.4.1 Sweet cherry breeding objectives

Fogle (1975) noted that sweet cherries are not well adapted to many climates, therefore historical genetic improvement efforts have been localized to areas with significant production. With the rise of global trade and postharvest technologies for long-distance shipping, genetic improvement efforts in the 21st century have become, to a great extent, similar the world over. The most commonly cited sweet cherry breeding objectives are listed in Table 1 and discussed briefly below.

Self-compatible (“self-fertile”) varieties can increase orchard area productivity, since less valuable pollenizer varieties (usually about 10% of the orchard) are not needed. Similarly, variations in bloom overlap of primary and pollenizer varieties can be eliminated, thereby improving fruit set and yield consistency. Extension of the ripening season is critical for more competitive marketing, as well as for more efficient use, and better retention, of seasonal harvest labor.

Reduced susceptibility to rain-induced fruit cracking is a universal goal even in the arid regions that dominate world production, since a single rain event at the wrong time can render a high-value cherry crop unmarketable. Fruit cracking is a complex trait (which is also influenced by temperature), as genotypes can vary both in their sensitivity to rain, as well as in the developmental stage at which they are most sensitive; for example, some varieties are more susceptible to cracking early in stage III (color change from green to yellow) and others later in stage III (color change from pink to red). Consequently, this is

Table 1 Most common sweet cherry breeding objectives

Breeding objective	Breeding programs and/or reviews
Self-compatibility	Blazková, 1996; Nikolic <i>et al.</i> , 1996; Saunier, 1996; Granger, 1998; Olmstead <i>et al.</i> , 2000b; Apostol, 2005a; Kappel, 2005; Sansavini and Lugli, 2005
Extended ripening	Saunier, 1996; Olmstead <i>et al.</i> , 2000b; Apostol, 2005a; Kappel, 2005; Sansavini and Lugli, 2005
Cracking susceptibility	Blazková, 1996; Nikolic <i>et al.</i> , 1996; Saunier, 1996; Andersen, 1998; Granger, 1998; Kappel, 2005; Sansavini and Lugli, 2005
Fruit quality traits	Blazková, 1996; Saunier, 1996; Andersen, 1998; Granger, 1998; Olmstead <i>et al.</i> , 2000b; Apostol, 2005a; Kappel, 2005; Sansavini and Lugli, 2005
Yield traits	Nikolic <i>et al.</i> , 1996; Andersen, 1998; Kappel, 2005; Sansavini and Lugli, 2005
Pest resistance	Blazková, 1996; Nikolic <i>et al.</i> , 1996; Lang <i>et al.</i> , 1998; Apostol, 2005a
Climatic adaptations	Blazková, 1996; Brown <i>et al.</i> , 1996; Nikolic <i>et al.</i> , 1996; Andersen, 1998; Lang <i>et al.</i> , 1998
Fruit color traits	Olmstead <i>et al.</i> , 2000b; Sansavini and Lugli, 2005
Mechanical harvest	Saunier, 1996

a trait perhaps most likely to be improved where the selection pressure is greatest due to higher incidence of rain events during harvest, rather than in the more important production areas where such events are infrequent. The complexity of the trait has, thus far, made it difficult to adequately screen for in laboratory assays.

High-quality fruit is a complex of multiple traits including large size, firm flesh, intense sweet–tart flavor, and good postharvest performance. Fogle (1975) advocated a minimum average diameter of 30 mm as a strong selection criterion, since large fruits command significantly higher market prices (though not in the absence of at least threshold levels of the other traits). Firm flesh is universally desired by both shippers and consumers. The intense sweet–tart flavor of premium sweet cherries is a balance of high soluble solids, relatively high acidity, and a slight astringency; a combination of pleasing sweetness with refreshing modest tartness that results in a desire for continued consumption rather than rapid satiety. There is no standard set of measures against which quantitative data can be compared, but rather a subjective balance of sugars, acids, and subtle flavor components that must be evaluated by the breeder or a trained taste panel as a complete sensory experience in combination with flesh texture and juiciness.

Yield traits include precocity, productivity, and consistency. Precocity, or the time to first fruiting, can be altered in a number of ways—planting at higher densities (Robinson *et al.*, 2005; Seavert, 2005), use of precocity-inducing rootstocks such as Gisela 5 (Lang, 2000), use of precocity-inducing tree training or orchard management strategies (Long, 2005), use of growth regulators (Elfving *et al.*, 2003), or via genetically improved precocity of scion varieties, such as that imparted by “Van” (Fogle, 1961). The pressure for breeding programs to increase the efficiency of the breeding cycle tends to lead to selection of more precocious varieties simply to avoid the expense of maintaining seedling trees for 8–10 years to obtain initial fruiting data. Productivity is a trait best determined on a comparative basis, depending on production region. High yields tend to inversely affect fruit size; however, where yields often are limited by climatic factors (e.g., spring frosts, low winter chilling, and poor pollination conditions), genotypes with a tendency to set excessive crops

under ideal conditions may be highly desirable after all.

Resistance or high tolerance to diseases, such as brown rot (*Monilia/Monilinia* spp.), leaf spot (*Blumeriella jaapii* Rehmi), powdery mildew (*Podosphaera clandestina* (Wall.:Fr.) Lev.), *Cytospora* spp., and bacterial canker (*Pseudomonas* spp.), is increasingly a concern as synthetic pesticide use is curtailed by product availability or by the demands of the market place. While few breeders have noted insect resistance as a sweet cherry breeding objective, genetic avoidance of such pests as cherry fruit flies (*Rhagoletis* spp.), black cherry aphid (*Myzus cerasi* Fab.), and plum curculio (*Conotrachelus nenuphar* Herbst) certainly would be welcome by growers.

Traits for adaptation to specific climates vary by location, and include late bloom for frost avoidance and/or tolerance of flower buds to low spring temperatures, survival of low winter temperatures (particularly in concert with resistance to the canker-causing bacterial and fungal diseases that often interact with sublethal low temperatures to kill trees), resistance to high-temperature-induced fruit doubling in hot climates, and adaptability to climates with very little of the winter chilling temperatures required for breaking dormancy.

Although sweet cherry flesh colors range from a near lack of pigment to pure yellow to pink to bright red to dark purple, currently varieties with dark red (mahogany) flesh and skin predominate for fresh market production. However, diverse fruit color traits can expand marketing opportunities. In the past 15 years, the so-called blush-type sweet cherries—having yellow flesh and yellow skin with patches of red “blush”—have become increasingly important as a “super-premium” product that commands the highest price in many markets, thereby generating interest in this and other variations that would stand apart from standard mahogany-colored fruits.

Suitability for mechanical harvest, including uniform ripening and ease of stem detachment from the fruit, may be increasingly important as harvest labor becomes more difficult and expensive to procure. Such traits would have to be combined with other critical fruit traits like firmness, and resistance to bruising and pitting as well as perhaps with changes in tree architectural traits and improved mechanical harvest technologies.

1.4.2 Sour cherry breeding objectives

Due to their primarily industrial use, sour cherry traits such as fruit size, flavor balance, and fresh appearance are not nearly as critical as for sweet cherries (Fogle, 1975). Certainly, threshold levels of flavor components and fruit size are necessary, but high productivity and adequate fruit texture (to withstand mechanical harvest, pitting, and processing) are of greater importance. As worldwide sour cherry production and value is less than that for sweet cherries, there are fewer breeding programs actively involved in sour cherry improvement. The most commonly cited breeding objectives for sour cherry are listed in Table 2 and discussed briefly below.

Important diseases for which resistance or high tolerance would be desirable include leaf spot, *Cytospora leucostoma* (Pers.) Fr. and *Cytospora rubescens* Fr., brown rot, *Prunus* necrotic ringspot virus (PNRSV), and sour cherry yellows disease. Leaf spot is the most important disease in worldwide sour cherry production (Iezzoni *et al.*, 1991), sometimes causing complete defoliation during mid-to-late summer that can prevent normal ripening of the crop as well as predispose trees to subsequent winter injury. Extension of the ripening season facilitates more efficient utilization of harvest machinery and industrial processing capacities as well as diversifies production risks from adverse climatic events. The advantages of self-compatibility, and increased and more consistent yields, are similar to those noted above for sweet cherry.

Key traits for high-quality sour cherry fruit include having high soluble solids, dark red juice and flesh, round pits, and good flavor. Traits that confer suitability for mechanical

harvest include varieties having dwarf stature when planted on their own roots (i.e., without grafting to rootstocks), uniformity of ripening, and ease of stem detachment from the fruit. Smaller trees increase the potential options for mechanical harvest technologies as well as can lead to improved quality of mechanically harvested fruit, but they also must exhibit high productivity due to the reduction in light interception by dwarf canopies on a per area basis. Finally, desirable climatic adaptations include late bloom for frost avoidance and cold-hardiness/survival of low temperatures.

1.4.3 Cherry rootstock breeding objectives

Rootstocks are a key component of the composite plant system common to most tree fruit production, in which a fruit-bearing scion (having commercial fruit quality) is grafted onto a genetically separate rootstock having additional important traits like the ability to reduce vigor, increase productivity, or adapt to a specific soil type. Thus, the evaluative step of genetic improvement for rootstocks requires the additional step of grafting and outgrowth/fruiting of a scion, adding a minimum of several additional years to the breeding-to-release (or use as an improved parent) cycle. Programs for genetic improvement of cherry rootstocks have been relatively few, often of secondary focus, and a number of the most successful are no longer active (e.g., the Giessen program at Justus Liebig University). The most commonly cited breeding objectives for cherry rootstocks are noted in Table 3 and discussed briefly below.

The ability to confer reduced vigor to the scion variety (“dwarfing” rootstocks) is an extremely

Table 2 Most common sour cherry breeding objectives

Breeding objective	Breeding programs and/or reviews
Pest resistance	Fogle, 1975; Apostol, 2005b; Budan, 2005; Iezzoni <i>et al.</i> , 2005b; Rozsnyay and Apostol, 2005; Schuster and Wolfram, 2005
Extended ripening	Fogle, 1975; Apostol, 2005b
Self-compatibility	Apostol, 2005b; Schuster and Wolfram, 2005
Yield traits	Apostol, 2005b; Bors, 2005; Iezzoni <i>et al.</i> , 2005b; Schuster and Wolfram, 2005
Fruit quality traits	Bors, 2005; Iezzoni <i>et al.</i> , 2005b; Schuster and Wolfram, 2005
Mechanical harvest	Fogle, 1975; Bors, 2005; Schuster and Wolfram, 2005
Climatic adaptations	Fogle, 1975; Bors, 2005

Table 3 Most common cherry rootstock breeding objectives

Breeding objective	Breeding programs and/or reviews
Reduced vigor	Gruppe, 1985; Callesen and Vittrup, 1996; Hrotkó, 1996; Wolfram, 1996
Yields traits	Gruppe, 1985; Callesen and Vittrup, 1996; Hrotkó, 1996; Wolfram, 1996
Edaphic adaptability	Gruppe, 1985; Wolfram, 1996
Ease of propagation	Callesen and Vittrup, 1996; Hrotkó, 1996; Wolfram, 1996
Graft compatibility	Wolfram, 1996
Disease resistance	Gruppe, 1985; Brown <i>et al.</i> , 1996; Wolfram, 1996; Lang and Howell, 2001

important goal for fresh sweet cherry production. In the wild, sweet cherry is a large forest tree completely unsuitable for fruit harvest, so attaining good productivity in a small-stature tree canopy is vital for better management of leaf-to-fruit ratios, fruit quality, protection from pests, and labor efficiency (Lang, 2000). Precocity and high yields are critical for the same reasons (and including the same caveats, such as negative impact on fruit size) as noted above for these traits in scion varieties. Edaphic adaptability, such as good anchorage across soil types, tolerance of waterlogged, drought-prone, or high-pH soils, and survival (cold-hardiness) in areas with low winter temperatures are desirable rootstock traits.

Improved rootstocks should be propagated easily by cuttings (to improve the economics of producing a grafted tree), have resistance to soil-borne diseases such as *Phytophthora* and *Armillaria* root rots, and have broad graft compatibility across varieties. This latter trait, along with tolerance to pollen-borne viruses, can be problematic when hybridizations involve a broad range of *Prunus* species like *P. canescens* L., *P. maackii* Rupr., *P. fruticosa* L., *P. dawydensis* Sealy, *P. subhirtella* Miq., *P. pseudocerasus* Lindl., *P. incisa* Thunb., and *P. tomentosa* Thunb., are needed.

1.4.4 Cherry breeding tools and strategies

Standard cherry breeding techniques and strategies have changed little since being reviewed by Fogle (1975). The average breeding-to-release time for sweet cherry varieties released since 2001 (Lang, 2006b) was 20 years, with most ranging from 12 to 18 years, but some taking as long as 64 years. Pollen collection, flower emasculation, hand-pollination, protection of

hybridizations from spring frosts, etc. continue to comprise the mechanical process for manipulating genetic improvement (Brown *et al.*, 1996), but the tools available to breeders for identifying valuable parental germplasm and/or progenies have advanced since Fogle's review. Unfortunately, combining ability information on potential parents for specific cherry fruit, tree, or rootstock characters remains negligible (Brown *et al.*, 1996).

The introduction of dwarfing, precocious rootstocks has created a potential new tool for cherry breeding, making feasible the maintenance of a potted population of maternal or paternal genotypes to more easily control flowering sequences and/or to protect hand-pollinated flowers from indiscriminate insect pollinators, frosts, and diseases. Grafting or micrografting of hybrid sweet cherry seedlings onto clonal dwarfing rootstocks also can be utilized to hasten first fruit evaluation and increase breeding cycle efficiency (H. Schmidt, personal communication). Similarly, girdling of the hybrid seedling main leader during spring of the third season of growth was reported by Fogle (1975) to advance time to first fruit. Alternatively, the use of 2–3 applications of ethephon (2-chloroethylphosphonic acid) during spring to temporarily halt growth and promote first flower bud formation on own-rooted hybrid sweet cherry seedlings was used (G. Lang and D. Elfving, unpublished data) to advance the fruit evaluation cycle in the field by 1–2 years. Elite genotypes were selected 4 years after crossing, budded to precocious rootstocks, and second test fruits from multiple test sites were available for confirmation 3 years hence (i.e., 7 years after crossing), making it theoretically possible to progress from hybridization to widespread testing to commercial release in less than 10 years.

The introduction of self-compatibility has increased the potential efficiency of backcrossing

to move a desirable trait into an otherwise high-quality gene pool (Fogle, 1975), though there have yet to be any examples of this. It also must be noted that the widespread use, over the last few decades, of a single genetic source (JI 2420) for creating self-compatible sweet cherry genotypes is fraught with potential for detrimental inbreeding outcomes (Choi and Kappel, 2004).

Colchicine has been used on several occasions to double the chromosome number in sweet cherry for crossing with sour cherry (Brown *et al.*, 1996). It also has been used with *in vitro* shoot culture to create hexaploid clones of the triploid “Colt” (*P. avium* × *P. pseudocerasus* Lindl.) rootstock, for use in future breeding as well as an attempt to impart a reduction in vigor to scions (James *et al.*, 1987). The induction of mutations by irradiation has been used in both sweet and sour cherry to develop germplasm with self-compatibility (Lewis and Crowe, 1954), spur-type bearing habits, and/or compact growth habits (Fogle, 1975; Brown *et al.*, 1996).

To date, few molecular markers associated with important tree or fruit traits have been reported for marker-assisted selection in cherry scion (Iezzoni, 1996) or rootstock (Beckman and Lang, 2004) breeding efforts. Most markers developed to date have been associated with genotype identification (e.g., Clarke *et al.*, 2004). *S*-allele genotyping has made it possible to select self-compatible progeny at the seedling stage (Yamane *et al.*, 2001; Williams *et al.*, 2004), though this has not been reported to be a primary selection criterion. However, the increasingly extensive identification and molecular “fingerprinting” of many of the self-incompatibility *S*-alleles in sweet (Iezzoni *et al.*, 2005a) and to a lesser extent sour (Hauck *et al.*, 2006) cherry germplasm provide an important tool for improving breeding efficiency. Besides assuring fertility of cross-pollinations (i.e., avoiding cross-incompatibilities), this knowledge also can be used in selecting maternal and paternal parents to yield 100% progeny with self-compatibility. Should linkages ever be found between specific *S*-alleles and important traits, parental selection by *S*-allele may likewise yield efficient development of desired progeny.

Sources of disease-resistant germplasm for cherry breeding have been difficult to find. Since Fogle’s (1975) outlook on breeding for disease resistance, commercial-quality sweet and sour

varieties with partial resistance to *Monilinia* have been reported, though little resistance was found when conditions were optimized for epidemic-level colonization (Brown and Wilcox, 1989). New sour cherry varieties with improved resistance to (or tolerance of) brown rot, leaf spot, and bacterial canker have been reported recently (Schuster and Wolfram, 2004), but not yet tested in commercial settings. Several sources of resistance to foliar infection by cherry powdery mildew have been identified (Toyama *et al.*, 1993; Olmstead *et al.*, 2000a, 2001a, b). This has led to the demonstration and use of apparently durable single dominant gene resistance as a potentially useful breeding tool (Olmstead and Lang, 2002).

1.4.5 Cherry breeding achievements

In 1975, Fogle stated that breeding of cherries was at a comparatively early stage. Thirty years later, which is only 1–3 generations of perennial tree breeding cycles, there have been some important achievements, especially with regard to sweet cherry varieties having large fruit size and/or self-compatibility.

Significant advances in average sweet cherry fruit size have been made by imposing severe selection pressure, such as using a minimum average fruit weight of 10 g for retention of progeny. Consequently, a number of varieties from the Summerland (British Columbia) and Washington State University breeding programs consistently yield fruits with average weights of 11–13 g (and some individuals 18 g and larger), including “Summit”, “Glacier”, “Tieton”, “Selah”, “Sonata”, “PC 8011-3”, and “PC 7903-2” (Whiting, 2006). Recently, Olmstead (2006) has shown that fruit cell number is controlled very strongly by genetics rather than environment, suggesting that use of genotypes with high cell numbers (e.g., “Selah”) as parents may further improve sweet cherry fruit size.

The introduction of commercially important self-compatible sweet cherry cultivars began with the release of “Stella” in 1968 (Lapins, 1970), followed in 1984 by “Lapins” (Lane and Schmid, 1984), and “Sweetheart” in 1994 (Lang, 1999), all from the Summerland breeding program. Summerland has released more than a dozen self-compatible varieties thus far (Lang, 1999, 2006b),

including “Santina”, “Sandra Rose”, “Staccato” (the latest ripening self-compatible variety), “Sonata”, “Skeena”, “Symphony”, “Stardust” (a blush type), “Sunburst”, “Newstar”, “Celeste”, and “Samba”. Washington State University has released half a dozen self-compatible varieties, including “Benton” and “Selah” (designated as “Columbia” and “Liberty Bell” in Lang, 2002a), “Cashmere”, “Glacier”, “Index” (Andersen, 1997), and “PC 7147-9” (Whiting, 2006). Other self-compatible varieties that have been released in recent years include “WhiteGold” (a blush processing cherry) and “BlackGold” from Cornell University (Lang, 2002a); “Alex”, “Sándor”, “Pál”, and “Péter” from Hungary (Apostol, 2005a); “Tehranivee” and “Vandalay” from Vineland, Ontario (Lang, 1999); “Dame Nancy” (a blush type), “Dame Roma”, “Sir Don”, “Sir Douglas”, “Sir Hans”, and “Sir Tom” from South Australia (Lang, 2006b); and “Sweet Early” (the earliest ripening self-compatible variety, maturing before “Burlat”), “BlazeStar”, “EarlyStar”, “GraceStar”, “LalaStar”, and “BlackStar” from University of Bologna, Italy (Sansavini and Lugli, 2005).

The ripening season for high-quality sweet cherries has been expanded significantly during the past two decades. Dark-red mahogany cherry varieties of proven commercial export quality now encompass at least 5 weeks of ripening windows (from “Chelan” and “Tulare” to “Sweetheart” and “Staccato”), and up to 8 weeks with newly released varieties that have yet to be tested in the commercial export market chain. The potential ripening window for high-quality blushed yellow cherries also has expanded from the single mid-season variety “Rainier” to the recently released “Early Robin”, “PC 8011-3”, “Royal Rainier”, “Dame Nancy”, and late-ripening “Stardust”. These advances have strengthened international sweet cherry supply and demand due to improved competition for retail shelf space, a more orderly supply for marketing, and a risk-diversified product portfolio for growers. In other words, the success of new sweet cherry cultivars that are large, often self-fertile, and spread economic risks have most certainly helped to drive the strong international growth in sweet cherry planting noted above in Section 1.3.

Advances in resistance to rain-induced fruit cracking of sweet cherries have been only

slight, with Andersen (1998) noting “Adriana”, “Regina”, and “Sam” as significant. Likewise, only a few new sweet cherry varieties have been developed thus far with fruit that form abscission zones for easy detachment at the pedicel–spur junction (“Andersen”) or at the fruit–pedicel junction (“WhiteGold”), which are critical for improving mechanical harvest efficiency and postharvest quality.

Advances in cherry disease resistance also have been incremental at best. Monogenic resistance to cherry leaf spot has been introgressed from *P. maackii* into a selection known as “Atlant” that has good fruit size and can be hybridized with either sweet or sour cherry (Brown *et al.*, 1996). The same authors also reviewed the reported progress in developing varieties with bacterial canker resistance to at least some pathovars of *Pseudomonas morsprunorum*. However, the causal agent of bacterial canker in North America is *P. syringae*, and no broad source of resistance to multiple *Pseudomonas* species and pathovars has yet been identified. Recently, Olmstead and Lang (2002) identified a sweet cherry source of dominant monogenic immunity to foliar infection by powdery mildew, which they have used to create elite breeding selections with resistance and commercial-level fruit quality that are now in advanced stages of evaluation for commercial industry release (M. Whiting, personal communication).

Brown *et al.* (1996) noted that “the impact of sour cherry improvement in Europe and Russia has been dramatic. . . (yet) sour cherry breeding in the United States has not yet impacted the industry, which is still heavily reliant upon “Montmorency”. This remains the case in 2006. Of particular impact in Europe has been the expansion of the ripening period to 40 days (Iezzoni *et al.*, 1991) and the adoption of varieties that are suitable for both processed products and fresh markets.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

The periodic Brooks and Olmo Registers of newly released fruit and nut varieties (largely in North America) indicate that commercial cherry variety releases during the past 15 years include only five new varieties of sour cherry (Andersen, 1994, 1997;

Iezzoni, 2002; Bors, 2005) while there have been 63 new sweet cherries (Andersen, 1994, 1997; Lang, 1999, 2002a, 2006b) and 21 new cherry rootstocks, most of which originated in European rootstock breeding programs (Andersen, 1997; Lang, 2002b, 2006a). Improved sweet and sour cherry genetic output is similar in Europe (Apostol, 2005a, b). This is a reflection of both the greater number of sweet cherry breeding programs, and the more extensive and valuable sweet cherry industries worldwide, relative to that for sour cherries. However, to some extent, it may also reflect greater genetic challenges for improvement of the tetraploid sour cherry (A. Iezzoni, personal communication) compared to the diploid sweet cherry. The number of active cherry breeding programs has declined worldwide during the past half-century, ostensibly a result of declining public funding and institutional support for long-term genetic improvement of what is a relatively minor fruit crop on an international level (O'Rourke, 2006). At present, the only active public breeding programs remaining in North America for sweet cherries are in Washington and British Columbia, with two active private programs of significance (both in California), and the only active public programs for sour cherries are in Michigan and Saskatchewan.

Even though the development of improved sweet cherries has been relatively prolific, the genetic diversity of these new varieties is fairly narrow. In North America, coefficients of co-ancestry are more than twice as high for self-compatible varieties as for those that are self-incompatible (Choi and Kappel, 2004), since all self-compatible varieties have been derived from a single progenitor source of self-compatibility ("JI 2420"). Yet, nearly one in five self-incompatible varieties also have a greater than half-sibling (0.125) relationship to self-compatible varieties. Continued use of such parents will increase the potential for inbreeding depression and limit long-term genetic improvement (Andersen, 1998) unless greater genetic diversity cannot only be accessed, but utilized efficiently. This can be a major challenge due to the inefficiencies of traditional breeding technologies for long-lived perennials such as cherry trees, the continuing decrease in government support for long-term public breeding programs, and the prohibitive costs for (and economic pressures on) private

breeders to undertake extensive introgression of new genetic diversity from germplasm having potential negative traits along with valuable genetic potential.

In 1998, Andersen stated, "Cherry breeders have failed miserably in improving some critically important traits. Why? ... they have not culled rigorously enough in large enough populations, ... have not used diverse enough base populations, ... have lacked sufficient knowledge of the physiology and genetics of some primary traits." To date, precise knowledge of the heritability of many key cherry traits remains incomplete—for example, winter hardiness, fruit shape, most disease susceptibilities, precocity, graft compatibility, and rain-induced fruit cracking, to name but a few. Improvement in many of these quantitatively inherited traits is confounded by the continuing lack of physiological knowledge underlying each phenomenon, such as the case for fruit cracking or adaptation to localized climatic limitations like low winter chilling or heat-induced fruit doubling. Breeding programs that have been most successful—for example, Summerland, Washington State University, and Cornell University—have benefited from close interactions between breeders, physiologists, pathologists, and horticulturists.

Fogle (1975) suggested that most advances in disease resistance would likely have to come from species other than sweet or sour cherry. Twenty years later, Brown *et al.* (1996) reported few advances in either disease or insect resistance. As noted above, other than for powdery mildew, advances in disease resistance remain slight, and no genetic resistance to insects has yet been reported. In sweet cherry, sources of durable resistance have yet to be identified for brown rot, bacterial canker, *Verticillium* wilt, cherry fruit fly, or plum curculio as well as viruses (such as little cherry or rugose mosaic) or viruslike diseases (such as X-disease). Some interspecific rootstock genotypes are lethally sensitive or hypersensitive to Prune dwarf virus (PDV) and/or PNRSV (Lang and Howell, 2001), but these diseases can cause subtle changes in tree productivity even when the genotype is tolerant. In sour cherry, resistance to leaf spot has yet to be transferred to commercially viable genotypes.

Adaptability of cherry rootstocks to a broader range of soil types, moisture profiles, and fertility

levels has yet to be addressed significantly. Broadening climatic adaptations of scion varieties or rootstocks is likely to be a breeding efficiency challenge, requiring many breeding cycles before significant advances can be made in concert with commercially viable fruit qualities or desirable rootstock traits. In the case of interspecific hybrid cherry rootstocks as well as attempts to introgress traits such as certain disease resistances or climatic tolerances from other *Prunus* species into sour or sweet cherry varieties (e.g., Downey, 1999), fertility and fruit set can be a major impediment to traditional breeding strategies. Beckman and Lang (2004) have noted “many rootstock programs release materials that are functionally genetic dead-ends.”

The potential for modifying currently important and distinctive commercial varieties, such as “Bing” or “Rainier” sweet cherry or “Montmorency” sour cherry, with secondary traits important for future production strategies, such as mechanical harvest, or the need for resistances against difficult-to-control insects and diseases to better facilitate reduced pesticide or organic production systems, remains distant. Many of these potential improvements may well be dependent on biotechnological approaches to overcome genetic and/or breeding efficiency barriers.

2. CHERRY IMPROVEMENT THROUGH GENETIC TRANSFORMATION

Of more than 30 cherry species, genetic transformation has been reported only for several commercially important genotypes, including sour cherry, chokecherry (*P. virginiana* L.), and the cherry rootstocks “Rosa” (*P. subhirtella autumnno*), “Gisela 6” (*P. cerasus* × *P. canescens*), “Colt” (*P. avium* × *P. pseudocerasus* Lindl.), “Inmil” (*P. incisa* Thunb. × *P. serrula* Franch.), and “Damil” (*P. dawcyckensis* Sealy) (da Cãmara Machado *et al.*, 1995; Druart *et al.*, 1998; Gutiérrez-Pesce *et al.*, 1998; Dolgov and Firsov, 1999; Song and Sink, 2005, 2006a, b). The majority of cherry species, including sweet cherry, are still considered recalcitrant to transformation due principally to their recalcitrance to *in vitro* regeneration. Reliable transformation methodologies require efficient gene delivery, effective selection, and efficient plant

regeneration systems, and generally need to be optimized for individual genotypes.

2.1 Cherry Plant Regeneration

Although the floral dip method allows the production of transgenic *Arabidopsis* without plant tissue culture or regeneration (Clough and Bent, 1998), *in vitro* tissue culture and plant regeneration are requisite steps in methodologies for stable transformation of other plant species, including cherry. There have been several early reports on plant regeneration from callus suspensions and/or protoplasts of sour cherry (Durzan, 1985; Ochatt and Power, 1988; Ochatt, 1990), wild sweet cherry (Ochatt, 1991), and “Colt” rootstock (Ochatt *et al.*, 1987). However, these suspension cell- and protoplast-based regeneration systems have not led to eventual successful transformations. With the development of gene delivery technology, *Agrobacterium*- and biolistic-mediated approaches, the application of the protoplast-based method has diminished (Ochatt and Patat, 1995; Davey *et al.*, 2005). Thus, cherry regeneration systems independent of protoplasts set the basis for further studies on *Agrobacterium*- and biolistic-mediated transformation.

Cherry regeneration is determined by factors that are both biotic factors, including genotype and explant type, and abiotic, such as culture media and environmental conditions. Although plant cell totipotency theoretically enables any of the cells to retain the ability to regenerate whole new plant through organogenesis and/or somatic embryogenesis after induction by a complex number of stimuli, regeneration capacity of plant cells usually varies between species, cultivars, and explant types due to the fact that such cells might not be competent to express totipotency under a particular set of experimental inductive conditions (Ganeshan *et al.*, 2002; Su, 2002). As summarized in Table 4, 48 genotypes from 6 cherry species regenerated plants from cotyledons (4), leaf explants (45), and internode sections (5). Only 8 of the 48 genotypes had over 50% regeneration frequencies and the mean number of shoots per explant varied between 0.6 and 4.7, thereby indicating that cherry plant regeneration varied among genotypes and most might not be amenable for *in vitro* plant regeneration. Accordingly, further

Table 4 Cherry regeneration systems

Common name	Species	Genotype ^(a)	Explant	Orientation	Regeneration		Medium	Reference
					Highest regeneration rate (number of shoots per explant)	Maximum shoot number per explant (shoot regeneration rate)		
Black cherry	<i>Prunus serotina</i> Ehrh.	A, D, and F	Leaf section wounded by removal of leaf edges	Abaxial side up (Ab-up)	38.3% (4.1) on medium 1	5.1 (28.3%) on medium 2	Medium 1: Woody plant medium (WPM) (Lloyd and McCown (1980) + 2.27 μM thidiazuron (TDZ) + 0.54 μM naphthaleneacetic acid (NAA); medium 2: WPM + 6.81 μM TDZ + 1.07 μM NAA	Espinosa <i>et al.</i> , 2006
Black cherry	<i>P. serotina</i> Ehrh.	Seedling A, PSB, 2322 , 2339, and “Pavia E”	Furled leaf with two transverse cuts along the midrib	Abaxial side down (Ab-down)	78% (NA)	–	WPM + 4.4 μM TDZ + 0.54 μM NAA	Hammatt and Grant, 1998
Cherry rootstock	<i>P. cerasus</i> L. × <i>P. canescens</i> L.	“Gisela 6”	Whole leaf with four cuts perpendicular to the midrib	Ab-down	68.3% (2.3)	2.3 (68.3%)	WPM + 8.9 μM 6-benzylaminopurine (BAP) + 4.9 μM indole-3-butyric acid (IBA)	Song and Sink, 2006a, b
Hybrid	<i>P. avium</i> L. × <i>P. sargentii</i>	Hybrid 1 and Hybrid 2	Furled leaf with two transverse cuts along the midrib	Ab-down	33% (NA)	–	WPM + 4.4 μM TDZ + 0.54 μM NAA	Hammatt and Grant, 1998
Sour cherry	<i>P. cerasus</i> L.	“ Gerema ”, “Scharö”, and “Schattenmorelle”	Cotyledon		10% (0.5)	0.5 (10%)	Murashige and Skoog (1962) medium + 9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.49 μM IBA + 4.7 μM kinetin (KT)	Tang <i>et al.</i> , 2000

Sour cherry	<i>P. cerasus</i> L.	“Beutal Spacher Rexelle” and “Morellenfeuer”	Half leaf with a cut perpendicular to the midrib Cotyledon	Ab-down	40.6% (2.0)	2.0 (40.6)	WPM + 8.9 μ M BAP + 2.2 μ M NAA	Tang <i>et al.</i> , 2002
Sour cherry	<i>P. cerasus</i> L.	“Montmorency”			64% (4.7) on medium 1	5.5 (58%) on medium 2	Medium 1: MS + 10.0 μ M TDZ + 2.5 μ M IBA; medium 2: MS + 7.5 μ M TDZ + 2.5 μ M IBA	Mante <i>et al.</i> , 1989
Sour cherry	<i>P. cerasus</i> L.	“Komsomolskaya”, “Vladimirskaia”, and 4 unlisted cultivars	Leaf piece	Ab-up	20% (NA)	–	Not clear	Dolgov, 1999
Sour cherry	<i>P. cerasus</i> L.	“Montmorency”	A whole leaf with four cuts perpendicular to the midrib	Ab-down	54.4% (2.4)	2.4 (54.4%)	Pretreatment medium: MS + 0.45 μ M TDZ; regeneration medium: Quoirin and Lepoivre (1977) medium (QL) + 13.3 μ M BAP + 2.7 μ M NAA	Song and Sink, 2005
Sour-sweet cherry hybrid	<i>P. fruticosa</i> Pall. × <i>P. avium</i> L.	“Black Eagle”	Leaf piece	Ab-up	60% (NA)	–	MS + 8.9 μ M BAP + 5.4 μ M NAA + 5.7 μ M IAA	Dolgov, 1999
Sweet cherry	<i>P. avium</i> L.	“Büttner”, “Burlat”, “Stella”, “Star”, and “Regina”	Leaf		30% (0.6)	0.6 (30%)	QL + 1 μ M TDZ + 2.5 μ M IBA	Yang and Schmidt, 1992
Sweet cherry	<i>P. avium</i> L.	“Burlat”, “Early” “Burlat”, “Hedelfinger”, “Napoleon”, and “Schneiders”	Half leaf with a cut perpendicular to the midrib	Ab-down	30.2% (1.2)	1.2 (30.2%)	WPM + 8.9 μ M BAP + 2.2 μ M NAA	Tang <i>et al.</i> , 2002

(Continued)

Table 4 Cherry regeneration systems (*Continued*)

Common name	Species	Genotype ^(a)	Regeneration				Reference
			Explant	Orientation	Highest regeneration rate (number of shoots per explant)	Maximum shoot number per explant (shoot regeneration rate)	
Sweet cherry	<i>P. avium</i> L.	"Kordia", "Regina", "Starking Hardy Giant", "Schneiders", and "Sweetheart"	Internode section ^(b) and whole leaf wounded by three transverse cuts along the midrib	Ab-down	50% (1.5) on medium 1	5 (25%) on medium 2	Medium 1: QL + 22.2 µM BAP + 2.5 µM IBA; medium 2: half Driver and Kuniyuki (1984) medium (DWK) + half WPM + 7.7 µM TDZ + 2.5 µM IBA Matt and Jehle, 2005
Sweet cherry	<i>P. avium</i> L.	"Lapins" and "Sweetheart"	Whole leaf wounded by five transverse cuts along the midrib	Ab-up	71% (NA)	–	WPM + 2.27 µM TDZ + 0.27 µM NAA Bhagwat and Lane, 2004
Wild cherry	<i>P. avium</i> L.	"F12/1", "Charger", and "1908"	Furled leaf with two transverse cuts along the midrib	Ab-down	62% (0.7)	0.7 (62%)	WPM + 22.2 µM TDZ + 0.54 µM NAA Hammatt and Grant, 1998
Wild cherry	<i>P. avium</i> L.	"F12/1", "Charger", "1904", "1905", "1906", "1908", "1909", "1912", "1919", and "2474"	A whole leaf with three cuts across the midrib	Ab-down	84% (3.5)	3.5 (84%)	WPM + 4.4 mM 1-phenyl-3-(1,3,5-trihydroxybenzene) + 0.54 mM NAA + 8.1 µM tween-20 Grant and Hammatt, 2000

^(a) Bold genotype yielded the best regeneration^(b) Explant type that yielded the best regeneration

optimization of regeneration protocols is still required for most cherry genotypes.

2.1.1 Genotype and explants

As reviewed by Ganeshan *et al.* (2002) and Su (2002), both genotype and explant type are very important factors and should be considered prior to plant regeneration studies. The choice of genotype is not usually as flexible as that of explant type due to the consideration of improving cultivars already in production. Cotyledons and hypocotyls are usually regenerative, readily available, and thus widely used to regenerate herbaceous plants. There have been two reports on cherry plant regeneration using cotyledons as explants (Mante *et al.*, 1989; Tang *et al.*, 2000). For seedling-propagated genotypes, cotyledon and hypocotyls are excellent for regeneration as well as for later transformation. However, most commercial genotypes of cherry are propagated vegetatively.

Therefore, leaves have been the major explant for cherry regeneration (Table 4). In the only regeneration attempt using internode sections, Matt and Jehle (2005) recently reported that the regeneration efficiency was 2x (“Regina”) and 5x (“Schneiders”) higher when using internode sections instead of leaf explants. In terms of regeneration ability, there is no question that tender internode sections are a potential explant for efficient regeneration due to the active dividing cells in its vascular tissue. Internode sections have been found to be preferable to leaf explants for regeneration, as well as for transformation, in sweet potato (Song *et al.*, 2004). Although internode sections of cherry, 2.0–3.0 mm in length \times 1.0–2.0 mm in diameter as used by Matt and Jehle (2005), are not easily available from *in vitro* plants or shoots, their high regeneration potential makes them more attractive for future attempts on regeneration and transformation.

In vitro cultures of cherry were the only source of leaf explants in previous reports and will continue to be an excellent source in the future (Table 4). Leaf explants from actively proliferating shoots showed high regeneration potential. Several reports have addressed the importance of leaf size, orientation, and wounding for adventitious shoot regeneration (Yang and Schmidt, 1992; Grant

and Hammatt, 2000; Bhagwat and Lane, 2004; Song and Sink, 2005). Whole-leaf explants with wounded midribs were used in 7 of 12 reports and showed higher regeneration efficacy than leaf sections (Bhagwat and Lane, 2004; Song and Sink, 2005). Incubation of whole-leaf explants with the abaxial surface down enabled the midribs to contact the medium and thus was used in most of the reports. In addition, early studies have shown that wounding of the midrib of leaf explants tends to promote adventitious shoot production. Shoot regeneration from wounded margins of leaf sections from “Montmorency” sour cherry was not successful (Song and Sink, 2005), whereas Espinosa *et al.* (2006) reported that adventitious shoot regeneration occurred at wounded margins as well as at the leaf midrib of black cherry.

Based on these previous reports (Table 4), the characteristics of leaf explants that are amenable for cherry regeneration can be described: (1) whole-leaf explants, 0.5–2.0 cm in length, are excised from *in vitro*, actively proliferating shoots; (2) 3–5 slices on leaf explants are made partially transverse and equidistant through the midrib; (3) leaf explants are placed with abaxial surface in contact with the medium.

2.1.2 Culture medium

The composition of the basal media plays a major role in both successful *in vitro* shoot cultures and plant regeneration. For shoot proliferation, two major basal media, Murashige and Skoog (1962) (MS) and Quoirin and Lepoivre (1977) (QL), usually are used. MS was used in 7 of 10 reports (Hammatt and Grant, 1998; Grant and Hammatt, 2000; Tang *et al.*, 2002; Bhagwat and Lane, 2004; Matt and Jehle, 2005; Espinosa *et al.*, 2006; Song and Sink, 2006a), and QL was used in the others (Yang and Schmidt, 1992; Dolgov, 1999; Song and Sink, 2005). QL works well for efficient proliferation of “Montmorency” sour cherry, and both QL and MS work for “Gisela 6” rootstock. MS was used to maintain 11 sweet cherry and 6 rootstock genotypes, although it did not lead to efficient shoot proliferation for each genotype (G. Song and K. Sink, unpublished data). In addition, woody plant medium (WPM) (Lloyd and McCown, 1980) may have potential for further improvement of shoot proliferation. For

shoot regeneration from leaf explants, WPM was the major basal medium used in 7 of 10 reports (Table 4).

Plant growth regulators (PGRs) are important stimuli for both shoot proliferation and regeneration. For shoot proliferation, 6-benzylaminopurine (BAP) has been used either alone or supplemented with additional auxins, such as indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) and gibberellic acid (GA₃), for most cherry genotypes tested. The working concentrations of these PGRs are usually 4.4 μM for BAP, 0.49 μM for IBA, 0.54 μM for NAA, and 1.45 μM for GA₃. For shoot regeneration, either thidiazuron (TDZ) or BAP, each combined with NAA or IBA, is used in most regeneration media (Table 4). Most of the previous studies have shown that TDZ was more effective for shoot regeneration than BAP (Hammatt and Grant, 1998; Bhagwat and Lane, 2004; Matt and Jehle, 2005; Espinosa *et al.*, 2006), whereas two other reports indicated BAP was more effective than TDZ in promoting shoot regeneration in some genotypes (Figure 1a) (Tang *et al.*, 2002; Song and Sink, 2006a). A low level (0.45 μM) of TDZ-pretreatment of leaf explants stimulated adventitious shoot regeneration of “Montmorency” sour cherry (Figure 1b) (Song and Sink, 2005). Variations in culture responses of leaf explants to TDZ or BAP might be related to genotype.

Carbohydrate, agar, and pH also are important factors for *in vitro* culture. To date, sucrose

(58.4–116.8 μM) has been the major carbon source used for cherry proliferation and regeneration, although sorbitol was found to be more effective than sucrose in promoting shoot regeneration from internode sections instead of leaf explants of “Sweetheart” sweet cherry (Matt and Jehle, 2005). Different types of agar affected shoot proliferation (G. Song and K. Sink, unpublished data); thus, attempts on different agar types might be an option to optimize shoot regeneration. According to previous reports, the suitable pH for cherry cultures generally ranges from 4.8 to 5.8.

Addition of timentin, cefotaxime, or carbenicillin in regeneration medium is usually required for *Agrobacterium*-mediated transformation. However, little attention has been given to the effect of these antibiotics on cherry shoot regeneration. Further studies are still required to determine suitable antibiotics that result in no or minimal inhibition of shoot regeneration at its working concentration.

2.1.3 Environmental conditions

Although light and temperature are important factors in tissue culture, they have not been given much attention for cherry shoot proliferation or regeneration. Culture temperature generally was set to 25 °C in most of the previous reports. Several studies have shown that a 16/8 h light/dark photoperiod regime yielded higher regeneration frequencies than continuous darkness (Yang and

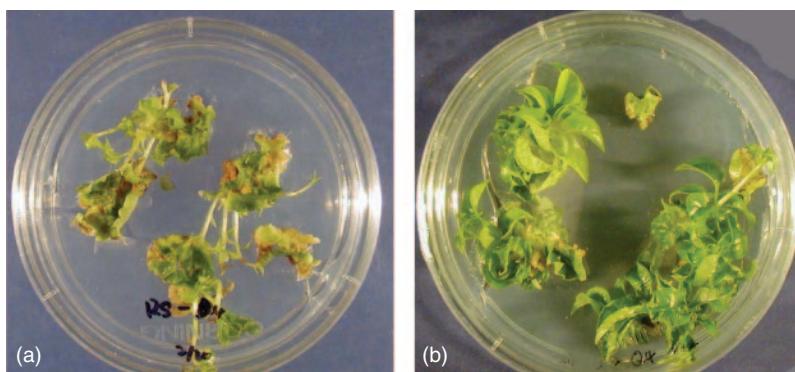


Figure 1 Optimal adventitious shoot regeneration from leaf explants of cherry rootstock “Gisela 6” (a) and sour cherry “Montmorency” (b); (a) eight-week culture on WPM + 8.9 μM BAP + 4.9 μM IBA; (b) eight-week culture on QL + 13.3 μM BAP + 2.7 μM NAA following a 24-h TDZ-pretreatment in liquid MS + 0.45 μM TDZ [Reproduced from Song and Sink (2005, 2006a)]

Schmidt, 1992; Bhagwat and Lane, 2004; Matt and Jehle, 2005). Espinosa *et al.* (2006) found that leaf explants incubated on medium in the dark for 3 weeks before transferring to a 16-h photoperiod yielded the best regeneration results for black cherry. A few reports have documented the effect of light quality on cherry regeneration. More investigation seems required to determine optimal temperature and light conditions for efficient shoot regeneration.

2.1.4 Rooting

Although rooting of adventitious shoots derived from leaf explants was achieved for several cherry genotypes, it has not been a major focus in most cherry regeneration reports (Tang *et al.*, 2002; Bhagwat and Lane, 2004; Song and Sink, 2005; Espinosa *et al.*, 2006). In general, rooting frequency is related to genotype as well as culture conditions. Rootstocks are generally amenable for rooting. Half MS, half QL, or WPM combined with IBA or NAA appears effective for rooting of most cherry genotypes.

2.2 Cherry Transformation

Similar to most woody fruit species, genetic transformation of cherry species is currently limited to only a few genotypes (reviewed by Petri and Burgos, 2005). Since the first transgenic cherry rootstock, “Rosa”, was obtained in 1995 (da Cãmara Machado *et al.*, 1995), there have been several publications on genetic transformation of cherry species (Table 5) (Druart *et al.*, 1998; Gutiérrez-Pesce *et al.*, 1998; Dolgov and Firsov, 1999; Song and Sink, 2005, 2006a, b). These studies have shown the possibility of genetic modification of cherry species through transformation and indicate that additional efforts are required to achieve successful stable transformation protocols for the wide range of cherry cultivars.

2.2.1 Gene delivery systems

Two major methodologies, *Agrobacterium*- and biolistic-mediated transformation, have been attempted to deliver foreign genes into cherry cells

(Table 5). The biolistic method, which allows efficient gene delivery to plant cells independent of plant species, is generally associated with problems such as the requirement for expensive devices and occurrence of multiple copies of transgenes in the transgenic plants. Thus, it is used mostly for transformation of species that are recalcitrant to infection by *Agrobacterium*. In the only report on biolistic-mediated transformation of three cherry genotypes, the transgenes were delivered into meristems but no stable transgenic plants were obtained (Druart *et al.*, 1998). Compared to the biolistic method, the multiple advantages of *Agrobacterium*-mediated transformation, such as low cost, ease of handling, and allowing for production of transgenic plants with single copy insertions, have made it the favored option to produce transgenic plants. Both *A. tumefaciens*- and *A. rhizogenes*-mediated transformation have yielded stable transgenic cherry plants (Table 5).

Using *A. rhizogenes*, Gutiérrez-Pesce *et al.* (1998) obtained transgenic plants of the cherry rootstock “Colt” containing the nondisarmed pRi1855 T-DNA (transfer DNA), but the possibility of chimera transformants was not excluded. In contrast, Druart *et al.* (1998) obtained only chimera regenerants from the cherry rootstocks “Damil” and “Inmil” (Druart *et al.*, 1998).

A. tumefaciens strains have yielded the most successful transformations of cherry species (Table 5). Da Cãmara Machado *et al.* (1995) obtained the first transgenic cherry rootstock, “Rosa”, using *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983), and stable transformation was confirmed by Northern blot analysis. Following the method described by da Cãmara Machado *et al.* (1995), transformation of “Inmil” rootstock was reported although no information is available to confirm stable transformation. Similarly, four *A. tumefaciens* strains were tested for transformation of a sour-sweet hybrid; 3 of 58 regenerants selected with 5 mg l⁻¹ hygromycin were β -glucuronidase (GUS) positive, but molecular studies of the regenerants were not reported (Dolgov and Firsov, 1999). Using transient GUS gene (*gusA*) expression studies, three different opine-type *A. tumefaciens* strains, EHA105 (Hood *et al.*, 1993), LBA4404 (Hoekema *et al.*, 1983), and GV3101 (Koncz and Schell, 1986), were evaluated for gene delivery to cherry explants

Table 5 Summary of cherry transformation systems

Transformation methodology	Genotype	<i>Agrobacterium</i> strain	Explant type	Selectable marker gene	Selection agent	Target gene or screenable marker	Transgene detection	Reference
Biolistic	Rootstock "Inmil" (<i>P. incisa</i> Thunb. × <i>P. serrula</i> Franch.) and "Damil" (<i>P. dawydensis</i> Sealy), sweet cherry (<i>P. avium</i> L.) "Summit"	—	Meristem	<i>bar</i>	None	<i>gusA</i>	No stable transformants were confirmed	Druart <i>et al.</i> , 1998
<i>A. rhizogenes</i>	Rootstock "Inmil" and "Damil", sweet cherry (<i>P. avium</i> L.) "Summit"	ATCC pRi15834	Meristem	<i>bar</i>	None	<i>gusA</i>	No stable transformants were confirmed	Druart <i>et al.</i> , 1998
<i>A. rhizogenes</i>	Rootstock (<i>P. avium</i> × <i>P. pseudocerasus</i>) "Colt"	NCPPB pRi1855	Shoot	None	None	pRi1855 T-DNA	Transformants were confirmed by Southern blot	Gutiérrez-Pesce <i>et al.</i> , 1998
<i>A. tumefaciens</i>	<i>P. subhirtella</i> Miq. "Rosa"	LBA4404	Embryogenic callus	<i>nptII</i>	75 mg l ⁻¹ Km	<i>gusA</i>	Plants from one transgenic event were confirmed by northern blot	Da Câmara Machado <i>et al.</i> , 1995
<i>A. tumefaciens</i>	Sour cherry (<i>P. cerasus</i> L.) "Montmorency"	LBA4404 GV3101 EHA105	A whole leaf with four cuts	<i>nptII</i>	None	<i>gusA</i>	Transient GUS expression	Song and Sink, 2005
<i>A. tumefaciens</i>	Sour cherry "Montmorency" and rootstock (<i>P. cerasus</i> L. × <i>P. canescens</i> L.) "Gisela 6"	EHA105	A whole leaf with four cuts	<i>nptII</i>	1) 50 mg l ⁻¹ Km or 2) Km at 25 mg l ⁻¹ followed by 50 mg l ⁻¹	<i>gusA</i>	Transgenic events were confirmed by Southern blot	Song and Sink, 2006a, b
<i>A. tumefaciens</i>	Sour-sweet cherry hybrid (<i>P. fruticosa</i> Pall. × <i>P. avium</i> L.)	GV3101	Leaf piece	<i>hpt</i>	10 mg l ⁻¹ Hyg	Antifreeze protein gene (<i>afp</i>)	<i>Hpt</i> and <i>AFP</i> were detected by PCR in one transgenic line	Dolgov, 1999; Dolgov and Firsov, 1999
<i>A. tumefaciens</i>	Sour-sweet cherry hybrid (<i>P. fruticosa</i> Pall. × <i>P. avium</i> L.)	GV3101	Leaf piece	<i>hpt</i>	None selection for 3 weeks followed by 5 mg l ⁻¹ Hyg	<i>gusA</i>	Transgenic events were PCR- and/or GUS positive	Dolgov, 1999; Dolgov and Firsov, 1999

(Song and Sink, 2005). Cherry explants were found susceptible to *A. tumefaciens* (C58C1 pGV3850) carrying the p35SGUSIntron binary vector (Pratesi *et al.*, 2004). Cherry leaf explants, including “Montmorency” sour cherry, “Gisela 6” cherry rootstock, and all five sweet cherry varieties tested, were susceptible to all strains. Similar to reports in rice (Li *et al.*, 1992), chrysanthemum (Boase *et al.*, 1998), and conifer (Humara *et al.*, 1999), the agropine-type strain EHA105 yielded larger expression areas, stronger intensity of GUS staining and more GUS foci per explant than the other two strains. Recently, stable transgenic plants of “Montmorency” and “Gisela 6” were obtained after inoculation of leaf explants with strain EHA105 (Song and Sink, 2006a).

Numerous factors influence transient *gusA* expression including *Agrobacterium* strain, plasmid type, time of inoculation and/or co-cultivation, and temperature and environmental conditions at various stages. A 2-day co-cultivation time was used for *A. rhizogenes* transformation of the cherry rootstock “Colt” by Gutiérrez-Pesce *et al.* (1998). A 3-day co-cultivation time was adopted for *A. tumefaciens* transformation of selected sour and sour-sweet hybrid cherry cultivars (Dolgov, 1999). Song and Sink (2005) used transient expression studies to determine optimal parameters for *A. tumefaciens* gene delivery systems. Using a bacterial density of 0.5 (OD₆₀₀) in the presence of 100 µM acetosyringone and a 4-day co-cultivation yielded efficient transient *gusA* expression without causing leaf explant necrosis or resulting in bacterial overgrowth in subsequent culture (Song and Sink, 2005). These results offer the following guidelines for conducting *A. tumefaciens* stable transformation of cherry genotypes: (1) explants: freshly prepared leaf explants; (2) *Agrobacterium* strain: EHA105, LBA4404, or GV3101; EHA105 is preferable; (3) bacterial suspension: bacterial pellet is suspended to OD₆₀₀ = 0.5 in liquid co-cultivation medium supplemented with 100 µM acetosyringone; (4) infection: 30 min at 28 °C; (5) co-cultivation medium: the same as the optimized regeneration medium, or a medium that can lead to active cell division; (6) co-cultivation: leaf explants are placed on filter paper overlaid on agar-solidified co-cultivation medium plus 100 µM acetosyringone for 4 days at 25 °C in the dark.

2.2.2 Tissues competent for transformation and regeneration

Competent cherry plant cells for transformation should be easy to regenerate, susceptible to *Agrobacterium*, and readily available. Three explant types, embryogenic calluses, shoot tips, and whole leaves, have been used to produce transgenic cherry plants (Table 5). Embryogenic callus is not easily available for most cherry species. Shoot tips might yield chimeral regenerants. Whole-leaf explants are the major explant type due to the fact that wounded midribs contain competent cells for transformation, and regeneration is adventitious (Song and Sink, 2005, 2006a). In addition, tender stem sections are a potential explant source of competent plant cells for both transformation and regeneration. For cherry species that are proliferated through seedlings, the cotyledon is an excellent explant.

2.2.3 Selection of transformed tissues

Recovery of transgenic plants requires an effective selection system by which the shoots are derived from a single to a few transformed cells. Regardless of the rapid development of strategies for production of marker-free transgenic plants, marker gene-assisted selection is usually required for successful transformation of most plant species. Recently, application of different selectable marker genes for plant transformation has been well documented (reviewed by Miki and McHugh, 2004). Three major selectable marker genes, including that for neomycin phosphotransferase II (*nptII*)-kanamycin (Km) resistance, the hygromycin phosphotransferase gene (*hpt*)-hygromycin (Hyg) resistance, and the bialaphos resistance gene (*bar*)-phosphinothricin, usually are used for selection. For cherry transformation, *nptII* yielded stable transgenic plants (Table 5). In the only report where *hpt* was used as a selectable marker for cherry transformation, Dolgov (1999) obtained polymerase chain reaction (PCR)-positive transformants using *hpt* instead of *nptII* under different selection regimes. However, in this case, no evidence indicated that *hpt* was preferable to *nptII*. Druart *et al.* (1998) attempted to transfer the *bar* gene into two cherry rootstock cultivars using biolistic and *A. rhizogenes* methods;

however, *bar* was not used as a selectable marker during the production of transformants since no screening agent was applied. All three major selectable marker genes, *hpt*, *nptII*, and *bar*, could be used for cherry transformation.

An effective selection system depends on not only selectable marker genes, but also suitable selection regimes. In general, selectable marker genes are under the direction of constitutive promoters. Different promoters might yield different expression levels of marker genes. In addition, expression of a marker gene is also affected by selection conditions such as culture medium, dose of a selection agent, and environmental conditions. Determination of the optimal dose of selection agent should be carried out under the optimized regeneration conditions, and antibiotic(s) used to eliminate residual *Agrobacterium* cells. Using $75 \text{ mg l}^{-1} \text{ Km} + 250 \text{ mg l}^{-1}$ carbenicillin, da Câmara Machado *et al.* (1995) obtained one transgenic event for “Rosa” rootstock. When cefotaxime at 500 mg l^{-1} was used, selection with Km at 25 mg l^{-1} followed by 50 mg l^{-1} yielded no transgenic plants, whereas selection with Hyg at either 10 mg l^{-1} or 5 mg l^{-1} resulted in the production of transgenic plants (Dolgov, 1999). Using 250 mg l^{-1} timentin, a one-step selection with 50 mg l^{-1} Km and a two-step selection using Km at 25 mg l^{-1} for 4 weeks followed by 50 mg l^{-1} both yielded stable transgenic plants from “Montmorency” sour cherry and “Gisela 6” cherry rootstock (Figure 2) (Song and Sink, 2006a, b). The two-step selection method eventually improved transformation frequencies although it was associated with an increase in the number of escapes (Song and Sink, 2006b). The following factors might have contributed to the successful selection: (1) several washes of leaf explants using 500 mg l^{-1} timentin after co-cultivation is an effective option to remove excess bacterial cells, thereby allowing a low level of timentin (250 mg l^{-1}) for effective control of bacterial overgrowth during selection; (2) the whole selection process is performed under optimal regeneration conditions; (3) a selection regime starting with a lower level of Km followed by a higher level is an option to improve transformation frequency.

There are also some screenable marker genes that can assist selection of transformed tissues without addition of selectable agents (Miki and

McHugh, 2004). For cherry transformation, transgenic plants of “Colt” rootstock were produced from the roots obtained after using *A. rhizogenes* nondisarmed pRi1855 T-DNA (Gutiérrez-Pesce *et al.*, 1998). However, this method can lead to production of chimera transformants (Druart *et al.*, 1998).

2.2.4 Regeneration of whole plants and characteristics of transgenic plants

For a regeneration system based on somatic embryogenesis, whole transgenic plantlets can be produced after inoculation, selection, and the germination of somatic embryos. Three previous reports have documented regeneration of transgenic cherry plants through somatic embryogenesis following Km selection (da Câmara Machado *et al.*, 1995; Druart *et al.*, 1998) or nonselection (Gutiérrez-Pesce *et al.*, 1998). For regeneration systems through callus-mediated production of adventitious shoots, a necessary step for obtaining transgenic plants is to root those selected shoots. These transformation systems allowed production of Km-resistant shoots after selection on regeneration medium (Song and Sink, 2006a, b). When these shoots were proliferated on stock culture medium (QLBI) + 50 mg l^{-1} Km + 250 mg l^{-1} timentin, transgenic shoots continued to grow and proliferate, whereas the escapes stopped growing. In addition, proliferated shoots could be rooted easily on WPM + $5.4 \mu\text{M}$ NAA + 50 mg l^{-1} Km (Song and Sink, 2006a, b).

Morphology of the transgenic plants is related partly to the introduced transgenes. Expression of the nondisarmed pRi1855 T-DNA in the “Colt” rootstock yielded the expected abnormal phenotypes such as enhanced rooting capacity, shortened internodes, and wrinkled leaves (Gutiérrez-Pesce *et al.*, 1998). Abnormal phenotypes associated with the expression of marker genes such as *nptII*, *hpt*, and *gusA* have not been reported in cherry plants. No studies to date have investigated the stability of transgenes in the sexual progeny of cherry plants. Using histochemical GUS assay, staining was detectable in all tissues of transgenic shoots even after five subcultures (G. Song and K. Sink, unpublished data), indicating the stable expression of *gusA* during vegetative proliferation.

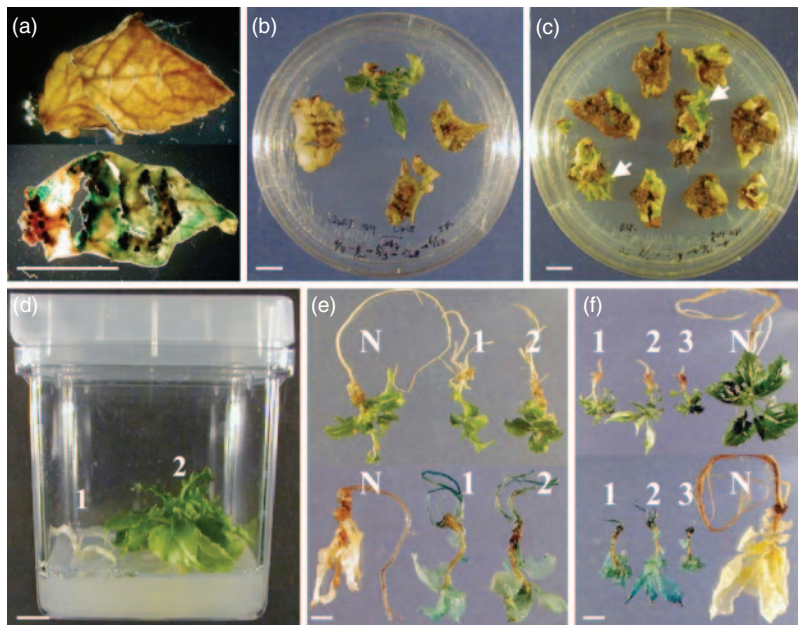


Figure 2 Histochemical GUS assays in transformed plant tissues after one-step selection with 50 mg l^{-1} Km; (a) GUS expression in uninoculated (upper) and inoculated (lower) leaf explant tissues of “Montmorency” after 8-week culture on selection medium; (b) recovery of Km-resistant shoots of “Montmorency” after 12-week selection; (c) recovery of Km-resistant shoots of “Gisela 6” after 12-week selection (arrows—adventitious shoot(s)); (d) regrowth of shoots regenerated from an inoculated leaf explant of “Gisela 6” on QLB1 (QL+ $2.2 \text{ } \mu\text{M}$ BAP + $0.25 \text{ } \mu\text{M}$ IBA) containing 50 mg l^{-1} Km and 250 mg l^{-1} timentin—(1) a shoot with GUS-negative leaves and (2) a shoot with GUS-positive leaves; (e) rooted transgenic plants (1 and 2) and nontransformed plant (N) of “Montmorency” before (upper) and after (lower) GUS staining; (f) rooted transgenic plants (1, 2, and 3) and nontransformed plant (N) of “Gisela 6” before (upper) and after (lower) GUS staining; bars: 1 cm [Reproduced from Song and Sink (2006a)]

To date, there has been no information yet available on field evaluation of transgenic cherry plants.

2.2.5 Strategies for cherry improvement through genetic transformation

Due possibly to emphasis on devising efficient transformation protocols, few reports have yet focused on cherry improvement by introducing novel useful traits. Transformation of the nondisarmed pRi1855 T-DNA yielded the expected dwarf characteristic in the rootstock “Colt”, and was further expected to reduce canopy growth of sweet cherry grafted onto it (Gutiérrez-Pesce *et al.*, 1998; Gutiérrez-Pesce and Rugini, 2004). To obtain cold resistance, the antifreeze protein gene (*afp*) was transformed into a sweet-sour cherry hybrid “Black Eagle” (Dolgov, 1999). One transgenic line was PCR positive for *afp*; however, the expected characteristic, cold resistance, was not expressed

(Dolgov, 1999). Transformation systems for two cherry genotypes have been established and are now transferring several virus-resistant genes into them (Sink and Song, personal communication).

3. FUTURE ROAD MAP

Industry input on possible biotechnology needs was obtained at the Cherry Marketing Committee meeting, Hart, Michigan in June 2001. Next, interviews were held (by K. Sink) with pertinent Michigan State University scientists to gain their perspective on the status of the suggested priorities and possible transgenes. Subsequently, the priorities were reviewed and updated at the 2006 Art Mitchell Symposium (<http://mitchellsymposium.org>).

These priorities will include the most important target traits to be addressed in near future and those are delineated below.

3.1 Diseases

3.1.1 Cherry leaf spot

This disease is prominent throughout cherry-producing regions worldwide and currently is controlled with fungicides (Wharton *et al.*, 2003). These authors tested sour cherry cultivars for resistance, but only the seedlings of “Almaz” (o.p.) were resistant. Attempts to transfer this resistance by traditional breeding to sour cherry are underway. It has been suggested to test *NPRI* homologs, since the homolog *MpNPRI* from *Malus* was used to confer fire blight resistance in apple (Malnoy *et al.*, 2004). It may be easy to isolate a similar homologous gene from cherry and test it. Otherwise, no genes specific for resistance to this pathogen have been isolated.

3.1.2 Armillaria

Three species, *mellea*, *ostoyae* (more virulent in northern Michigan), and *bulbosa* are involved. This root rot is mostly a problem for cherry production in Michigan, though it affects other stone fruits in many production regions (Beckman and Lang, 2004). An extensive screening of *Prunus* spp. and rootstocks is underway (Lang, personal communication). No resistance genes have yet been reported.

3.1.3 Brown rot

Monilinia fructicola (G. Wint.) Honey (Ogawa *et al.*, 1995) and *Monilia laxa* (Aderhold and Ruhland) Honey cause blight on twigs and blossoms, cankers, and fruit rot. The problem is worldwide, with sweet cherry generally more sensitive than sour cherry. Cultural practices and fungicides are used for control. No genes directly involved are yet available via breeding or genomics.

3.1.4 Cherry yellows

This disease is a complex of at least two viruses, PDV and PNRSV (Vasková *et al.*, 2001). Even when planting stock is virus free, the virus can be transmitted via infected pollen; thus, flowers often are removed from young trees to prevent infection

during tree establishment. Although the viruses usually are not lethal, annual GA₃ applications may be required later to reduce the symptoms of leaf and bud chlorosis, and increase yield by about 30% over nontreated infected trees. There is no control for this disease once trees are infected. Gene sequences, in PTRAP6i, are available for both viruses (Liu, 2005). But, variants of these viruses exist (Ogawa *et al.*, 1995). A proof of concept for the virus resistance crossing a graft union is underway at MSU using RNA interference (RNAi) technology.

3.1.5 Bacterial canker

This disease is incited by *Pseudomonas syringae* pv. *syringae* van Hall and *P. s.* pv. *morsprunorum* (Wormald) Young *et al.*, causing cankers on limbs, branches, and trunks as well as spots on leaves and fruit, and flower blast and spur death at bloom (Ogawa *et al.*, 1995). Bacterial canker is a limiting factor for tree survival in some regions and can be a significant problem in most. The disease is most prevalent on sweet cherry: “Royal Ann”, “Bing”, “Lambert”, and “Van” are very susceptible, while “Corum”, “Sam”, and “Sue” appear to have reasonable tolerance to canker. Use of copper compounds for control is limited due to the development of resistant strains. Promising strategies may be to test lytic proteins, *hrp*, and *NPRI* genes similar for resistance to fire blight.

3.1.6 Powdery mildew

The dominant gene *Pmr1* for cherry powdery mildew resistance has been reported (Olmstead and Lang, 2002). If this gene could be isolated and cloned, the possibility would exist to transfer it to sensitive commercial sweet cherry cultivars like “Bing”, “Rainier”, and “Lapins.”

3.2 Insects

3.2.1 Plum curculio

This beetle lays eggs under the fruit skin, and larvae eat the fruit flesh, leaving an ugly crescent-shaped mark (<http://www.msue.msu.edu/vanburen/plumcurc.htm>). There is zero tolerance for the

insect. Control is considered difficult and more so because the availability of organophosphate pesticides may be in jeopardy in the United States by the Food Quality Protection Act. No target genes are presently available.

3.2.2 Cherry fruit fly (CFF)

There are two fruit flies on cherry in the eastern US, the Eastern fruit fly (*Rhagoletis cingulata* Loew) (Compton *et al.*, 2005) and the Black CFF (*Rhagoletis fausta* Osten Sacken) (<http://pesp.org/mcc98-final.htm>). The Western CFF (*Rhagoletis indifferens* Curran) (Smith, 2005) is the major insect pest in the Pacific Northwest. There is zero tolerance for all fruit flies. CFFs lay their eggs under the fruit skin, so larvae hatch already in the flesh. Insecticides must be ingested by the adult for control, since contact insecticides are not allowed. These insects are a more widespread threat than plum curculio, since they occur later in the growing season when pesticide residues can be a problem due to multiple applications. A possible control approach may be via pheromone engineering to change the host attractiveness, a scheme that would mimic the natural host marking system. No breeding attack or transgene(s) are presently possible.

3.3 Chilling and Freezing of Flowers

In *Prunus* spp., the pistil is susceptible to freezing spring temperatures. This results in flower losses and concomitant reduced yields. In sour cherry, fluctuating annual yields are due largely to this factor. Sour cherry has good cold acclimation in all tissues but the pistil and a cherry homolog of the *CBF1* gene has been found (Owens *et al.*, 2002). Further expression studies are needed. Suggested gene constructs to test include pistil promoters *SK2* and *PsTLL1* in conjunction with *CBF* genes.

3.4 Fruit Traits

3.4.1 Pitless fruit

The hard cherry pit can escape detection during processing and wind up damaging the teeth of the

consumer. The cherry fruit is a drupe, having an ovary that is composed of the exocarp, mesocarp, and endocarp (the pit containing the embryo). The pit is more precisely the outer portion of the endocarp where the cells become highly lignified and thickened during development as the fruit matures. The pit is about 4–5 mm, and some varieties may have a small tip at the pit base that may break off during processing, but this is not a problem with “Montmorency.” The developing embryo promotes growth of the developing fruit. At the end of stage II, the embryo is fully developed and subsequently extensive fruit enlargement takes place. If the promoter of the endocarp lignification genes could be discovered, it may be possible to pursue a change to a softer endocarp. The embryo is soft and could be considered edible, so that if pits break or escape during processing, it would not be the problem of present. No soft pit mutants have been found for any stone fruit crop. Almonds have varying degrees of pit strength, but no soft-pit almonds that can be eaten are known. Although genes involved in lignification are probably in current EST libraries, the problem is probably more complex and problematic to solve using transgenes.

3.4.2 Firmer cherry fruit at harvest

Although sour cherries generally have high-quality fruit at harvest, the shaking process, conveyer belt, and bulk handling all lead to bruising of the fruit. Wind whipping of fruit on trees also causes bruising. Some Michigan cherry orchards have a problem with unusually soft fruit most years, whereas soft fruit occurs less frequently in other orchards. This is complicated by the fact that there may be sport clones of “Montmorency”, and an environmental interaction may play a role. In the western United States, GA₃ is applied to increase sweet cherry fruit firmness. Similar GA₃ tests have been undertaken on sour cherries in Michigan. Thus, transgene GAs may be of interest with fruit-specific promoters. No single gene/enzyme is likely to significantly impact texture (firmness). Such genes are likely to function in concert with additional factors (Giovannoni, 2001). The gene expansion may represent a candidate that could be tested immediately. For polygalacturonases, which

are highly redundant among species, knock-out technology may not have the desired effect.

3.4.3 Browning of bruised flesh

Elimination of bruise-induced flesh browning in either sweet or sour cherry would be desirable. A transgenic approach would be through knock-out or antisense control of the enzyme polyphenol oxidase, coupled to a fruit promoter.

3.4.4 Red fruit flesh

The ripe fruit of “Montmorency” have a red skin, but clear flesh. “Montmorency” also has a high level of melatonin, a desirable component in cherry juice. “Balaton” has red color throughout the fruit, but a lower level of melatonin. Although some genes for anthocyanin are available, a transgenic “Montmorency” having red fruit and red flesh, produced strictly for juice, probably would not be a profitable venture.

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Grapes

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1. INTRODUCTION

1.1 Origin of Grapevine and the History of Viticulture

The domestication of grapevine (*Vitis vinifera* L.) is closely linked to the discovery of wine that was considered by ancient cultures as a gift from the Gods. The mythology of wine and its mystical significance has given the grapevine a privileged position among cultivated plants and the growing of grapes has a prominent place in the history of western civilization (Johnson, 1989). Although wild grapes were present in many places of Eurasia during the Neolithic period, it is generally considered that cultivation of the grapevine existed by 6000–5000 BC along the eastern shores of the Black Sea in the region known as Transcaucasia where the greatest genetic diversity is found and very early archaeological evidence, including grape pips and artefacts of a “wine culture” were excavated (Zohary, 1995; McGovern, 2003). Uncertainty remains about the occurrence of a secondary domestication center in western Mediterranean (Grassi *et al.*, 2003; Arroyo-Garcia *et al.*, 2006; Imazio *et al.*, 2006) with paleobotanical findings in Spain dated 3000 BC (Nunez and Walker, 1989). The genetic relationship between wild and cultivated grapes is still not resolved but progress is being made using nuclear and plastid DNA markers (Perret *et al.*,

2000; This *et al.*, 2001, 2006; Sefc *et al.*, 2003; Carreno *et al.*, 2004).

By 4000–2000 BC grape growing extended to Asia Minor, through the Fertile Crescent, into the Nile delta, and Balkan Peninsula. During the 1st millennium BC, the Phoenicians and Greeks introduced viticulture into the western Mediterranean (McGovern, 2003). It is likely that cultivated grapes were also brought to China from Asia Minor during the Han Dynasty (200 BC) but the cultivation of grapevine is historically proven in Japan only from 1200 AD. Under the influence of Rome, the growing of grapes extended throughout Europe from the shores of the Atlantic to the valleys of the Rhine and the Danube despite the edict of the Emperor Domitian (92 AD) to limit the growth of provincial viticulture and to protect the export of wine from Italy. With the fall of the Roman Empire the wine trade and grape growing went into decline, but the spread of Christianity to northern Europe and the need for wine for sacramental purposes led to the establishment of a new international trade in wine (Johnson, 1989).

By 600 AD the consumption of wine had been prohibited under Islamic law and this promoted the cultivation of table grapes in the Middle East and North Africa. This led to the introduction of table grapes into Spain, from where they spread to France, Italy, and the New World. The restoration of wine grape cultivation in North Africa did not

occur until the colonization of Algeria by the French in 1830.

By the end of the Middle Ages, wine drinking was a firmly established social custom over most of Europe, and viticulture grew steadily from the 16th to the 19th centuries despite a series of calamities such as the frost of 1709 and the phylloxera plague, which appeared in France in 1868 and then spread progressively through all the grape-growing regions of Europe (Johnson, 1989).

In 1525, Cortez ordered the planting of grapevine in Mexico and by 1550 the growing of grapes had spread to Peru, Chile, and Argentina. In 1616, viticulture started in South Africa by Dutch settlers at the Cape of Good Hope. The first grapes were planted in California in 1697 and the development of viticulture extended with the establishment of numerous Spanish missions from 1769 to 1810. Immigration played a large part in the development of commercial viticulture in California during the second half of the 19th century. Viticulture in Australia began in 1788 with the founding of a penal colony. In the 1850s German Lutherans founded the wine industry of the Barossa Valley and at the end of the 19th century, settlements were established along the Murray River in South Australia and Victoria and the growing of grapes for drying became the predominant industry of this region. In New Zealand, grapes were planted early in the 19th century by French settlers and religious orders (Johnson, 1989).

1.2 Diversity, Taxonomy, and Evolution of Grapevine

The cultivated vine (*V. vinifera* L.) belongs to the family Vitaceae that is primarily intertropical in its distribution and consists of perennial plants that take the form of woody or herbaceous liana (tree-climbing plants) or shrubs with lianalike stems. The family shows considerable morphological variability and a large range of adaptation to highly varied environments. The systematics of the Vitaceae is based on the classification of Planchon (1887) who identified 10 genera. After many revisions, the family currently consists of 17 genera from which only the genus *Vitis* produces edible fruit. Until recently, Vitaceae were included with the Rhamnaceae

and Leeaceae in the Rhamnales order within the Eudicots classification. Improved classification is expected to occur over time as more molecular information becomes available. The taxonomy browser at National Center for Biotechnology Information (NCBI) currently lists the Vitaceae as a core eudicotyledon in the order Vitales (<http://www.ncbi.nlm.nih.gov/Taxonomy>).

According to cytotaxonomy studies (Shetty, 1959; Lavie, 1970), the family ancestor may belong to the *Cissus* genus (367 species) that is typically intertropical, distributed throughout Africa and Asia, and consists of plants with tetramerous flowers and a chromosome karyotype of $2n = 24$. The genera *Cyphostemma* (258 sp.) and *Tetrastigma* (132 sp.) occur respectively in Africa and Asia and appear to be closely related. They have morphological and anatomical similarities and a common karyotype ($2n = 22$) with many cases of polyploidy. The genera *Ampelocissus* (90 sp.), *Vitis* (71 sp.), *Ampelopsis* (31 sp.), and *Parthenocissus* (18 sp.) consist of plants with pentamerous flowers and a common karyotype of $2n = 40$, with the exception of *Vitis* ($2n = 38$). They are distributed in the temperate climates of America, Asia, and Europe, with the exception of *Ampelocissus* that is typically found in the tropical warm areas including Africa. The number of species indicated in brackets for each genus corresponds to a compilation of the references found in the literature (J.-M. Boursiquot, personal communication). Due to problems of synonyms, the numbers may be overestimated).

Phylogenetic studies based on the study of chloroplast DNA confirmed the relationships of Vitaceae and Leeaceae (nonclimbing vines), but showed that these two families were distant from the Rhamnaceae and close to the base of the large clade of Rosids (Savolainen *et al.*, 2000; Soltis *et al.*, 2000). Other studies dealing specifically with the Vitaceae (Ingrouille *et al.*, 2002; Soejima and Weng, 2006) suggest that these became separated from the Rosids at the end of the Cretaceous period about 100 million years ago (mya) and confirm the ancestral character of *Cissus* and the closeness of *Cyphostemma* and *Tetrastigma*, forming a large clade including also the genus *Cayratia* (65 sp.), which had already been previously claimed (Rossetto *et al.*, 2002). The recent completed sequencing of the grapevine chloroplast genome has placed the Vitaceae as sister to the other

rosids (Jansen *et al.*, 2006). Among the Vitaceae with $2n = 40$ chromosomes, the *Ampelopsis* genus would be ancestral, the genera *Parthenocissus* and *Ampelocissus* would be intermediary, and the *Vitis* genus, including the subgenera *Euvitis* ($2n = 38$) and *Muscadinia* ($2n = 40$), would be more recent.

The hypothesis that *Ampelopsis* may be older than *Parthenocissus* and *Vitis* is supported by the work of Lodhi and Reisch (1995), who observed that the nuclear genome of *Ampelopsis brevipedunculata* is made up of 666 Mpb (mega base pair), which is significantly higher than the genome of *Parthenocissus tricuspidata* (516 Mpb) and the average in the species of *Vitis* (475 Mpb). Throughout a diverse range of organisms, retrotransposon copy number appears to be correlated with genome size. When active, these elements play a role in genetic evolution with replication and insertion increasing genome size and creating polymorphisms (Bennetzen, 2000). Several retrotransposons have been identified in *V. vinifera* (Verriès *et al.*, 2000; Pelsy and Merdinoglu, 2002; Kobayashi *et al.*, 2004). The level of retrotransposons in the genome was estimated to be 41% (Tomkins *et al.*, 2001) and it is likely they have played a considerable role in the evolution of the Vitaceae family.

The phylogenetic distance between *Vitis* and *Ampelopsis* shown by cladistic analysis practically nullifies any hopes of genetic hybridization between these two genera by conventional breeding. The same is probably true for hybrid production between *Vitis* and *Parthenocissus* species. The use of genetic resources from "Virginia creepers" therefore lies in the development of somatic hybridization methods using protoplast technology. However, this technique is a long way from being mastered in vines, despite some success in the regeneration of protoplasts from somatic embryos (Reustle *et al.*, 1995).

Conversely, the phylogenetic proximity of the subgenera *Euvitis* and *Muscadinia* explains why the karyotype differences have not been an insuperable obstacle in crossings between *V. vinifera* and *V. rotundifolia* (Wylie, 1871; Detjen, 1919; Dunstan, 1962; Olmo, 1971; Bouquet, 1980). Given its anatomical, morphological, and karyological characteristics, the subgenus *Muscadinia* must be elevated to the genera level as proposed one century ago by Small (1903), though this proposal has not yet achieved general acceptance.

Although there is no doubt about the close phylogenetic relationship between *Euvitis* and *Muscadinia*, the hypothesis of a relatively recent origin of the genus and its resulting division into two subgenera, is weakened by the discovery of fossil pips in 55-million-year-old northern European tertiary sediments. Some seeds (*Vitis rectisulcata*) are globular with a smooth chalaza, similar to the pips of the actual *Euvitis* and others (*V. longisulcata*) are oblong with a wrinkled chalaza, similar to the pips of the actual *Muscadinia* (Fairon-Demaret and Smith, 2002). These recent findings confirm the distinction of two types (*Vitis teutonica* and *Vitis ludwigii*) made in 1939 by Kircheimer (cited by Rives, 1975) between fossilized pips assignable to *Vitis* and suggest that the genus *Vitis* was widely distributed throughout the northern hemisphere before the advent of the ice ages. The separation of *Euvitis* and *Muscadinia* may date from the Tertiary period and it is probable that *Muscadinia* became extinct in Europe during the Quaternary. *Muscadinia* could be regarded as transitional between *Vitis*, which are adapted to temperate climates, and *Ampelocissus*, which are adapted to tropical climates (Mullins *et al.*, 1992). *Ampelocissus* has marked similarities in morphology, anatomy, and karyotype with *Vitis* and *Muscadinia* but its ability to hybridize with them is unknown.

The *Muscadinia* is currently represented by only three species (*V. rotundifolia*, *V. munsoniana*, and *V. popenoei*) of which only the first two have been properly described and whose natural range is limited to the Southeast of the United States. Cytological observations relating to F_1 hybrids *V. vinifera* \times *V. rotundifolia* could lead to the assumption of an allo-polyploid origin of the vine (Patel and Olmo, 1955). However, this hypothesis has not yet been confirmed either by *in situ* hybridization results from *V. vinifera* chromosomes, which have only revealed a single ribosomal (5.8S, 18S, and 26S rRNA) locus (Haas *et al.*, 1994; Haas and Alleweldt, 2000), or by the data acquired during development of genetics maps. If an auto- or allo-polyploidization process really occurred, then there is no doubt that it was very old and followed by many rearrangements. Unexplained is why the *Muscadinia*, apparently widely scattered across the entire northern hemisphere during the tertiary, did not become diversified like the *Euvitis* and

had a considerably reduced natural range during the quaternary eventually becoming limited to the North American continent.

The genus *Vitis*, formerly *Vitis* subgenus *Euvitis* Planch., contains about 70 species. These are found mainly in the temperate zones of the northern hemisphere and are distributed almost equally between America and Asia. Only one species, *V. vinifera* L., originated in Eurasia. It has been spread throughout the world by man. The systematics of *Vitis* has been a subject of controversy for more than a century. The determination of the number of “real” species of grapes, and their proper names, has considerable significance for plant improvement. So far, the most comprehensive treatment of the genus is that of Galet (1967) who listed 59 species, including 25 species from Asia, grouped into 11 series (Mullins *et al.*, 1992).

Many species of *Euvitis* are thought to have arisen during the Quaternary ice ages. Their distribution is consistent with the breaking up of large populations by the ice fronts and the survival of small populations in refuges. This isolation and differing environmental conditions of the refuges provided ideal circumstances for speciation. The formation and dissolution of refuges probably occurred several times during the Quaternary period. At the end of the ice ages, the species of *Euvitis* acquired a remarkable diversity in morphological and physiological characters, but this was not associated with significant genetic differentiation. There are no genetic barriers and the species of *Euvitis* are interfertile. They are really ecospecies and can be defined as populations of grapevines that are easily distinguishable by morphological characters and which were/are isolated by geographical, ecological, and phenological barriers (Mullins *et al.*, 1992).

The high sensitivity of *V. vinifera* to many pests and pathogens has hampered its universal cultivation since the 19th century and led particularly to the domestication of wild species in the United States, *Vitis labrusca* in the Northeast (Hedrick, 1908) and *V. rotundifolia* in the Southeast (Olien, 1990). There is no doubt about the native origin of the *Muscadinia* grapevines. But it is a practical certainty that the *labrusca* varieties, and notably the Concord variety, are natural hybrids between this wild species and the varieties of *V. vinifera* introduced in

the first colonies (Booth, 1911; Tukey, 1966). Due to their hybrid origin, these varieties were named “*Vitis × labruscana*” by Galet (1967).

There is considerable diversity within *V. vinifera*. The number of different varieties held in germplasm collections around the world is estimated at approximately 10 000 (Alleweldt and Dettweiler, 1994) but an accurate knowledge of this diversity is hampered by a complex synonymy. The same variety may have different names and different varieties may have the same name. However, there is a general acceptance that the number of “real” varieties properly identified in the world is closer to 5000. Included are cultivars that are widely grown in many grape growing countries, those that are of strictly local importance and those which have practically disappeared from vineyards. The classical method of ampelography based on morphological descriptions to distinguish cultivars is now nearly completely replaced by the use of molecular markers, especially the microsatellite DNA markers (Thomas and Scott, 1993; Thomas *et al.*, 1993, 1994; Dangl *et al.*, 2001; This *et al.*, 2004) coupled with computer technology.

1.3 Economic Importance

Grape berries are eaten fresh (table grapes) or dried (raisins), but the main use is wine making. Total world grape production in 2005 was estimated at 66 million tons, from which 65.7% was used for wine production estimated to be 277 million hectoliters, 26.7% consumed as table grapes, and 7% transformed into raisins. The worldwide surface area of vineyards was estimated to be 8 million hectares distributed between Europe (58.9%), Asia (21.7%), America (12.1%), Africa (4.9%), and Oceania (2.4%) (Organisation of Viticulture and Wine (OIV) Statistics 2005).

In 2005, the 10 highest wine producing countries were Italy (50.6 million hectoliters), France (50.5 million hectoliters), Spain (35.3 million hectoliters), the United States, Argentina, Australia, China, Germany, South Africa, and Chile. The main consumers of wine are the wine-producing countries, but during the last 50 years, there has been a marked and continuous drop in consumption in many of the traditional wine-drinking nations. In France, for example, consumption per capita was 1271 in 1963, 741

in 1988, and 531 in 2005. This gap between production and consumption has been offset to some extent by an increase of wine consumption in northern European countries, the United States and China, as well as the growing international trade in wine. In 2005, 28.9% of world wine production was exported with the three leading wine-producing countries responsible for half of all exports.

In some countries large quantities of wine are distilled to produce high-proof spirits (cognac, armagnac, brandy, or grappa) and fortifying spirits for the making of sherry, port, dessert wines, or other aperitif beverages. Since the “French paradox” was brought to the fore (Renaud and De Lorgeril, 1992), there is epidemiological and biological evidence that moderate consumption of wine protects against cardiovascular diseases (Rotondo and Gaetano, 2000). Recently, the discovery of melatonin in berries of several cultivars suggests that the well-established pharmacological properties of the grapevine are due not only to the presence of polyphenolic compounds, such as resveratrol, anthocyanins, and proanthocyanidins, but also to the powerful antioxidant activity of this compound (Iriti *et al.*, 2006). While the negative impacts of excessive alcohol consumption have been well documented, research into the nutritional value and health benefits of wine and grape products are relatively new study areas.

It is difficult to state precisely the worldwide production of table grapes. Certain producing countries do not provide statistics and others are hampered in their efforts because of indifferent reporting from individual producers. Summarizing 2003 OIV statistics, Asia has the highest production, with China as leading country (3.4 million tons), followed by Turkey (1.5 million tons), Iran (1.4 million tons) and India (1 million tons). In America, table grapes are produced mainly in Chile (0.9 million tons), the United States (0.7 million tons), and Brazil (0.6 million tons). In Europe, table grapes are produced mainly in Italy (1.5 million tons) and Spain (0.7 million tons). In Africa table grapes are produced mainly in Egypt (1 million tons). Despite the perishability of the fruit and the high cost of transportation, table grapes support a significant international trade with 2.9 million tons imports (mainly into United States, Germany, Russia,

and the United Kingdom) and 3.1 million tons exports (mainly from Chile and Italy). Annual per capita consumption of table grapes is low and does not exceed 10 kg in most producing countries. In Europe and North America table grapes represent less than 5% of annual per capita consumption of fresh fruits. Fresh grapes have important nutritional qualities. They provide about 700 cal kg⁻¹ and in addition contain calcium, phosphorous, and vitamins.

For raisins (OIV 2005 statistics), the world's largest producers are Turkey with a production of nearly 3.4 million quintals, the United States (3.2 million quintals), and Iran (2.1 million quintals). Raisins are a high-energy food, rich in sugars providing 3340 cal kg⁻¹.

Grape berries have many other uses, such as canned fruit after pasteurization. The fresh juice of pressed grapes can be bottled for consumption, converted to jellied products or concentrated (such as the “*pecmez*” of Turkey). In addition there are many industrial and miscellaneous uses of grapes and grape products including the production of ethanol, wine vinegar, grape-seed oil, anthocyanin pigment, food additives, cosmetics, or pharmaceuticals. Finally the residues of wine making (marcs) can be processed for the industrial production of tartaric acid and the waste can be further utilized for fertilizer production.

1.4 Traditional Breeding: Objectives, Constraints, Tools, and Strategies

The breeding of woody perennial fruit plants, such as grapevines, presents considerable difficulties. Although cultivated grapes bear self-fertile flowers, they do not breed true from seed. In their wild state, all the species of *Vitis* (including *V. vinifera*) are dioecious resulting in strict allogamy that maintained a high level of individual genetic heterozygosity. The heterozygosity of cultivated grapes was confirmed by DNA microsatellite analysis (Thomas and Scott, 1993; Sefc *et al.*, 2000) and recently by single nucleotide polymorphism (SNP) markers (Salmaso *et al.*, 2004). The lower level of heterozygosity observed in the remaining wild grapevines (Aradhya *et al.*, 2003) may be attributed to some inbreeding existing in small and isolated populations. Grapevine cultivars represent gene combinations that are disrupted by

the sexual process and the fixed heterozygous state of cultivars have been maintained and perpetuated for centuries by vegetative propagation. Thus, modern wine production is based primarily on traditional cultivars. The styles of wine produced by these cultivars enjoy a high level of consumer acceptance with marketing focused on traditional cultivars. Appropriate management technologies have been developed to grow these traditional cultivars, and they have become firmly entrenched by custom or by law in some European countries and regions effectively limiting what cultivar can be grown in specific regions. As a consequence of these special circumstances, clonal selection, the exploitation of genetic variation within traditional cultivars, has become the most widely used procedure for improvement of wine grapes.

Most wine grapes are of ancient origin and are thought to have arisen from natural crosses within *V. vinifera*. These cultivars might be a mixture of more than one genotype, due to propagation of closely related and phenotypically similar plants. This concept is known as the polyclonal origin of grapes (Rives, 1961). However, molecular analysis based on random amplified polymorphic DNA (RAPD) markers did not detect genetic polymorphism among different clones of Pinot noir despite this phenotypically heterogeneous cultivar being grown since the early Middle Ages (Ye *et al.*, 1998). Whatever their origin, grapevine cultivars are likely to have accumulated by now a substantial amount of somatic mutations (Franks *et al.*, 2002). These are more difficult to detect using molecular markers, but amplified fragment length polymorphism (AFLP) techniques (Scott *et al.*, 2000), microsatellites (Franks *et al.*, 2002; Riaz *et al.*, 2002), or more recent techniques based on retrotransposons (Pelsy *et al.*, 2003) and SNPs (Salmaso *et al.*, 2004) now make it possible to detect genetic polymorphism derived from mutations among clones of the same variety. Though the selection of bud sports has been an important element in genetic improvement of pome and stone fruits, the occurrence of such bud sports is less well documented in grapes, except for some visual mutants (Boss and Thomas, 2002; Franks *et al.*, 2002; Fernandez *et al.*, 2006; Walker *et al.*, 2006). A considerable amount of work has been done for the generation of mutants in fruit crops, mostly using combinations of radiation and tissue culture techniques (Predieri, 2001) but

results on grapevine are few. Moreover, most of the expected mutations are usually recessive and are only revealed in the M₂ generation, which can take several years for grapevine.

Efficient tools for genetic mapping have been developed during the last 10 years and are now used for the studying the genetics of important traits. In 1998, the international scientific community decided to join its effort to develop a large set of microsatellite (= simple sequence repeat, SSR) markers through a consortium, the Vitis Microsatellite Consortium (Adam-Blondon *et al.*, 2004; This *et al.*, 2004; Merdinoglu *et al.*, 2005). Since Lodhi *et al.* (1995), several genetic linkage maps have been published for grape (for a review, see Adam-Blondon *et al.*, 2007; Riaz *et al.*, 2006). Recently, in the framework of the International Grape Genome Program (<http://www.vitaceae.org>) an integrated SSR map was constructed using segregation data from five full-sib progenies (Doligez *et al.*, 2006a). This consensus map, comprising 502 SSR loci has a total length of 1647 cm (Kosambi) covering the 19 linkage groups. Physical maps are under construction and will greatly facilitate the mapping of genes and candidate gene approaches through their integration with genetic maps (Lamoureux *et al.*, 2006). Several bacterial artificial chromosome (BAC) libraries are now available in grape, opening the road for structural genomics in this species (Tomkins *et al.*, 2001; Adam-Blondon *et al.*, 2005, 2007). Expressed sequence tag (EST) sequencing projects have provided a basic resource for the study of the molecular mechanisms involved in most of the traits of interest of the grapevine (Ablett *et al.*, 2000; Goes da Silva *et al.*, 2005; Terrier *et al.*, 2005) but also for an efficient transfer of knowledge from model species to grapevine. Over 400 000 sequences, mostly ESTs, have been deposited at NCBI (<http://www.ncbi.nlm.nih.gov>) corresponding to more than 16 000 UniGenes (Build #19; <http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=29760>) or more than 34 000 unique sequences as determined by the DFCI grape gene index (Release 5; <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape>) Recently, grape berry biochemistry was investigated using proteomic analysis (Sarry *et al.*, 2004).

Two grape genome DNA sequencing projects are being undertaken. One is conducted at IASMA (Italy) and involves the sequencing of the Pinot Noir genome (Velasco *et al.*, 2006). The second project conducted at Genoscope in collaboration with URGV (France), is based on a near-homozygous line PN40024 (Bronner and Oliveira, 1990) that has Pinot Noir as one parent. This project is also associated with the genome sequencing of grape pathogens. The availability of the sequence of a pathogen and of its host would offer many opportunities to understand the molecular mechanisms underlying the dynamics of host–pathogen interactions.

The complete grapevine sequence will provide a resource that will open new avenues of research to answer many fundamental questions, and also will provide a new tool to stimulate the breeding of this economically important species. Indeed *V. vinifera* wine grape cultivars of commercial value were obtained by controlled crosses during the 19th and 20th centuries. In France, the variety Alicante Bouschet was bred by Louis and Henri Bouschet from 1829 to 1855 and was obtained from a tripartite cross between Aramon, Teinturier du Cher, and Grenache noir (Snyder, 1937). In Germany, the variety Muller-Thurgau was obtained in 1892 from a cross between Riesling and Madeleine royale (Dettweiler *et al.*, 2000). In South Africa, the variety Pinotage was obtained in 1925 from a cross between Pinot noir and Cinsaut (Orffer, 1979). However, plantings of wine grape cultivars recently developed from traditional breeding programs still remain limited, despite considerable work carried out during the second half of the 20th century (Alleweldt and Possingham, 1988). New cultivars are always assigned new names, contributing generally to their slow acceptance in the market place. The current interest in France about the cultivar Marselan, obtained recently from a cross between Cabernet Sauvignon and Grenache noir (Bouquet and Boursiquot, 1999), was likely stimulated by the disclosure that the two worldwide leading varieties (Cabernet Sauvignon and Chardonnay) were in fact obtained several centuries ago from crosses between older varieties (Bowers and Meredith, 1997; Bowers *et al.*, 1999).

Table grapes are not subject to the same constraints as wine grapes with regard to tradition and consumer acceptance, and conventional

breeding programs have produced numerous outstanding new cultivars during the 20th century, most of them seedless (Ledbetter and Ramming, 1989). Over the last 20 years, the efficiency of breeding for seedless berries was considerably improved with the use of *in vitro* embryo rescue (Cain *et al.*, 1983; Spiegel-Roy *et al.*, 1985). Molecular markers and genetic maps have been developed for the detection of quantitative trait loci (QTL) involved in seedlessness (Striem *et al.*, 1996; Lahogue *et al.*, 1998; This *et al.*, 2000; Adam-Blondon *et al.*, 2001; Doligez *et al.*, 2002; Mejia and Hinrichsen, 2003) and other berry characteristics, such as muscat flavor (Doligez *et al.*, 2006b).

But it is with rootstocks that breeding has had the greatest impact on viticulture. Until the end of 19th century, grapevine was propagated by layering or by hardwood cuttings. Since the introduction of the root louse Phylloxera in Europe during 1868, *V. vinifera* varieties are grafted on resistant rootstocks derived from several North American wild species, mainly *Vitis riparia*, *Vitis rupestris*, and *Vitis berlandieri*. This is probably the first and most significant example of a successful genetic solution against a major pest of cultivated perennial plants and the breeding of Phylloxera-resistant rootstocks adapted to different edaphic and climatic conditions, such as limy soils or drought, is the foundation of modern viticulture (Pouget, 1990). Most of these rootstock cultivars were selected at the end of 19th century, but breeding work is continuing in France, Germany, California, Brazil, and Australia. Resistance to lime chlorosis is still a major problem in some French vineyards (Pouget, 1980) and in Germany rootstock breeding is based on the use of complete Phylloxera resistance from *Vitis cinerea* (Schmid *et al.*, 2003). One of the current objectives is to improve resistance against the root-knot nematode *Meloidogyne incognita* (Cousins and Walker, 2002) and the soil-inhabiting nematode *Xiphinema index* that is the carrier of the grape fanleaf virus (Walker *et al.*, 1994; Bouquet *et al.*, 2000a). Molecular breeding holds great promise for the genetic improvement of rootstocks. Linkage maps have been constructed from a cross between two half-sib genotypes *V. rupestris* × *V. arizonica* that carry resistance to Pierce's Disease (PD) and *X. index* (Douceff *et al.*, 2004; Riaz *et al.*, 2006). Lowe and Walker (2006) have also developed a genetic map

from progeny of a cross between *V. champini* and *V. riparia* that may be useful for studying the genetics of many traits including nematode resistance and salt tolerance. In France, INRA scientists from Bordeaux, used progeny from a cross between *V. vinifera* cv. Cabernet-sauvignon and *V. riparia* and constructed a map for identifying QTLs involved in lime chlorosis tolerance and scion vigor (S. Decroocq and N. Ollat, personal communication).

If the use of interspecific hybridization in rootstock breeding was a considerable success, it is still controversial in wine grape breeding. In the genus *Vitis*, species other than *V. vinifera* produce berries with unfamiliar flavors. These flavors are evident in the wines of numerous interspecific hybrids, for example the “foxy” wines of the *Vitis* × *labruscana* cultivars of the New York State vineyards. Hundreds of hybrids with complex pedigrees involving *V. vinifera*, *V. rupestris*, *V. lincecumii*, *V. labrusca*, *V. riparia* and several other North American species were produced in France during the first half of the 20th century, firstly as phylloxera-resistant cultivars (“Direct Producer Hybrids”), then as downy and powdery mildew-resistant cultivars. Their wines were unfamiliar and were not widely accepted, although these hybrids were cultivated on more than 400 000 ha, due to their high productivity and hardiness. Several of these hybrids were prohibited in France as far back as 1934 and the majority of the others in 1955. This led to a drastic change of strategy in France resulting in the use of *Muscadinia* since 1975 (Bouquet, 1980, 1986; Merdinoglu *et al.*, 2003) in breeding efforts. Nevertheless, it should not draw attention away from the accomplishments in other countries, particularly in Germany, to breed resistant cultivars using some of these “French hybrids” (Chambourcin, Villard blanc, Seyval). The success of a cultivar such as Regent (Eibach and Töpfer, 2003) shows that there is still potential in the use of *Euvitis* germplasm for genetic improvement in the framework of an environmental-friendly sustainable viticulture. Molecular breeding, such as genetic mapping and marker-assisted selection, is currently being developed for disease resistance (Dalbo *et al.*, 2001; Pauquet *et al.*, 2001; Donald *et al.*, 2002; Fischer *et al.*, 2004; Akkurt *et al.*, 2006; Wiedemann-Merdinoglu *et al.*, 2006) especially with an aim to pyramid genes from *Muscadinia*

and *Euvitis* (Kozma and Dula, 2003; Eibach *et al.*, 2006; Moreira *et al.*, 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

The evolution of the range of grapevine varieties can be characterized by two words: shifting and simplification (Doazan, 2000). Throughout history, and particularly during the 18th and 19th centuries for which detailed and reliable documents are available, changing (shifting) varieties has always been a means of adapting to changes of viticulture practices imposed either by nature or by man. During the 20th century, however, we have observed a slowing down, if not a freezing, of this evolution concomitant with the emergence of the “Appellation d’Origine” notion, particularly in France. In addition, there has also been a simplification of the varietal range due to the development of an international wine trade based on a few traditional cultivars. This simplification has resulted in widespread ignorance about the genetic diversity that ensured the success of viticulture in the past and its long-term effects in the future have not been carefully considered. It seems to be a paradox that newly bred wine cultivars are not rapidly adopted by industry although their agronomic and enological potential have been intensively investigated using sophisticated methods not available during the adoption of traditional cultivars. The present generation within the wine industry may be too presumptuous to believe that success of traditional cultivars, based on today’s context, will continue forever on such unchanged basis. In fact, there is a general industry perception in some countries that to be successful a new wine cultivar must be very similar to, or if possible indistinguishable from the traditional cultivar that it is designed to replace except for the addition of a desirable trait such as disease or pest resistance. Thus, biotechnology offers a means of inserting new characters into the genome of traditional cultivars without changing any of their other characteristics, particularly wine typicity and quality (Bisson *et al.*, 2002). The best example of the rationale of transgenic breeding versus conventional breeding is the introduction in new wine cultivars of the genes *Run1* and *Rpv1* that

confer resistance to powdery mildew and downy mildew (Bouquet *et al.*, 2000b). Despite their great potential, the future of these “*Muscadinia*-derived” varieties is not ensured, and the positional cloning and sequencing of the gene *Run1* is currently under way, for direct insertion into the genomes of traditional premium wine cultivars (Barker *et al.*, 2005, 2006).

Rootstock cultivars currently used in viticulture worldwide were obtained from conventional breeding and this traditional approach is still practiced in several countries. The advantage with the transgenic approach for grapevine improvement lies in the potential to improve rootstocks particularly adapted to some types of soil and/or climate. The best example is the genetic transformation of the 41 B rootstock, perfectly adapted to the lime soils of the Champagne vineyards, for resistance to grape fanleaf virus (Mauro *et al.*, 2000). Furthermore, grapevine cultivars often have some characteristics or deficiencies that need improvement or correction but where the natural genetic variability in the *Vitis* germplasm is lacking or unusable in conventional breeding. The best example is the susceptibility of much, if not all *V. vinifera* cultivars to wood diseases such as Eutypa dieback, Esca (black measles), or black dead arm. In the case of Eutypa dieback a potential source of resistance may exist in *Vigna radiata* (Guillen *et al.*, 1998).

Despite the focus on the production of improved plants for commercial release (Colova-Tsolova *et al.*, 2001; Kikkert *et al.*, 2001; Bouquet *et al.*, 2003) it should not be forgotten that the ability to produce transgenic grapevines is a valuable research tool for studying and understanding the genetics and function of the genes and processes involved in disease and pest resistance, plant development, primary and secondary metabolism, and response to biotic and abiotic stress (Thomas *et al.*, 2000; Vivier and Pretorius, 2000; Gollop *et al.*, 2002; Mezetti *et al.*, 2002a; Pratelli *et al.*, 2002; Tesnière *et al.*, 2006). However, the lack of high-throughput (HTP) methods for reverse genetics approaches in grapevine functional genomics is a real bottleneck to be overcome in the future (Adam-Blondon *et al.*, 2007). An interesting way is to use transgenic technology and insertion mutagenesis that enhances gene

expression (Pereira, 2000). An Ac/Ds transposon enhancer mutagenesis system has been tentatively applied to grapevine for this purpose (Perl *et al.*, 2000) but is far from being applied routinely. In the future, homology-dependent gene silencing (HDGS) will offer a new tool to shut down the expression of endogenous genes in grapevines. Post-transcriptional gene silencing (PTGS) has been recently suggested in plants using constructs containing repeats that specifically confer aberrant or double stranded RNAs and could likely induce predictable silencing of homologous genes in the host genome (Wesley *et al.*, 2001). However, until now the RNA interference (RNAi) technology has been used in grapevines only for inducing virus resistance (Jardak-Jamoussi *et al.*, 2004) and silencing the berry polyphenol oxidase gene (M.R. Thomas, personal communication). Another HTP technology proposed for silencing genes in grapevines is virus-induced gene silencing (VIGS) that employs virus vectors carrying fragments from the exons of plant host genes that can be introduced by transformation or viral infection into the host and result in the suppression of endogenous gene expression (Baulcombe, 1999). Attempts to use VIGS are currently being investigated at the INRA at Colmar (O. Lemaire and P. Mestre, personal communication).

2. DEVELOPMENT OF TRANSGENIC GRAPES

2.1 Donor, Marker, and Reporter Genes

In grapevine genetic engineering, such as in other crop plants, three types of genes are commonly introduced into the plants; (i) selectable marker genes which are crucial for the development of plant transformation technology because they allow scientists to identify and isolate transformed cells from nontransformed cells and to monitor and select for the transformed progeny, (ii) visual marker genes or reporter genes, which enable direct observation of transformation events and result in a more precise and easier evaluation of various treatments and procedures, and (iii) genes of interest corresponding to the traits being modified.

2.1.1 Selectable marker genes

Until now, approximately 50 selectable marker genes have been used for transgenic plant research or crop development and evaluated for efficiency, safety, scientific applications, and commercialization (Miki and McHugh, 2004). Selectable marker genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or nonconditional on the presence of external substrates. The selectable marker genes currently used for grapevine are few and correspond to the first category as they promote the growth of transformed tissue and are conditional on the use of toxic agents, such as antibiotics or less frequently herbicides. All these genes have bacteria as the donor sources.

The neomycin phosphotransferase II (*nptII*) gene confers resistance to the antibiotic kanamycin or other aminoglycoside antibiotics such as neomycin and paramomycin and is the most widely used (see Tables 1 and 2). It is generally placed under the control of the promoter (pNOS) of the bacterial nopaline-synthase gene with the terminator (tNOS) of the same gene. In some cases, it has been used with the manopine synthase promoter (Agüero *et al.*, 2006), with the widely used constitutive 35S promoter from the cauliflower mosaic virus (Olah *et al.*, 2003; Wang *et al.*, 2005; Agüero *et al.*, 2006) or even with the Ubiquitin 11 promoter from *Arabidopsis thaliana* (Vidal *et al.*, 2003). The *nptII* gene has been experimentally fused with reporter genes, such as *uidA* and/or *gfp* (see Section 2.1.2) and placed under the control of a double enhanced

Table 1 Transgenic plants obtained with *Vitis* species and rootstocks

Cultivar	Selectable marker	Promoter	Gene product	References
<i>V. rupestris</i>	NPTII	NOS, 35S	GUS	Mullins <i>et al.</i> , 1990
110 Richter	NPTII, HPT	NOS, 35S	GCMV coat protein, GUS	Le Gall <i>et al.</i> , 1994
<i>V. rupestris</i>	NPTII	NOS, 35S	GUS	Martinelli and Mandolino, 1994
<i>V. rupestris</i> , 110 Richter	NPTII	NOS, 35S	GFLV coat protein, GUS	Krastanova <i>et al.</i> , 1995
41 B, SO4	NPTII	NOS, 35S	GFLV coat protein, GUS	Mauro <i>et al.</i> , 1995
Freedom, 101-14, Teleki 5C	NPTII	NOS, 35S	GNA lectine (homopteran insect resistance)	Viss and Driver, 1996
110 Richter	NPTII	NOS, 35S	GUS	Soloki <i>et al.</i> , 1998
Georgikon 28	NPTII	NOS, 35S	GUS	Mozsar <i>et al.</i> , 1998
<i>V. riparia</i> , 3309 Couderc, 101-14, 110 Richter	NPTII	NOS, 35S	Translatable, antisense, untranslatable coat protein (GFLV, GLRaV), GUS	Krastanova <i>et al.</i> , 2000
41 B	NPTII	NOS, 35S	GFLV coat protein + replicase + proteinase	Mauro <i>et al.</i> , 2000
110 Richter	NPTII	NOS, 35S	GFLV replicase, GUS	Barbier <i>et al.</i> , 2000
<i>V. rupestris</i>	NPTII	NOS, 35S	ArMV coat protein	Spielmann <i>et al.</i> , 2000
<i>V. rupestris</i>	NPTII	NOS, 35S	GVA and GVB movement protein (sense and antisense)	Martinelli <i>et al.</i> , 2000
110 Richter	NPTII	NOS, 35S	GFLV coat protein	Golles <i>et al.</i> , 2000
<i>V. rupestris</i> , 110 Richter	NPTII	NOS, 35S	GFLV coat protein, Antifreeze proteins, GUS	Tsvetkov <i>et al.</i> , 2000
41 B	NPTII	PR10	Vst I gene (resveratrol production)	Gutoranov <i>et al.</i> , 2001
<i>V. rupestris</i>	NPTII	NOS, 35S	GVA movement protein (sense and antisense)	Coutos-Thevenot <i>et al.</i> , 2001
110 Richter	NPTII	NOS, 35S	Eutypine-reducing enzyme	Martinelli <i>et al.</i> , 2002
<i>V. rupestris</i> , 110 Richter	NPTII	NOS, 35S	GUS	Legrand <i>et al.</i> , 2003
110 Richter	NPTII	NOS, 35S	Truncated virE2	Olah <i>et al.</i> , 2003
110 Richter	NPTII	NOS, 35S	Ferritin (ironbinding protein)	Holden <i>et al.</i> , 2003
41 B	NPTII	NOS, 35S	GFLV coat protein and movement protein	Olah <i>et al.</i> , 2004
				Valat <i>et al.</i> , 2006

Table 2 Transgenic plants obtained from *V. vinifera* cultivars and hybrids

Cultivar	Selectable marker	Promoter	Gene product	References
Koshusanjaku	NPTII	NOS, 35S	GUS	Matsuta <i>et al.</i> , 1993
Koshusanjaku	NPTII	NOS, 35S	GUS	Nakano <i>et al.</i> , 1994
Table grape cultivars	HPT	35S	Yeast invertase	Perl <i>et al.</i> , 1994
Chardonnay	NPTII	NOS, 35S	GFLV coat protein, GUS	Mauro <i>et al.</i> , 1995
Thompson seedless (synonym Sultana)	NPTII	NOS, 35S	Shiva-1, TomSRV coat protein	Scorza <i>et al.</i> , 1996
Chancellor (<i>hybrid</i>)	NPTII	35S	GUS	Kikkert <i>et al.</i> , 1996
Superior seedless	HPT, Bar	35S		Perl <i>et al.</i> , 1996a, b
Cabernet-Franc	NPTII	NOS, 35S	Fe-superoxide dismutase (freezing tolerance)	Rojas <i>et al.</i> , 1997
Sultana	NPTII, HPT	NOS, 35S	GUS	Franks <i>et al.</i> , 1998
Chardonnay	HPT	35S	GUS	Thomas <i>et al.</i> , 2000
Sultana	NPTII, HPT	NOS, 35S	Polyphenol-oxidase (antisense)	Thomas <i>et al.</i> , 2000
Merlot	NPTII	35S	Chitinase	Kikkert <i>et al.</i> , 2000
Neo Muscat	NPTII	NOS, 35S	Rice chitinase (RCC2)	Yamamoto <i>et al.</i> , 2000
Riesling, Dornfelder	NPTII	NOS, 35S	Glucanase and chitinase (fungal disease resistance)	Harst <i>et al.</i> , 2000
Superior seedless, Red Globe	NPTII, HPT	NOS, 35S	Chitinase, glucanase, RIP, Barnase	Perl <i>et al.</i> , 2000
Superior seedless	NPTII	NOS, 35S	GVA and GVB movement protein (sense and antisense)	Martinelli <i>et al.</i> , 2000
Koshusanjaku	NPTII, HPT	NOS, 35S	GUS	Hoshino <i>et al.</i> , 2000
Podarok Magaracha	NPTII, Bar	NOS, 35S	Basta (herbicide) resistance	Levenko and Rubstova, 2000
Russalka seedling	NPTII	NOS, 35S	GFLV coat protein, ArMV coat protein, GVA and GVB coat protein	Golles <i>et al.</i> , 2000
Russalka seedlings	NPTII	NOS, 35S	GFLV coat protein, antifreeze proteins, GUS	Tsvetkov <i>et al.</i> , 2000; Gutoranov <i>et al.</i> , 2001
Cabernet-Sauvignon, Chardonnay, Syrah, Sauvignon blanc, Chenin, Riesling, Muscat Gordo blanco	NPTII	NOS, 35S	GUS, GFP	Iocco <i>et al.</i> , 2001
Thompson seedless	NPTII	35S, CsVMV	GFP	Li <i>et al.</i> , 2001
Thompson seedless, Silcora	NPTII	35S	DefH-iaaM (parthenocarpic development)	Mezetti <i>et al.</i> , 2002b
Superior seedless	NPTII	Dfr	GUS	Gollop <i>et al.</i> , 2002
Chardonnay, Syrah, Portan	NPTII	35S	GFP	Torregrosa <i>et al.</i> , 2002a
Fredonia (<i>hybrid</i>)	NPTII	NOS, Agl5	GUS	Motioike <i>et al.</i> , 2002
Pusa seedless, Perlette, Beauty seedless	NPTII	NOS, 35S	GUS	Das <i>et al.</i> , 2002
Chardonnay	NPTII	35S, Ubq3, Ubq11	Natural and synthetic magainins (antimicrobial peptide)	Vidal <i>et al.</i> , 2003
Portan	NPTII	SIRK, 35S	SOR K+ channel, GUS	Pratelli <i>et al.</i> , 2003
Thompson seedless, Chardonnay	NPTII	35S	Pear PGIP (tolerance to Pierce'disease and Botrytis)	Agüero <i>et al.</i> , 2006
Nebbiolo, Lumassina, Blafränkisch	NPTII	NOS, 35S	GFLV coat protein full length, sense and antisense	Gambino <i>et al.</i> , 2005
Seyval (<i>hybrid</i>)	NPTII	NOS, 35S	Chitinase and RIP	Bornhoff <i>et al.</i> , 2005
Russalka	NPTII	35S	GFLV coat protein (translatable, untranslatable, and truncated forms)	Maghuly <i>et al.</i> , 2006

(Continued)

Table 2 Transgenic plants obtained from *V. vinifera* cultivars and hybrids (*Continued*)

Cultivar	Selectable marker	Promoter	Gene product	References
Red Globe	NPTII	35S	GUS	Wang <i>et al.</i> , 2005
Chardonnay,	NPTII	MAS, 35S	Pear PGIP, GUS, GFP	Agüero <i>et al.</i> , 2006
Thompson seedless				
Chardonnay	NPTII	Ubq3	Synthetic magainin (MS199) (in minimal cassette)	Vidal <i>et al.</i> , 2006a
Chardonnay	NPTII	Ubq3	Natural and synthetic magainins (crown gall tolerance)	Vidal <i>et al.</i> , 2006b
Thompson seedless	NPTII	35S,	GFP	Li <i>et al.</i> , 2006
		CsVMV		
Portan	HPT	35S	Alcohol dehydrogenase, sense and antisense	Tesnière <i>et al.</i> , 2006

35S promoter (Kikkert *et al.*, 1996; Li *et al.*, 2001; Vidal *et al.*, 2003). Two other selectable marker genes have been used with success in grapevine, the hygromycin phosphotransferase (*hpt*) gene conferring resistance to the antibiotic hygromycin and the phosphinothricin aminotransferase (*pat*) gene conferring resistance to the Basta[®] herbicide. They were generally placed under the control of the 35S promoter with a tNOS terminator (Perl *et al.*, 2004; Bouquet *et al.*, 2006). Procedures and efficiency of selection are different according to the type of marker genes used (see Section 2.3). Though the *nptII* gene product was determined to be safe in the United States and its use permitted in genetically engineered food (Redenbaugh *et al.*, 1994), the use of antibiotic marker genes in potential commercial products has become unacceptable in Europe because of public distrust. Thus, alternative methods of selection for grapevine transgenic breeding are currently under investigation (see Section 3.3).

2.1.2 Visual marker or reporter genes

The bacterial enzyme β -glucuronidase (GUS), which is coded by the *Escherichia coli* gene *uidA* (Jefferson *et al.*, 1987) has been the first and the more extensively used visual marker used in genetically modified (GM) plants in general and in grapevines in particular because of the enzyme stability and versatility allowing histochemical localization and fluorometric quantification at low cost. The major drawback is that the assays are destructive to the plant cells. A feature of GUS is its capacity to generate fusions with other

proteins that extended its usefulness for gene-tagging experiments. In grapevine genomics, it has been used for functional analysis of the resveratrol synthase gene promoter (Schubert *et al.*, 1997) and the dihydroflavonol reductase gene promoter (Gollop *et al.*, 2002). Though the low level of GUS protein from GM plants is not a concern with regard to toxicity or allergenicity (Gilissen *et al.*, 1998), its use in commercial transgenic cultivars is prohibited in Europe.

The luciferase enzyme (LUC) that is coded by the firefly *Photinus pyralis* gene *luc* and uses luciferine as a short-life substrate is a visual marker that offers several advantages including the capability of monitoring gene expression patterns nondestructively in real time with great sensitivity (Ow *et al.*, 1986). However, probably because of substrate cost and the need of specific detection equipment, LUC has been used infrequently in grapevine for promoter functional analysis via transient transformation (Torregrosa *et al.*, 2002b; Verriès *et al.*, 2004). The same group stably introduced *VvAdh2* or 35S promoter fusions to *luc* into grapevine plants and observed strong *luc* expression (P. Chatelet and L. Torregrosa, personal communication).

The green fluorescent protein (GFP) from jellyfish (*Aequora victoria*) has become a powerful reporter as it allows visualization of gene expression in living tissue in real time without invasive procedures such as the application and penetration of cells with substrate and products that may diffuse within or among cells. Both advantages provide a significant improvement over *uidA* and *luc* reporter genes. It is now used by many groups and has a low cost (Haseloff *et al.*,

1997; Harper *et al.*, 1999; Stewart, 2001; Hraska *et al.*, 2006). In grapevine, GFP has been used to characterize the activity of three constitutive promoters using a bifunctional fusion marker containing the *gfp* and the *nptII* genes (Li *et al.*, 2001, 2006) and stability of expression (Iocco *et al.*, 2001). It was also used to analyze in transgenic tobacco plants, the promoter of the grapevine *mrip1* gene coding for the Merlot ripening-induced protein 1 (Burger *et al.*, 2006) and currently the promoters of several transcription factors involved in berry development (Fernandez *et al.*, 2007). It is also a very useful tag for monitoring intracellular localization and transport of fusion proteins. GFP is particularly useful to visualize putative transformed events in embryogenic tissues (Torregrosa *et al.*, 2002a; Li *et al.*, 2006) because reading is not hampered by chlorophyll fluorescence and GFP positive cells can be selected and extracted from the remaining tissues without damage. Using *in situ* GFP-assisted selection with a selection agent (Li *et al.*, 2006), it was possible to moderate the level of the selection agent or the length of selection period and reduce the number of false positive or chimeric structures (see below).

2.1.3 Genes of interest

Since the first reports of transformation based on reporter genes and embryogenic cultures, the production of transgenic vines has become routine in private and publically run laboratories, worldwide. Some advanced work continues on the improvement of methods and procedures (Wang *et al.*, 2005; Li *et al.*, 2006), and applications in grapevine genomics are currently developing in some laboratories (Tesnière *et al.*, 2006) but much effort has shifted to the transfer of potentially beneficial genes into rootstock and scion cultivars. Among the latter, fungal or bacterial disease resistance has been of paramount interest, while in rootstocks, virus resistance has been the focus. Although the major objective of many groups is to obtain GM grapevine cultivars adapted to a sustainable and environmental-friendly production system, some projects have also focused on product quality, especially in table grapes and raisins.

Until now, few sequences of *V. vinifera* genes have been introduced into grapevine. They include

the coding sequence of the *Vst1* (*Vitis* stilbene synthase 1) gene (Wiese *et al.*, 1994) that was fused to a fungal-inducible promoter belonging to a class 10 PR (pathogenesis related) gene expressed in alfalfa and introduced in the rootstock 41 B to improve tolerance to *Botrytis cinerea* (Coutos-Thevenot *et al.*, 2001). The coding sequence of the Shakerlike outward rectifying potassium channel SOR was cloned from a complementary DNA (cDNA) library of *V. vinifera* cv. Pinot noir and a dominant negative mutant was introduced into *V. vinifera* cv. Portan for a reverse genetics study (Pratelli *et al.*, 2003). At the same time, the promoter sequence (3 kbp, kilo base pair) of the inward rectifying potassium channel SIRC was fused to *uidA*, introduced into Portan, and showed strong GUS levels in guard cells (Pratelli *et al.*, 2002). The grapevine *VvAdh2* (alcohol dehydrogenase 2) coding sequence (1.3 kbp) was cloned from a *V. vinifera* cv. Danuta genomic DNA library and introduced in the sense and antisense orientation into Portan (Tesnière *et al.*, 2006). The promoter sequence (2.2 kbp) of the *dfr* (dihydroflavonol reductase) gene was isolated from a *V. vinifera* cv. Red Globe genomic DNA library. The 2.2 kbp sequence and deletion fragments of 757 and 265 bp were fused with *uidA* and introduced in *V. vinifera* cv. Superior seedless (Gollop *et al.*, 2002). Recently, some resistance gene analogs (RGAs) were cloned from a cDNA library of a *V. vinifera* × *Muscadinia rotundifolia* hybrid bearing the *Run1* gene that provides complete resistance to the powdery mildew (Barker *et al.*, 2006) and are currently introduced with their own promoters into *V. vinifera* cvs. Portan and Chardonnay (A. Bouquet, personal communication).

However, most genes of interest that were inserted into grapevine come from other plants or other living organisms including animals, fungus, bacteria, and virus. With regard to genes coming from plants, monocots or dicots, a fragment (1.1 kbp) of the coding sequence of the rice chitinase gene (*RCC2*) classified as class I chitinase (Nishizawa and Hibi, 1991) was introduced into *V. vinifera* cv. Neo Muscat and enhanced resistance to powdery mildew and anthracnose (Yamamoto *et al.*, 2000). Genes encoding a class II chitinase, a class II 1,3 glucanase, and a ribosome inactivating protein (RIP) were cloned from barley (*Hordeum vulgare*) and proven to enhance fungal resistance of

tobacco transgenic plants (Jach *et al.*, 1995). They were introduced into *V. vinifera* cvs. Dornfelder and Riesling (Harst *et al.*, 2000) and in the *Vitis* hybrid "Seyval" (Bornhoff *et al.*, 2005). The full-length coding sequence (975 bp) of the *Vr-ERE* gene (eutypane-reducing enzyme) was cloned from a *V. radiata* cDNA library (Guillen *et al.*, 1998) and introduced into the rootstock 110 R (Legrand *et al.*, 2003) and *V. vinifera* cv. Portan (J.-P. Roustan and A. Bouquet, personal communication) in the hope of conferring resistance or tolerance to the Eutypa dieback disease. The use of the *Vr-ERE* gene as an alternative selectable marker using structural analogs of the toxin Eutypin as selection agents are currently being investigated in grapevine (A. Bouquet, M.C. Cutanda, and L. Torregrosa, personal communication), apple, and pear (E. Chevreau, personal communication). The *GNA* gene from the snowdrop *Galanthus nivalis*, encodes a lectin protein that may repel sap-sucking insects such as phylloxera. Before being used in *V. vinifera*, it was experimentally inserted into the rootstocks Freedom, 101-14 and Teleki (Viss and Driver, 1996) but the results of the resistance tests have not yet been published. A gene encoding the iron-binding protein ferritin was cloned from *Medicago sativa* (Deak *et al.*, 1999) and introduced into the rootstock cultivar 110 R (Olah *et al.*, 2004). The coding sequence (1 kbp) of a polygalacturonase-inhibiting protein (PGIP) gene cloned from pear (Stotz *et al.*, 1993) was introduced into *V. vinifera* cvs. "Thompson seedless" and "Chardonnay" and reported to confer some degree of resistance to *Botrytis* and PD (Agüero *et al.*, 2005).

Plant promoter sequences used for grapevine transformation studies include a pathogen-inducible PR 10 promoter from alfalfa (Coutos-Thenot *et al.*, 2001). Perl *et al.* (1994) constructed a synthetic gibberellin (GA)-inducible promoter using a common motif appearing in promoter regions of different GA-regulated plant genes, which were then placed downstream to a truncated minimal 35S promoter. The ovule-specific regulatory regions from the *DefH9* gene of *Antirrhinum majus* was shown to drive the expression of an auxin-synthesis (*iaaM*) bacterial gene conferring parthenocarpic fruit development in tomato and strawberry transgenic plants (Ficcadenti *et al.*, 1999; Mezetti *et al.*, 2002a). This synthetic gene *DefH9-iaaM* was also inserted into table grape cultivars (Mezetti *et al.*, 2002b). The

ubiquitin 3 promoter coming from *A. thaliana* and a signal peptide coming from the pea vicilin protein were used to drive the expression of magainin genes in grapes (Vidal *et al.*, 2003). The flower-specific and developing fruit-specific promoter *Ag15* from *A. thaliana* proved to be efficient in grapevine when fused with the *uidA* reporter gene (Motioike *et al.*, 2002). In contrast the ACT2 promoter from *A. thaliana*, previously shown to be a strong constitutive promoter in this and other species, failed to promote significant expression of a *gfp* reporter gene in grapevine (Li *et al.*, 2001).

Few genes coming from animals, vertebrates and invertebrates, have been used for grapevine genetic engineering. The gene *Shiva-1* encoding the antibacterial immune protein cecropin was cloned from the moth *Hyalophora cecropia* (Xanthopoulos *et al.*, 1988) and introduced into *V. vinifera* cv. Thompson seedless (Scorza *et al.*, 1996) in an attempt to improve tolerance to bacterial diseases. Transformed plants were obtained, but the results of their assessment were not published. More recently, the sequence (72 bp) of the *mag 2* gene encoding a natural magainin was isolated from the skin of the African clawed frog *Xenopus laevis*, fused with a 48 bp signal peptide from the pea vicilin protein (Pang *et al.*, 1992) and placed under the control of the *A. thaliana* ubiquitin 3 promoter. Natural magainins or their synthetic derivatives are small peptides that have broad-spectrum antimicrobial activity, inhibiting the growth *in vitro* of bacteria and fungi including major grapevine pests such as *Agrobacterium tumefaciens* (Li and Gray, 2003). The *mag 2* gene, the synthetic magainin *MS199* gene, and a peptidyl-glycine-leucine gene also isolated from *X. laevis* were introduced in *V. vinifera* cv. Chardonnay (Vidal *et al.*, 2003). Expression of the *mag 2* and *MS199* genes in transgenic lines induced significant diminution of crown gall symptoms. However, only *mag 2* induced measurable symptom reduction but not strong resistance to powdery mildew (Vidal *et al.*, 2006a).

Two genes coming from fungi have been used for grapevine genetic engineering. The gene *suc2* from the yeast *Saccharomyces cerevisiae* coding for an invertase was inserted into table grapes to investigate if an alien invertase expressed in developing grape berries could alter the sucrose level and the accumulation of glucose and fructose, improving the fruit sweetness of early ripening

cultivars (Perl *et al.*, 1994). The gene *ThEn42* encoding an endochitinase from the biocontrol fungus *Trichoderma harzianum* (Hayes *et al.*, 1994) was inserted by biolistics into *V. vinifera* cvs. Merlot and Chardonnay (Kikkert *et al.*, 2000). For Chardonnay, the results of greenhouse and field tests were indicative of a relationship between chitinase expression and reduced incidence and severity of *Botrytis* bunch rot. However, the level of powdery mildew resistance observed did not correlate with chitinase expression and was not substantial enough to warrant further trials (Reisch *et al.*, 2003).

Few bacterial genes have been introduced into grapevine. Truncated *virE2* genes isolated from *A. tumefaciens* strains C58 and A6 and from *Agrobacterium vitis* strain CG450 were inserted into the rootstock 110 R. Transgenic lines with the *virE2* gene from C58 expressed resistance to crown gall, but not transgenic lines with the *virE2* gene from A6 and CG450 (Holden *et al.*, 2003). The *barnase* gene that codes for a ribonuclease of *Bacillus amyloliquefaciens* was introduced into *V. vinifera* cv. Red Globe under the control of a seed-coat-specific promoter and it was claimed to cause ablation of developing seeds resulting in berries having soft seed rudiments but not complete seedlessness (Perl *et al.*, 2003). The *iaaM* gene from *Pseudomonas savastanoi* was inserted into table grapes with an ovule-specific regulator (Mezetti *et al.*, 2002b). The gene encoding the streptavidin protein from *Streptomyces avidinii*, placed under the control of the embryo-specific promoter Tob-RB7, controls the availability of biotin and induced depletion of biotin during early stages of seed development, leading to almost total seed degeneration in transgenic table grapes (Perl *et al.*, 2003).

Due to the problems caused by viruses, most of the donor gene sources reported in grapevine transgenic studies have been of virus origin. Since the first report of resistance to the tobacco mosaic virus (TMV) using transgenic tobacco plants (Powell-Abel *et al.*, 1986), similar results have been obtained for a broad range of viruses in different plant species. Virus-resistant transgenic plants acquire the so called pathogen-derived resistance (Sanford and Johnston, 1985) through the expression of a viral sequence, such as a coat protein (CP), replicase or movement protein genes (Baulcombe, 1996). Viral resistance was obtained

by introducing the grape chrome mosaic virus (GCMV) CP gene into the rootstock 110 R (Le Gall *et al.*, 1994). This success was concomitant with the transformation of the rootstocks 110 R and *V. rupestris* cv. du Lot (Krastanova *et al.*, 1995) with the grape fanleaf virus (GFLV) CP gene. The same plasmid p1660, bearing CP, *uidA*, and *nptII* genes, was used to obtain transgenic plants from the rootstocks 41 B, SO4, and *V. vinifera* cv. Chardonnay (Mauro *et al.*, 1995). Since then, the GFLV CP gene in the translatable or untranslatable form has been inserted with success into a *V. vinifera* cv. Russalka seedling (Golles *et al.*, 2000) and in several other rootstock cultivars such as *V. riparia* cv. Gloire, 3309C and 101-14 (Krastanova *et al.*, 2000). Preliminary screening by graft inoculation *in vitro* and in the greenhouse suggested that some lines were resistant (Krastanova *et al.*, 2000). The GFLV CP gene was inserted equally into *V. vinifera* cultivars in sense and antisense orientation (Gambino *et al.*, 2005) or in translatable, untranslatable, and truncated forms (Maghuly *et al.*, 2006). But CP was not the only one gene from GFLV to have been used in grapevine. The GFLV replicase, proteinase, and movement protein genes were inserted in the rootstocks 110 R and 41 B (Barbier *et al.*, 2000; Mauro *et al.*, 2000; Valat *et al.*, 2006). With regard to other nepoviruses, the tomato ring spot virus (TomRSV) CP gene was inserted into *V. vinifera* cv. Thompson seedless (Scorza *et al.*, 1996). The introduction of the arabis mosaic virus (ArMV) CP gene into grapevine was also attempted (Golles *et al.*, 2000; Spielmann *et al.*, 2000) but transgenic *V. rupestris* plants expressing ArMV CP gene did not accumulate the CP at a detectable level and were not protected against ArMV infection under green-grafting conditions, whereas accumulation of the CP and protection was observed in *Nicotiana benthamiana* transgenic plants (Spielmann *et al.*, 2000). With regard to other grapevine viruses such as closteroviruses and vitiviruses, the grape leafroll-associated viruses 2 and 3 (GLRaV-2 and -3) CP gene was inserted in sense and antisense orientation into several rootstocks, such as *V. riparia* cv. Gloire, 3309 C, 101-14, and 110 R (Krastanova *et al.*, 2000) and the genes coding for the CP of grapevine virus A (GVA) and B (GVB) associated with different syndromes of the rugose wood complex, were inserted into a *V. vinifera* cv. Russalka seedling (Golles *et al.*, 2000). The gene

coding for the movement protein of GVA was inserted in sense and antisense orientation into the table grape cultivar Superior seedless and the rootstock *V. rupestris* cv. du Lot (Martinelli *et al.*, 2000, 2002).

The 35S promoter from cauliflower mosaic virus has been extensively used in grapevine transformation studies. Promoters derived from the subterranean clover stunt virus have been tested and found to be similar to the califlower mosaic virus (CaMV) 35S and NOS promoters (Thomas *et al.*, 2000). More recently, a bidirectional promoter with duplicated enhancer derived from the cassava vein mosaic virus (CsVMV) was found to have higher activity than the corresponding promoter from CaMV in transgenic grapevines (Li *et al.*, 2001).

2.2 Methods of Transformation Employed

The first experiments of *in vitro* grapevine inoculation by *Agrobacterium* were reported by Hemstad and Reisch (1985). By inoculating *Vitis* interspecific hybrids shoots with *A. tumefaciens* (C58C1 pTi37) or *Agrobacterium rhizogenes* strains (A4 and K37), these authors could induce the formation of galls and putative transformed roots, but the insertion of transfer DNA (T-DNA) in the tissues and organs recovered was not investigated. *Agrobacterium*-mediated transformation is the most widely used system to introduce transgene in grapevine.

2.2.1 *Agrobacterium*-mediated transformation

2.2.1.1 *Transgenic roots obtained with A. rhizogenes*

Gribaudo and Schubert (1990) were the first to report the production of properly characterized grapevine transformed roots. Using the wild type 8196 *A. rhizogenes* strain, they obtained roots of several *V. vinifera* cultivars exhibiting the right hairy root phenotype and producing opines. The same year, Guellec *et al.* (1990) were the first to obtain grapevine transgenic organs coding for engineered transgenes. Inoculating plantlets of *V. vinifera* cv. Grenache with C58C1 pRi 15834

or both pRi 15836 + pGV1850::1103, a co-integrate vector bearing the kanamycin resistance gene, they established hairy roots producing neomycin phosphotransferase protein (NPTII) but no plants were regenerated. Surprisingly, Nakano *et al.* (1994) introduced the *nptII* and *uidA* genes into embryogenic cultures of *V. vinifera* cv. Koshusanjaku with an *A. rhizogenes* strain and regenerated plants expressing these genes but exhibiting altered phenotypes, likely due to the presence of the oncogenes from the nondisarmed pRi. Similarly, Shimizu *et al.* (1994) reported in an abstract the successful adventitious shoot regeneration from Ri plasmid-induced hairy roots and the development of transgenic grapevines but no additional publication resulted. Torregrosa and Bouquet (1997) used *A. rhizogenes* and *A. tumefaciens* co-transformation to obtain grapevine hairy roots producing the GCMV CP but the regeneration of plants was not achieved. Despite problems with plant regeneration, this system is very useful for producing material for root pathogen studies or to investigate the effect of transgene expression on root metabolism and development (Lupo *et al.*, 1994; Torregrosa and Bouquet, 1997; Franks *et al.*, 2006). Moreover, the possibility to use the transgenic hairy roots as rootstocks (Torregrosa and Bouquet, 1997) provides an opportunity to study the interaction between a transgenic root system and a wild-type scion.

2.2.1.2 *Transgenic plants obtained with A. tumefaciens*

Though Baribault *et al.* (1989, 1990) obtained transformed cells of *V. vinifera* cv. Cabernet Sauvignon expressing *nptII* and shoots expressing GUS, the successful regeneration of transgenic grapevine plants was first achieved by Mullins *et al.* (1990), using *A. tumefaciens* LBA4404 bearing pCGN7320 encoding *uidA* and *nptII* genes. These authors observed GUS positive adventitious buds of *V. vinifera* cv. Cabernet Sauvignon and Chardonnay, but they could regenerate transgenic shoots only from hypocotyls from somatic embryos of *V. rupestris*. Since then a number of different genotypes, including *Vitis* species and hybrids (rootstocks) and several commercially important *V. vinifera* cultivars have been transformed (for review, see Kikkert *et al.*,

2001; Perl *et al.*, 2004). Currently, the most widely used system consists of the co-culture of disarmed *Agrobacterium* strains containing binary vectors with grapevine somatic embryogenic callus or cell suspensions (Perl *et al.*, 2004; Bouquet *et al.*, 2006). Embryogenesis has been induced from a range of organs: zygotic embryos, leaves, ovaries, and stamens (Martinelli and Gribaudo, 2001; Torregrosa, 1995), with stamens being the most reliable organ.

Using embryogenic competent explants, the system includes several steps:

1. Initiation and propagation of embryogenic tissues on solid or in liquid cultures.
2. Pretreatment of tissues to improve susceptibility to *Agrobacterium* (optional, see below).
3. Co-culture of the bacteria and grapevine tissues.
4. Selection and multiplication of transformed cells.
5. Differentiation of transformed embryolike structures.
6. Conversion of putatively transformed somatic embryos into plantlets.

Tissues produced by explants cultured on semi-solid embryogenic induction media are generally heterogeneous and contain friable structures with various rates of growth mixed with differentiating somatic embryos. After several cycles of subculture and selection on semi-solid media, the yellowish-friable tissues are preferably selected because this type of callus, formed of proembryogenic clusters, is very stable and allows easy long-term maintenance either on semi-solid or liquid media (Figure 1a). However for some cultivars, this type of callus is not very stable on semi-solid media and another type of tissue dominates composed of embryogenic cell clusters (generally in the core in contact with the medium) surrounded by embryolike structures and embryos at different stage of development (from globular to immature torpedo). Some authors have named the first type of callus, type I and the second one, type II. It is easy to shift from type I to type II by changing plant regulator balance, reducing the auxin concentration or replacing a strong auxin 2,4-dichlorophenoxyacetic acid (2,4-D) by a weaker one *b*-naphthoxyacetic acid (NOA) or 1-naphthaleneacetic acid (NAA). It is not clear which kind of callus is more appropriate for

Agrobacterium transformation as the reports are contradictory on this point (Mauro *et al.*, 1995; Perl *et al.*, 1996a; Scorza *et al.*, 1996; Franks *et al.*, 1998; Torregrosa *et al.*, 2002a), probably because of the diversity of the culture conditions and selection strategy (Kikkert *et al.*, 2001). Because type I is more convenient for propagating the tissue but not necessary the best for co-culture with *Agrobacterium*, some preliminary experiments may be required to clarify this point.

Co-cultivating embryogenic callus or suspension with *Agrobacterium* is considered as a general method because it has been a successful method for many genotypes (Iocco *et al.*, 2001; Martinelli and Mandolino, 2001; Perl and Eshdat, 1998; Perl *et al.*, 2004) but efficiency and reliability vary considerably from one genotype to another even between cultivars from the same species. Some steps need modification for some highly recalcitrant *V. vinifera* cultivars, like the seedless grape Danuta (Torregrosa *et al.*, 2002a), the wine grape Pinot noir (Iocco *et al.*, 2001) or *Vitis* hybrid cultivars, like Niagara (Motioike *et al.*, 2002).

As an alternative to the use of embryogenic cultures, several groups have investigated adventitious organogenesis. The system is based on the use of explants that are able to produce adventitious budlike structures (Torregrosa, 1995), i.e. leaves, petioles, stem fragments or somatic embryo fragments. This regeneration process was developed for several grapevine including *Vitis* species (Tang and Mullins, 1990), *V. vinifera* (Colby *et al.*, 1991b), and intergeneric hybrids like *Vitis* × *Muscadinia* (Torregrosa and Bouquet, 1996). Organogenesis competence has to be induced at the time or soon after contact with *Agrobacterium*. Despite much effort, experiments did not succeed in demonstrating the utility of this method for obtaining transgenic plants (Colby *et al.*, 1991a). Recently, Mezetti *et al.* (2002b) reported the regeneration of transgenic plants of table grape cultivars using a system based on intense axillary and adventitious budding induced by high benzyladenine (BA) levels. The key to the procedure was the production of large amounts of meristematic tissue that implies tedious *in vitro* apex manipulations. However, it is difficult to determine the utility of this system for other genotypes as the original work focused on two *V. vinifera* cultivars and there has been no additional report.

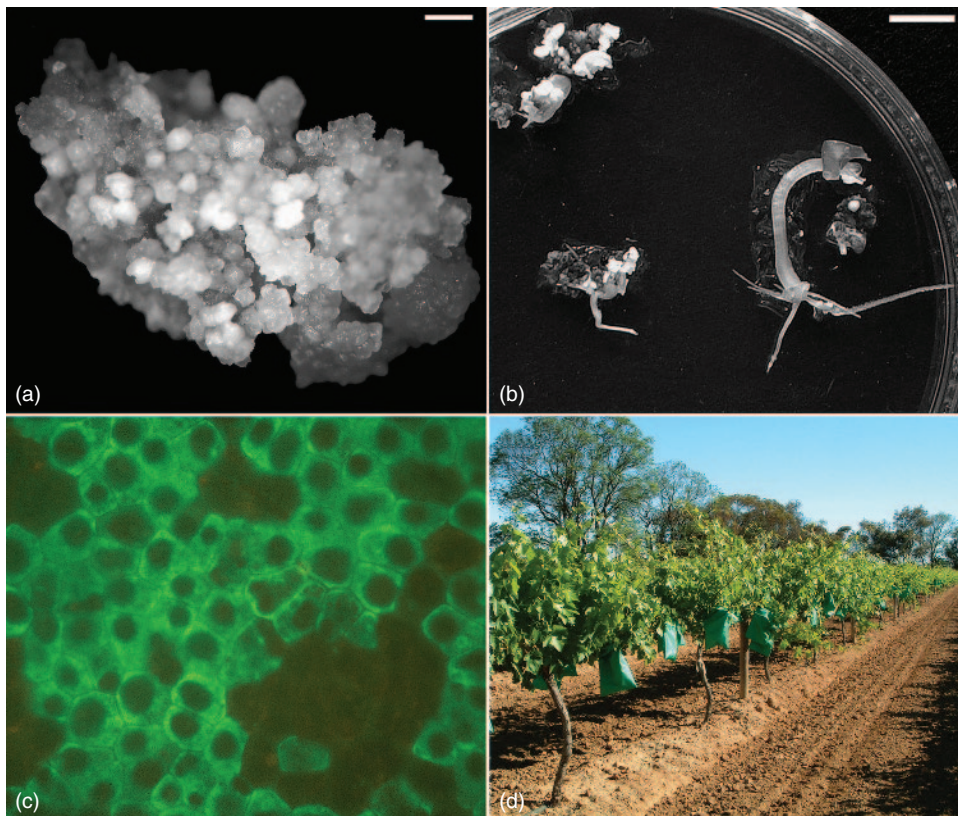


Figure 1 Grapevine transformation. (a) Friable embryogenic callus grown on semi-solid medium suitable for *Agrobacterium*-mediated transformation experiments (bar = 100 μ m). (b) Germinating transgenic somatic embryos after transformation (bar = 1 cm). (c) Variegated GFP pattern in the skin cells of postveraison berries. Expression of the *gfp* gene is controlled by the UFGT promoter. (d) A field trial of transgenic grapevines in Australia. Inflorescences are bagged to prevent pollen escape

2.2.1.3 *Agrobacterium* strains

There are three important points that need to be considered when using *Agrobacterium* to mediate transformation; one is in relation to the efficiency of the bacteria to transfer the T-DNA to the host, the second relates to the response of the plant tissue to co-culture with the bacteria and the third is related to the selection procedures.

The *Agrobacterium* strain used in transformation experiments can play a significant role in *Agrobacterium*-host tissue interaction. The chromosomal background is involved in some important features in the attachment between bacteria and the plant cell (Lorence and Verpoorte, 2004). Virulence genes of the pTi or pRi are also important as they support many aspects of the transformation process, i.e., bacteria-host

signaling, transaction on binary T-DNA borders, and supplying the protein machinery of T-DNA transport (Tzfira and Citovsky, 2003). Moreover, during the interaction with *Agrobacterium*, several changes occur in host gene expression leading to various molecular changes, i.e., the production of PR proteins, which may affect tissue development (Ditt *et al.*, 2006).

Several disarmed *A. tumefaciens* biovar 1 strains have shown some efficiency in transferring T-DNA into grapevine; LBA4404 (Hoekema *et al.*, 1983), GV2260 (Deblaere *et al.*, 1985), EHA101 (Hood *et al.*, 1986) and its derivative, EHA105. Grapevine is a natural host for *A. tumefaciens*, but *A. vitis* (previously named *A. tumefaciens* biovar 3) is the most frequently isolated strain from naturally infected grapevines (Burr and Katz, 1983). Compared to *A. tumefaciens*, *A. vitis*

shows unique properties in relation to T-DNA content (*vir* sequences), enzymatic properties, and epidemiology (Schrammeijer *et al.*, 1998; Burr and Otten, 1999). The question of the efficiency of the natural host *A. vitis* is still unresolved (Kikkert *et al.*, 2001) but because of the lack of disarmed strains, little attention was initially paid to the use of this species.

From fragmented shoot apices inoculated with Ag162 (*A. vitis*), Baribault *et al.* (1990) could recover putative transformed buds of cv. Cabernet Sauvignon. Süle *et al.* (1994), studying the effect of *A. vitis* on various *Vitis* genotypes found that *V. vinifera* gives the best response in terms of galling, *vir* gene induction, and GUS activity. Stover *et al.* (1996), who studied the response of several *Vitis* genotypes to CG49 containing p35SGUSINT, found Cabernet Sauvignon to be susceptible to *A. vitis*. Using the *gfp* gene (Haseloff *et al.*, 1997) as a reporter, Torregrosa *et al.* (2002a) compared the initial transformation efficiency of *A. tumefaciens* LBA4404 (Ach5 pTiAch5) and EHA105 (C58C1 pTiBo542), *A. rhizogenes* A4 (A4 pRiA4) and *A. vitis* K252, an octopine limited host range (LHR) Ag57 isolate (Panagopoulos and Psallidas, 1973). K252/*gfp* proved able to transform embryogenic tissues of all genotypes tested and GFP positive embryolike structures were recovered but at a much lower rate than with other strains. From all these studies, *A. vitis* appears competent to transfer the T-DNA from a binary vector to different types of grapevine tissues, including embryogenic cultures, but the efficiency is rather low compared to standard *A. tumefaciens* or *A. rhizogenes* strains.

LBA4404, probably the most widely used vector for grapevine transformation experiments during the 1990s is an efficient vector for transforming *V. vinifera* cultivars (Harst *et al.*, 2000; Yamamoto *et al.*, 2000) or *Vitis* hybrids (Mullins *et al.*, 1990; Martinelli and Mandolino, 1994; Motioike *et al.*, 2002). EHA105 carrying a disarmed supervirulent pTiBo542, which was used in later grapevine studies was shown to be a very efficient and versatile vector for transformation (Scorza *et al.*, 1996; Franks *et al.*, 1998; Perl *et al.*, 2004; Wang *et al.*, 2005; Bouquet *et al.*, 2006). The high transformation efficiency of EHA strains or *Agrobacterium* strains carrying A281-derived plasmids was also observed for other woody plants (Gill *et al.*, 2004).

2.2.1.4 Browning of the plant tissue

During the co-culture or soon after, grapevine tissues subjected to *Agrobacterium* become partially dark, with some cultivars being more susceptible than others (L. Torregrosa and M. Thomas, personal communication). The browning is caused by the preparation and the manipulation of the tissues during co-culture and also by the response to the bacteria. Several strategies have been used to reduce browning and include: preculture on fresh medium (Li *et al.*, 2006) or on activated-charcoal enriched medium (Iocco *et al.*, 2001; Bouquet *et al.*, 2006), addition of antioxidants before, during or after co-culture, like PVP and DTT (Perl *et al.*, 1996b) or DTT alone (Li *et al.*, 2006), reduction of bacteria concentration and washing of the bacterial culture before use (Iocco *et al.*, 2001).

2.2.2 Biolistic-mediated transformation

The Department of Horticultural Sciences at Cornell University has extensively utilized this technology for grapevine transformation (Kikkert *et al.*, 2004) and established a system based on the bombardment of fine embryogenic suspensions layered onto a filter disk. Biolistic-mediated transformation, also known as gene gun transformation, appears much simpler than *Agrobacterium*-based technology because it avoids some time-consuming steps including the construction of the binary vector, introduction of the vector into *Agrobacterium*, and culture and maintenance of bacterial strains. It is also much more rapid as transformation experiments can start as soon as the expression cassette is cloned inside any plasmid that is maintained in *E. coli*. Also, after transformation, there is no need for expensive bacteriostatic agents to control *Agrobacterium* growth and, because selection of putatively transformed cells can start in liquid culture, the amount of the selection agent can be lowered.

The Cornell team were the first to transform regenerative grapevine structures using this method. Initially, embryolike structures of the *Vitis* hybrid Chancellor expressing GUS were recovered (Hebert *et al.*, 1993) and Kikkert *et al.* (1996) later succeeded in regenerating these structures into nonchimeric plants. The technology was

very efficient for Chancellor as 100 putative transformed embryos could be recovered per bombarded plate but the rate of transformation with a *V. vinifera* cultivar was only 10–30% (Kikkert *et al.*, 2001). These authors reported that as the bombardment was set to higher pressure at a reduced distance, the number of transformation events increased but also cell damage. In order to obtain better transformation rates (number of transformed cells versus number of injured cells) several parameters required optimization for each new line. Attention to detail was also required for DNA coating procedures, microprojectile energy settings, and selection procedures (selection agent type and level). The biolistic method has been successfully used to introduce genes of interest into Chardonnay, an important *V. vinifera* cultivar (Vidal *et al.*, 2003, 2006b). But other groups' experiences with the biolistic method, Franks *et al.* (1998) with Sultana or Soloki *et al.* (1998) with 110 R rootstock, did not result in regenerated transformed plants. The regeneration of transformed plants from 110 R has not been successful. At the present time, for stable transformation followed by plant regeneration, the biolistic method appears more difficult compared to the use of *Agrobacterium*-mediated transformation. In particular, the fine embryogenic cell suspensions required for efficient biolistic transformation is a type of culture difficult to obtain and stabilize for some cultivars, especially as these cultures also have to maintain the capacity to regenerate plants.

A recent development for the gene gun approach is the minimal gene cassette strategy (Fu *et al.*, 2000). This strategy is based on the fact that for a physical method of transformation, no other DNA sequence apart from the expression cassette is needed for transgene integration and expression. Vidal *et al.* (2006b) tested this technology in grapevine comparing the effectiveness of the co-bombarding of two minimal cassettes (one coding for the MS199 antimicrobial peptide and one coding for *nptII*) or corresponding circular plasmids. Starting with the same amount of tissue, 1 year after the bombardment, they respectively recovered 16 and 28 Chardonnay plants from the mix of minimal cassettes and the mix of the corresponding plasmids. Several plants in both treatments expressed the transgenes. This technology appears very promising to produce

plants containing only sequences of agronomical interest.

2.2.3 Transient transformation methods

Grapevine protoplast technology has been reviewed by Papadakis *et al.* (2001). Compared to other plants, grapevine protoplasts are recalcitrant to propagation and regeneration (Papadakis *et al.*, 2005) possibly due to special features of oxidative metabolism. Wall-less cells are suitable material for experiments based on a single cell design and allow the introduction of DNA or viruses into grapevine plant cells by either electroporation (Valat *et al.*, 2006) or polyethylene glycol (PEG) treatment (Jardak-Jamoussi *et al.*, 2002). Brehm *et al.* (1999) transiently expressed fusions of stilbene synthase promoters to *uidA* in grapevine protoplasts to study the effect of fungal cell wall elicitors on transcription activity. Transient transformation of grapevine protoplasts was also reported by Jardak-Jamoussi *et al.* (2002) who incubated protoplasts in the presence of PEG and the plasmid pB1426 carrying the *uidA* and *nptII* genes, but no transformed tissues or organ were regenerated. Plants regeneration from grapevine protoplasts has been reported only once using embryogenic cultures as donor tissue (Reustle *et al.*, 1995).

Undifferentiated cell suspensions (Torregrosa *et al.*, 2002b) are another suitable tissue for transient approaches. Hawker *et al.* (1973) was the first to describe how to initiate a grapevine cell suspension from small pieces of callus. Callus from various explants (berry, petiole, stem) is used as starting material to establish cell suspension cultures. These cultures represent an interesting system to study grapevine secondary metabolism (Decendit and Merillon, 1996) and gene expression (Loulakakis, 1997). The approach was used by Torregrosa *et al.* (2002b) to study the promoters of *Adh* genes from *V. vinifera* and *A. thaliana*. Large differences in promoter activity were observed that corresponded to the *Adh* promoter leader sequence and the type of cell suspension system. Cell suspensions of *V. vinifera* were found more appropriate than those of *A. thaliana* to investigate grapevine *Adh* promoter functioning. Using this system, further research investigated the possible role of *Adh* 3' flanking sequences in transcript regulation

(Tesnière *et al.*, 2005) and the functioning of the *VvAdh2* promoter (Verriès *et al.*, 2004). Serial deletions of the *VvAdh2* promoter were fused to *luc* and transiently introduced into *V. vinifera* cell suspensions that were subjected to aerobic and anaerobic conditions. Reverse GT-boxes, within the proximal promoter region, involved in the response to anaerobic treatment and putative ethylene responsive elements (ERE) participating in the regulation of *Vvadh2*, could be identified. It appears that undifferentiated grapevine cell suspensions appear a more reliable technology for transient gene expression than protoplasts.

However, both types of tissues can be difficult to induce and maintain and *in planta* or *ex planta* direct organ transient transformation could be an alternative technique to circumvent the use of protoplasts or cell suspensions. The most promising methods of organ or plant transient expression use VIGS for repressing a gene (Robertson, 2004) or overexpressing a gene using agroinfiltration (Kampila *et al.*, 1997). VIGS and agroinfiltration are being investigated for grapevine (P. Mestre and O. Lemaire, personal communication), however, these approaches have not yet become standard for grapevine because there have been no protocols developed and published.

2.3 Selection of Transformed Tissues

2.3.1 Selection and regeneration of putatively transformed plants

After co-culture, an important balance between effective selection of putatively transformed cells and the minimization of the inhibitory effects on cell growth caused by the selective agent must be achieved. This balance is important when using *Agrobacterium*-based protocols as grapevine callus is particularly sensitive to browning caused by contact with *Agrobacterium* and general handling (Perl *et al.*, 1996b), releasing phenolic compounds that inhibit the growth of surrounding tissue. In order to determine the optimal level of selection, it is crucial to know the sensitivity of both transformed and nontransformed grapevine tissues to the relevant selection agent(s).

In decreasing order of use, the selectable marker genes used for grapevine transformation are: *nptII*, *hpt*, and *pat* (see Section 2.1.1). Results

with the phosphomannose-isomerase (*pmi*) gene as an alternative selectable marker were not encouraging (Reustle *et al.*, 2003; Kieffer *et al.*, 2004). Two stages in the regeneration process after transformation of embryogenic tissue have to be considered separately; (i) the selection of embryogenic tissues and the production of nonrooted embryolike structures, and (ii) germination of somatic embryos with roots and shoot production. The sensitivity of callus growth and adventitious shoot and root formation to kanamycin was studied by Colby and Meredith (1990) in several cultivars of *V. vinifera* and in *V. rupestris* cv. St. George, to investigate the suitability of kanamycin resistance as a selectable marker for grapevine transformation. The authors concluded that the high kanamycin sensitivity of adventitious shoot formation in grape exceeded that reported for any other plant species and could likely hinder the recovery of transformed plants. Torregrosa *et al.* (2002a) compared the effect of hygromycin, kanamycin, and phosphinothricin on embryogenic callus of three *V. vinifera* cultivars. Among the three selection agents, only kanamycin and hygromycin could significantly reduce growth without inducing major tissue necrosis. According to the genotype and the medium, 1.25–5 $\mu\text{g ml}^{-1}$ of hygromycin or 80–100 $\mu\text{g ml}^{-1}$ of kanamycin was necessary to significantly inhibit callus proliferation. Interestingly, for the same level of necrosis, hygromycin could reduce more callus growth than kanamycin. Some *Vitis* genotypes (Le Gall *et al.*, 1994) are less sensitive to hygromycin than *V. vinifera* and needed higher concentrations of hygromycin (10–16 $\mu\text{g ml}^{-1}$). The *pat* gene was found to be effective by other authors possibly because genotype-dependent effects (Perl *et al.*, 1996b). Peros *et al.* (1998) studying the effect of kanamycin and hygromycin on axillary bud development observed an interaction between the selection agent and genotype. Similar to embryogenic callus, hygromycin appeared more phytotoxic than kanamycin. Furthermore, the toxic effects of both antibiotics were found to be different. Kanamycin induced leaf chlorosis whereas the leaves of plantlets grown with hygromycin became stunted and turned red. Shoot development was completely inhibited at 0.8 $\mu\text{g ml}^{-1}$ hygromycin or 4 $\mu\text{g ml}^{-1}$ kanamycin. Torregrosa *et al.* (2000) evaluated the level of antibiotic resistance conferred by the *nptII* and

hpt genes using grapevine microcuttings bearing both resistance genes. Results showed that despite higher phytotoxicity of hygromycin compared to kanamycin, the transgenic cuttings exhibited a much better tolerance to hygromycin than kanamycin. Because of insufficient resistance levels conferred by the *nptII* gene, some transgenic clones showed poor growth similar to the nontransformed control cutting in the presence of kanamycin.

NptII was the first resistance gene successfully used to obtain transformed grapevine plants (Mullins *et al.*, 1990) or organs (Guellec *et al.*, 1990) and has been used in the transformation of many grapevine genotypes including *V. vinifera* cultivars or *Vitis* hybrids using kanamycin (Mullins *et al.*, 1990; Martinelli and Mandolino, 1994; Franks *et al.*, 1998; Iocco *et al.*, 2001; Torregrosa *et al.*, 2002a; Motioike *et al.*, 2002) or paromomycin (Mauro *et al.*, 1995; Perl *et al.*, 2004; Wang *et al.*, 2005) as the antibiotic. Nevertheless, because *hpt* confers a better level of tolerance to tissues and organ buds of grapevine than *nptII*, hygromycin selection represents a very good alternative to *nptII* (Le Gall *et al.*, 1994; Perl *et al.*, 1996a; Franks *et al.*, 1998; Pratelli *et al.*, 2002; Tesnière *et al.*, 2006).

Because sensitivity to antibiotic selection varies according to the genotype and the stage of development, 5.0–15 $\mu\text{g ml}^{-1}$ hygromycin or 75–150 $\mu\text{g ml}^{-1}$ kanamycin appears adequate for the selection of putative embryogenic structures whereas during regeneration of plants the level of selection could be reduced to 2.5–5.0 and 25–50 $\mu\text{g ml}^{-1}$ for hygromycin and kanamycin, respectively. Recently, Wang *et al.* (2005) showed that paromomycin could replace kanamycin for selecting *nptII* transformed structures of Red Globe. From this experience and from Perl *et al.* (2004), 5–20 $\mu\text{g ml}^{-1}$ paromomycin would appear to be appropriate to select putatively transformed somatic embryos.

2.3.2 Elimination of agrobacteria

Agrobacteria grow on grapevine tissues either on solid or liquid medium. After 48 h co-culture, it is not rare to observe bacteria growing between explants and medium. A few days later, agrobacteria progressively surround

the tissue and the tissue begins to undergo necrosis. To stop bacteria development after co-culture, tissues are carefully washed with a liquid solution often containing high levels of antibiotics (500–1000 $\mu\text{g ml}^{-1}$). After washing, the grapevine tissue needs to be maintained in contact with antibiotics during several subcultures (Perl *et al.*, 2004; Bouquet *et al.*, 2006) to kill the bacteria. A broad range of antibiotics applied alone or in combination can be used to kill the agrobacteria: carbenicillin, cefotaxime (claforan[®]), amoxicillin plus clavulanic acid (augmentin[®]), ticarcillin plus clavulanic acid (timentin[®]), with concentrations ranging between 200 and 1000 $\mu\text{g ml}^{-1}$. But the efficiency of the antibiotic can vary with the commercial supplier and also storage conditions. Most authors tend not to change the supplier when a system is working and try to use fresh, or recently prepared, antibiotic solutions with the first days after co-culture being particularly important. Another factor to be considered is the strain type, with strains such as EHA101 being particularly difficult to eliminate compared to LBA4404 (L. Torregrosa, personal communication). Antibiotics are generally maintained in the media for 2–3 months after co-culture until transfer of regenerative structures to the light for shoot growth.

2.4 Regeneration of Whole Plants

After the initial co-culture, embryogenic tissues are transferred to a selection medium allowing the differentiation of embryolike structures. There are two options for regeneration: (i) maintain embryogenic proliferation with a medium designed for long-term embryogenic maintenance, or (ii) induce embryo development from the embryogenic tissue by reducing auxin levels. The first option has the advantage to amplify the initial transformation events before regeneration of putative transformed embryos but increases the delay for plant recovery (6–12 additional weeks) and also increases the risk to recover multiple plants from the same transformation events. More generally for grapevine, callus is transferred onto a medium without plant growth regulators (Le Gall *et al.*, 1994) or with reduced auxin levels and/or auxin suppressors (Perl *et al.*, 1996b, 2004; Iocco *et al.*, 2001; Bouquet *et al.*, 2006) to encourage embryo differentiation. To avoid excess browning

during the process of embryogenic tissue selection, some authors delay the selection and increase progressively the amount of the selection agent through subculturing (Franks *et al.*, 1998; Perl *et al.*, 2004; Bouquet *et al.*, 2006).

After 2–3 subcultures of the embryogenic culture on regenerative and selection medium, small clusters of embryos are transferred onto plant growth regulator-free medium to encourage embryo development (selection agent being at the maximum level by this stage). Well-developed embryos (torpedo and further) are then transferred onto medium containing benzylaminopurine (2 μ M BAP (6-benzylaminopurine)) and incubated for 7–10 days under light for greening (Bouquet *et al.*, 2006). Developing embryos are returned to plant growth regulator (PGR)-free medium for 2 weeks under low light intensity to achieve embryo elongation and rooting (Figure 1b). This stage of selection is very important to avoid false positives, as nontransformed embryos are generally unable to root in the presence of the appropriate level of the selection antibiotic. Only germinating embryos are processed further. To stimulate caulogenesis of germinating embryos, whole root meristem regions plus cotyledon upper parts are discarded and a cotyledon basal part containing the shoot meristem is deeply scarified (Pratelli *et al.*, 2002). Explants are placed onto BFe2 medium (Torregrosa and Bouquet, 1995) with the meristem region in contact with the medium and incubated under moderate light intensity. At this stage, the level of selection antibiotic can be reduced to 5 μ g ml⁻¹ hygromycin or 50 μ g ml⁻¹ kanamycin. Emerging shoots are subcultured under the same conditions to encourage axillary branching. Shoots are isolated and converted into plants on root-induction medium containing 1 μ M indole-3-acetic acid (IAA) (Bouquet *et al.*, 2006).

The polymerase chain reaction (PCR) is a convenient method to systematically screen all putative transformed lines using DNA extracted from 100 mg fresh material (1–2 small leaves from *in vitro* plantlets). Depending on the experimental design, several clones of each PCR positive line are transferred into 10 cm diameter pots progressively acclimated to greenhouse or glasshouse conditions (Bouquet *et al.*, 2006). The average rate for successful transplanting and acclimatization depends on the variety but is generally higher than 90%. When

the transgenic plants are successfully established in the greenhouse, the stable integration of the gene of interest is confirmed by Southern blotting and further characterization and testing of the plants can start.

2.5 Testing

The stability of transgene expression and inheritance has not been extensively studied in grapevine. Expression stability has been assessed using the reporter genes *gfp* and *uidA* under the control of the constitutive CaMV 35S promoter (Franks *et al.*, 1998; Iocco *et al.*, 2001). These two reporter genes were used to track transgene expression during the transformation process and to confirm that transgene expression was stable. It was observed that while most regenerated plantlets and mature plants maintained a uniform reporter gene expression pattern, up to 35% showed variegated expression (Franks *et al.*, 1998; Iocco *et al.*, 2001). It was suggested that the variegated expression pattern could be due to a number of factors resulting in gene silencing and methylation of introduced transgenes was demonstrated (Franks *et al.*, 1998). Subsequent observations showed that the *gfp* expression pattern of regenerated grapevine plants was maintained under glasshouse and field conditions for more than 6 years (M. Thomas and P. Iocco, personal communication). This indicates that the expression pattern is fixed after plant regeneration and yearly cycles of winter dormancy, pruning, and bud burst followed by new growth do not change the expression pattern. The same gene expression pattern present in leaves was also found to be present in preveraison and postveraison berries. Stable leaf *gfp* transgene expression over 5 years for Sultana (Thompson Seedless) grown under glasshouse conditions has been reported (Li *et al.*, 2006). Variegated *gfp* gene expression in berry skin has also been observed when the promoter was from the *ufgt* (UDP glucose-flavonoid 3-*O*-glucosyltransferase) gene (P. Iocco and M. Thomas, personal communication). The UFGT protein represents the last enzyme in the anthocyanin pathway and results in the red color of berries and wine. It is not clear whether the variegated expression pattern was due to gene silencing effects as previously seen for the CaMV 35S promoter or was representative of cell-specific

activation of the UFGT promoter in the skin cells of postveraison berries (Figure 1c).

Information on the stability of transgene expression over a number of generations for grapevine is lacking due to the long generation times involved and the fact that cultivars are not propagated by seed but by vegetative cuttings. This means that selection of a superior transgenic vine will always be followed by clonal propagation either by tissue culture or vegetative cuttings. However, results from GFP expressing grapevine plants have demonstrated that transgene expression is transmitted to progeny in the expected Mendelian fashion with progeny showing GFP expression (M. Thomas, personal communication).

Phenotypic differences between transgenic vines and the original donor cultivar have been observed. Reported differences relate mostly to changes in leaf shape (Franks *et al.*, 1998; Iocco *et al.*, 2001). The change in leaf shape was also found in plants regenerated from embryogenic cultures indicating that the culture conditions and regeneration procedure were responsible for the new phenotype and not the transformation procedure. The lobed leaf phenotype reported by Franks *et al.* (1998) for transgenic Sultana vines was still present for the same vines even after 6 years growth, however, the leaves on canes that contained bunches had reverted to the typical nonlobed leaf Sultana phenotype (M. Thomas, personal communication). This suggests that the leaf phenotype may be developmentally controlled. A modified flower phenotype was observed for Chardonnay plants overexpressing the MADs-box gene *VvMADS1* (Boss *et al.*, 2003). The flowers showed extended growth of the calyx/sepals that encased nearly the whole cap structure formed by the petals. Fruit development for these transgenic Chardonnay vines was normal and it was suggested that the altered flower morphology may provide some level of resistance to *B. cinerea*, which infects at the flower cap abscission zone during anthesis (Boss *et al.*, 2003). No results on the effect of transgenes on yield and fruit and wine quality have been published for either transgenic rootstocks or scions as field trials spanning many years are required to obtain this information. Existing GM grapevine field trials may eventually provide us with this information.

The observed stability of gene expression over many years, as reported above, indicates that when

commercial plantings of transgenic vines occur the plants in these vineyards can be expected to maintain the phenotype conferred by the transgene for the life of the vineyard, which is usually more than 30 years.

2.6 Specific Regulatory Measures Adopted

There were no commercial plantings of GM vines in 2006 and therefore no specific regulatory measures have been put into place for commercial growing of transgenic vines or for the sale of the resulting fruit and wine. However, a number of experimental field trials have been carried out in different countries under the regulatory guidelines specific to each country and year of planting (Bouquet, 2006). Internet resources listing grapevine field trials include the United States (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>), Australia (<http://www.ogtr.gov.au/>), Europe (<http://gmoinfo.jrc.it/>), and the OECD (<http://webdomino1.oecd.org/ehs/biotrack.nsf>). Countries where field trials have been carried out include Australia, France, Italy, Canada, the United States, Chile, and Germany. These field trials have mostly been managed by research organizations, however, a few have involved commercial wine companies in France (Moët et Chandon), Canada (Chateau des Charmes wines), and the United States (Pebble Ridge Vineyards). The traits being investigated are largely covered in Tables 1 and 2 and can also be found on the above Internet sites and range from resistance to pests and pathogens to flowering and improved fruit quality. Of the many field trials initially established only a few were still active in 2006 with Australia, France, Italy, and Chile each having one field trial and the United States having 12 permits for trials that extended beyond 2006. Management of these GM vines also varied between countries. In Australia, procedures were used to prevent pollen and seed dispersal by bagging inflorescences (Figure 1d) and grape bunches while in Germany and the United States such precautions were not required. Public and government concern based on “Frankenwine” negative media reports have resulted in some field trials being terminated prematurely in a number of countries or specific regions within countries. GM vines have also caused past issues in Europe with

European Commission regulations controlling the marketing of vegetative GM material and derived products (Meldolesi, 2001).

The field trial in Germany has provided useful information on pollen flow from transgenic vines to surrounding non-GM vines (Harst *et al.*, 2006). Hermaphrodite grapevine cultivars were generally considered to be self-fertilizing due to the structure of the flower. Using GUS as a marker and pollen traps it was observed that wind blown pollen could travel at least 100 m from the pollen source. The study also looked at the effective distance of pollen travel in the direction the wind was blowing at the time of anthesis as determined by GUS positive seedlings germinated from seed collected from surrounding non-GM vines. It was found that 2% of seeds collected from non-GM vines in a radius of 10 m from GM vines germinated GUS positive seedlings and 2.7% within a 20 m sector. While the study only looked at pollen flow from 36 *uidA* expressing transgenic vines it does provide the first information about the potential for pollen flow in a commercial vineyard situation. The values found within the small area surveyed were above the European Union GM contamination compliance threshold of 0.9%. However, as the authors point out this threshold is only relevant for grapevine seed or seed-derived products and as berry tissue and wine made from it are derived from maternal tissue GM cross contamination by pollen flow may not be a major issue for wine products. In addition, wine is a highly processed product and methods to detect such low levels of contamination in wine do not currently exist.

3. FUTURE ROAD MAP

3.1 Expected Products

Future products can be divided into those that benefit the grower, winemaker, consumer, or the environment. Economic sustainability is the primary driver for grape growers and winemakers and any GM vine that improves productivity, reduces costs, or allows greater product differentiation will have an advantage over existing varieties. But these GM varieties will only be grown if there is a market for the end product. The globalization of the wine industry since the 1980s has also brought into sharper

focus the fickle nature of consumer preferences. The rapid changes in consumer preferences for wine often resemble that of a fashion industry. The factors that influence consumer preferences are many and varied but a large part is due to (i) marketing and media exposure about a particular wine region, wine style or variety and (ii) value for money. Consumers are also becoming more health conscious and aware of environmental issues and these may also have a future impact on what growers and winemakers produce.

The two major long-term issues confronting viticulture are global warming and the use of chemicals to control many pests and diseases in the vineyard. The application of gene technology to produce GM vines that reduce the use of chemicals to control pests and diseases is a high priority in many parts of the world (see Tables 1 and 2) as growers, the environment and the public will all benefit. The identification and positional cloning of a powdery mildew resistant gene (Barker *et al.*, 2005) for transfer into susceptible varieties is in progress and if successful will represent one of the first case studies where a beneficial grape trait is selectively transferred from *M. rotundifolia* to *V. vinifera*. At the present time a perception exists that industry and public acceptance of GM vines and wines may be more forthcoming if gene transfer is limited between closely related genera and species and the powdery mildew resistance gene is one such example. The use of transgenic rootstocks resistant to pests and virus diseases (Table 1) may also find easier industry and public acceptance since it is only the grafted scion that produces the fruit and therefore any fruit product is non-GM. Modeling of global warming (Jones *et al.*, 2005; White *et al.*, 2006) indicates that regions currently producing high quality grapes and wine will experience an increase in temperature and quality may decrease over time. Solutions to this problem are likely to be explored using a GM approach in the future.

Fruit quality traits such as decreased browning (Thomas *et al.*, 2000) of dried grapes and seedlessness of table grapes (Perl *et al.*, 2003) have been investigated with some success. No reports describing wine made from grapes modified for quality traits have to date been published. The recent discovery that tartaric acid in grape berries is synthesized from vitamin C (DeBolt *et al.*, 2006) opens up the possibility to genetically modify this

process to produce berries and wine with high levels of vitamin C.

The development of future GM grape varieties and products will largely depend on public research for the foreseeable future. This is due to the long period of time (10–25 years) required to develop new GM plants and evaluate the fruit and wine from replicated field trials in different environments, making it an unattractive investment for biotechnology companies. The current concern in many regions of the world about GM products has also reduced investment enthusiasm of wine companies. However, the advantage of the long period required for field evaluation is that all public concerns about GM products will have been addressed through market acceptance of other GM-derived products from other crop species by the time grapes or wine from GM vines are ready for marketing.

3.2 Risks and Concerns

Despite the potential benefits of this new technology to improve the reliability and quality of the world food supply, public and scientific concerns have been raised about the environmental and food safety of GM crops (Hails, 2000). It is feared by some sectors of society that DNA technology will harm people by undesired impacts on health and the environment. In Europe, consumer acceptance of commercial DNA technology plant products seems further away than ever, especially for grape products. Current discussions in western countries mainly focus on GM food and consumer safety. This topic has been reviewed in depth (Kuiper *et al.*, 2001). To some extent, this has diverted public attention away from ecological and environmental concerns about the impact of growing GM crops. With respect to the use of gene technology for grapevine improvement, the public concern is minimal since field experiments are very few. The issue is likely to come before consumers when a grape-derived commercial product is being considered for market release. As this will not occur for quite a few years the intervening years offer an opportunity to debate rationally the advantages and disadvantages of transgenic grapevines on human health, environmental health, and economic health at a regional, national, and global level.

3.2.1 Impact of GM grapevines on health

The safety aspects of the current products of grapevine growing, wine, table grapes, and raisins have always been based on long-term pragmatic experience that started many centuries, if not millenarians ago. The safety assessment of grapevine cultivars obtained from conventional breeding has primarily been based on limited analysis of known macro and micronutrients, antinutrients, and toxicants. Products with unusual tastes or harmful levels of specific compounds have been generally rejected from breeding programs, but sometimes rejection followed only after their release on the commercial market. That was the case in the beginning of the 20th century for some American interspecific hybrid varieties with low and still controversial levels of toxic methyl alcohol in their wines. An interesting example of wine market manipulation and associated trade barriers occurred with the ability to identify anthocyanin diglucosides. Though no scientific data support a possible harmful effect on consumer health, the presence of anthocyanin diglucosides in the berry skins and the wines of most French–American interspecific hybrids was an effective way to distinguish these hybrids from the *V. vinifera* cultivars and helped to eliminate them from the French market after the 2nd World War through planting regulations. These interspecific hybrids were more productive and had better disease tolerance than the original *V. vinifera* cultivars but at the time there was a significant oversupply of wine in France. Industry marketing, assisted by government, promoted low yielding traditional *V. vinifera* cultivars as producers of better quality wine and this perception soon dominated French thinking and spread to the rest of the world. At the end of the 19th century, the success of grafting *V. vinifera* cultivars onto American rootstocks resistant to Phylloxera was delayed for a long period due to baseless fears that some compounds toxic against the aphid, and possibly humans, could potentially be transferred from the roots to the scion and the grapes (Pouget, 1990). This suspicion was often reinforced by some temporary regulations in France that were in hindsight harmful, delaying adoption of best practice methods, and represented early examples of untoward application of the precautionary principle with regulations

based on perception rather than scientific facts.

Safety assessment of GM grapevine cultivars should be carried out on a case-by-case basis, using the concept of substantial equivalence developed from the idea that existing foods can serve as a reference for comparing the properties of foods derived from GM plants. But consensus on practical application of this principle should be further elaborated, particularly with respect to the detection and characterization of unintended effects, such as disruption, modification, or silencing of active genes or activation of silent genes, which may result in the formation of either new metabolites or altered levels of existing metabolites. In the future, the development and validation of new strategies such as a targeted (compound-specific) approach, or nontargeted (profiling) approach based on DNA microarray technology, proteomics, and metabolomics should offer adequate answers (Kuiper *et al.*, 2001). However, it should be emphasized that the occurrence of unintended effects is not specific for genome modification through DNA technology. It also occurs frequently in conventional breeding, but the effects are usually easier to identify when they concern morphological characteristics rather than biochemical pathways.

For GM grapevines, three scenarios are envisioned in which the product (wine or fruit) would be (i) substantially equivalent, (ii) substantially equivalent except for the inserted trait, or (iii) not equivalent at all. An example of the first scenario would be virus (GFLV) resistance induced in a transgenic rootstock by expression of viral CP or movement protein genes (Valat *et al.*, 2006). Even if proteins derived from the inserted genes move toward the scion and the berries, the wine will not be different from a wine obtained with naturally GFLV infected grapevines. The second scenario, likely the most frequent, could be illustrated by the insertion of genes encoding PR-proteins such as chitinase (Yamamoto *et al.*, 2000), antimicrobial peptides such as magainin (Vidal *et al.*, 2006a), PGIPs (Agüero *et al.*, 2005), or lectins such as GNA (Viss and Driver, 1996) in *V. vinifera* scions or rootstocks cultivars to protect them against fungal and bacterial diseases or pests. The potential toxicity of GNA lectin has been reported (Ewen and Pusztai, 1999) even if the conditions

of the test were controversial. Furthermore, a number of known plant-derived allergens are PR-like proteins (Niskanen and Dris, 2004). Thus, the potential allergenic or toxic features of newly introduced proteins have to be carefully evaluated. Furthermore, it should be noted that overexpressing PR-proteins in genetically modified grapevines will lead to increase protein-haze formation and risks in wine turbidity, whereas attempting to remove the PR-proteins from wines by silencing their expression in grapes may increase plant susceptibility to pathogens (Ferreira *et al.*, 2004). The safety assessment of selectable marker genes used in transformation is not specific to grapevine. Health aspects of marker genes encoding resistance to antibiotics have been largely dealt with, and there is a general agreement that newly expressed proteins do not show any risk of toxicity or allergenicity and that horizontal transfer of antibiotic resistance genes from plants to microorganisms residing in the human gastrointestinal tract is unlikely to occur, given the complexity of steps required for gene transfer, expression, and efficacy. Nevertheless, considering the public concern with existing problems with drug-resistant bacteria in human health, transformation technologies that do not result in antibiotic resistance genes, for instance use of alternative marker genes or gene excision methods, have been promoted in some cases (see Section 3.3). The third scenario could also be illustrated by modifications to characteristics of the berry induced for instance by the insertion and overexpression of the yeast invertase gene *SucII*, which aimed to change the ratio of fructose/glucose (Perl *et al.*, 1994) or by silencing the polyphenol oxidase (PPO) gene to produce light colored and low browning Sultana raisins after drying (Thomas *et al.*, 2000). In the past decade, progress has been made with respect to the identification and characterization of berry and wine constituents that may exert beneficial and/or adverse effects on human health. Several compounds have been identified with positive effects on the incidence of cardiovascular diseases (see Section 1.3). The overexpression of some compounds, such as resveratrol, could be interesting (Jeandet *et al.*, 2002). But beneficial compounds may also exert adverse effects, depending on the amount, conditions, and the presence of other agonists or antagonists, and

adequate safety assessments would need to be done.

3.2.2 Impact of GM grapevines on the environment

The ecological probabilities of harm attributed to the release and use of GM plants focuses on the invasion or modifications of ecosystems due to potential weediness, spread of transgenes by either vertical (outcrossing) or horizontal gene flow, secondary ecological impacts such as effects on nontarget or beneficial insects or undesired consequences on soil microbial communities, occurrence of super pests or diseases due to the “breaking down” of the introduced resistance genes, and loss of biodiversity. All these potential risks have been reviewed in depth (Conner *et al.*, 2003). With regard to GM grapevines, some are more important than others.

3.2.2.1 Potential “weediness” of GM grapevines

The ability of grapevines to escape from cultivation and revert to a wild and “weedy” condition alongside neglected vineyards is well known in countries with an old viticulture tradition. This event is more frequently observed with rootstock cultivars of recent origin. Indeed the ability of *V. vinifera* cultivars to revert to a wild condition is less common and may be related to their lower fitness compared to interspecific rootstock hybrids. The emergence of GM “weedy types” of *V. vinifera* could also be associated with chance hybridization with wild populations. For transgenes conferring resistance to herbicides, pests, diseases, and environmental stress, it can be suggested that this may result in increased fitness, survival, and spread. But gene flow between GM vine cultivars and wild vines is likely to be very low in Europe since there are very few remnant wild populations of *V. vinifera* spp. *sylvestris* (Arnold *et al.*, 1998; This *et al.*, 2006) left as a result of the diseases and pests introduced into Europe from America. This *et al.* (2001) did not find any trace of gene exchange in France between a local wild population and the cultivars growing in the vicinity. However, the situation could be different in the United States where large populations

of wild *Vitis* species are still observed, and the occurrence of gene exchange with *V. vinifera* cultivars has been historically proven (Tukey, 1966). For this reason, it is recommended to avoid using herbicide resistance genes as selectable markers and to evaluate carefully the benefits and drawbacks of breeding GM vines resistant to herbicides (Levenko and Rubstova, 2000).

3.2.2.2 Unintended effects on soil-borne microbial communities

Soil is a highly complex ecosystem of primary importance in nutrient cycling that influences primary production and vegetation dynamics. Furthermore, soil interaction with microbial populations of species with antagonistic properties (e.g., pseudomonads and *Trichoderma*) is a key factor in sustainable agriculture. Soil-borne microorganisms, accounting for >80% of the total biomass (excluding roots) are generally heavily exposed to GM plant material, either through root exudates, leaf shedding, or decomposition. Thus, investigating relevant effects of GM plants on soil-borne microbial communities is a major challenge for the future. Glandorf *et al.* (1997) reviewed the effects of GM plants expressing a range of antimicrobial proteins for plant pathogen control, such as chitinases and glucanases, on saprophytic soil microflora. In some cases, changes in the populations of bacteria, fungi, or soil invertebrates have been detected, even though no direct toxicity to the organisms has ever been demonstrated, and the effects can differ according to the plant or the transferred gene requiring assessment on a case-by-case basis (Kowalchuk *et al.*, 2003). Though the GM crops examined thus far appear to have little or no effect on crucial soil organisms and detected effects have been minor compared with other “normal” sources of variation, it may be necessary to have a system in place to screen for unwanted nontargeted effects. However, it should be noted that this has not been done with conventionally bred cultivars or those obtained by mutagenesis. In grapevine, there are considerable gaps in our knowledge on the role of soil-borne microbial communities. Arbuscular mycorrhizal fungi are an important group of beneficial soil microbes that form mutualistic symbioses with grapevines. Grapevine not only responds positively

to colonization (Bavaresco *et al.*, 2000; Linderman and Davis, 2001) but may also suffer in the absence of mycorrhizae (Menge *et al.*, 1983). The role of the grapevine rhizosphere bacteria is also likely significant (Waschkies *et al.*, 1994; Compant *et al.*, 2005).

3.2.2.3 Unintended effects on berry microflora

More is known about the microbial communities that develop on the berry skins. *S. cerevisiae* is by far the best-known yeast strain colonizing ripe berries under natural conditions. The natural pattern of distribution of populations of this yeast has not been clearly established, though many authors suggested that *S. cerevisiae* coexists initially with many other types of yeast on the surface of grape berries, and becomes predominant as the fermentation proceeds (Pretorius, 2000). An ecological study has shown that these yeast floras remain unchanged during successive years in the same vineyard, but vary from one region to another (Frezier and Dubourdiou, 1992). It cannot be excluded that the use of grapevines genetically engineered for disease resistance against fungi or bacterial diseases might cause significant differences in yeast ecology. For instance, the role of endopolygalacturonase was clearly demonstrated in the interactions between colonizing yeasts and grapevines (Gognies *et al.*, 2006) and GM cultivars expressing PGIP could likely alter these interactions. Kim *et al.* (2004) reported that strains of *S. cerevisiae* show different responses to eutypinol, a secondary metabolite produced by the degradation of eutypine, which is implicated as the main phytotoxin from *Eutypa lata*, the fungal disease causing the symptoms of “dying-arm disease”. It cannot be excluded that GM grapevines expressing the *V. radiata*—ERE (Legrand *et al.*, 2003) might modify the distribution of yeasts in favor of strains more tolerant to inhibition of mitochondrial respiration. Changes in wild yeast on berries as a result of an introduced gene is likely to have little effect during wine making as in most cases commercially available yeast strains are added for fermentation. However, it would be prudent for wine making and wine quality assessment to be carried out on grapes harvested from GM vines

prior to commercialization to alleviate concerns oenologists and others may have.

3.2.2.4 Evolution of viral populations

Environmental safety issues have been raised on the potential hazards related to the release of transgenic plants containing virus-derived genes (Tepfer, 2002). In grapevine, the two major concerns are (i) the risk of heterologous encapsidation of RNA in particles composed of CP of another virus and thus the risk to modify the vector specificity (Belin *et al.*, 2001; Andret-Link *et al.*, 2004), and (ii) the risk of genetic recombination between viral transgene transcripts and RNAs from viruses that infect transgenic plants and thus the risk of plant-to-virus gene flow. With regard to the risk associated with the largely used virus-derived CaMV 35S promoter, it has been treated in more detail elsewhere (Hull *et al.*, 2000).

It was first shown in the 1970s that plants infected by two related viruses could result in various forms of heterologous encapsidation (Rochow, 1970). This can in some cases affect viral transmission (Bourdin and Lecoq, 1991). Heterologous encapsidation has been observed in transgenic plants expressing a CP gene from several different viruses (Lecoq *et al.*, 1993; Tepfer, 2002). For grapevine, changes in vector specificity might be possible if a viral RNA of ArMV (transmitted by *Xiphinema diversicaudatum*) is encapsidated by a transgene-encoded CP of GFLV (transmitted by *X. index*). ArMV and GFLV are two distantly related viruses that cause a single disease phenomenon, the grapevine infectious degenerative disease called “court-noué”. The genome of ArMV has segments of sequence similarity common to the genome of GFLV (Loudes *et al.*, 1995), with amino acid comparisons between the proteins encoded by the RNAs of the two virus showing 75% identity (Wetzel *et al.*, 2004). Occurrence of heterologous encapsidation has never been observed in grapevine, either in greenhouse experiments or in field tests, but if there was any potential risk, it could be eliminated easily through modification of the CP gene (Jacquet *et al.*, 1998).

Along with mutation, genetic recombination is considered to be an essential source of variability

for viral genomes and thus play a key role in their evolution (Roossinck, 1997; Worobey and Holmes, 1999). Among many examples, experimental evidence obtained by Le Gall *et al.* (1995) showed template switching in the 3' untranslated region of pseudorecombinants constituted by RNA-1 of GCMV (*Grapevine chrome mosaic virus*) and RNA-2 of TBRV (*Tomato black ring virus*). There is strong evidence for recombination in transgenic plants expressing CP genes (Greene and Allison, 1994; Aaziz and Tepfer, 1999) and its potential effect on virus evolution has been discussed (Rubio *et al.*, 1999). In grapevine, Vigne *et al.* (2004b) screened for the presence of GFLV recombinants in 190 transgenic plants belonging to 18 lines of the 41 B rootstock expressing the GFLV CP gene and 157 nontransgenic plants. Their survey indicated that transgenic grapevines did not promote the emergence of viable GFLV recombinants to detectable levels in any of the two experimental field sites during the trial period nor did they affect the molecular diversity of indigenous GFLV populations. No GFLV variants from the field had features typical of strain F13 from which the CP transgene was derived.

The role of the 3' untranslated region of viral RNA in the recombination of grapevine nepoviruses (Le Gall *et al.*, 1995) has previously been mentioned. In transgenic *Nicotiana benthamiana* expressing the CCMV (*cowpea chlorotic mottle virus*) CP, the deletion of this 3' untranslated region has been shown to reduce or even abolish the recovery of recombinant viruses (Greene and Allison, 1996). In grapevine, the results of Vigne *et al.* (2004b) do not support the involvement of this region in the emergence of viable recombinants, since the transgenic plants contained the complete 3' untranslated region of GFLV RNA-2 (Serghini *et al.*, 1991) and expressed transgene transcripts. This may be explained by low transgene expression in the 18 transgenic grapevine lines with steady state accumulation of transgene messenger RNA (mRNA) orders of magnitude lower than GFLV RNA-2 in infected plants. It should be noted that in the grapevine survey several variants were identified in nontransgenic plants located outside the experimental field site, and the study of their genetic structure showed that heterogeneous populations of GFLV variants in a single vine are likely to result from mixed infections due to

repeated transmission of individual variants by viruliferous nematodes (Vigne *et al.*, 2004a; Vigne *et al.*, 2005). There is apparently strong sequence stability of the GFLV CP gene and the same low degree of diversity was observed among variants in the Champagne region, several European countries, the United States, and China. Thus, it is unlikely that the possible use of transgenic plants in the future will modify this natural variability. The potential problem of recombination between viral and plant genomes is complicated by the recent finding that sequences homologous to those of the nonretro RNA virus PVY (*Potato virus Y*) are integrated into the genome of several grapevine cultivars (Tanne and Sela, 2005). The hypothesis advanced was that the viral RNA recombined with a retrotransposable element, with the product subsequently inserted into the host cell genome as a "native transgene".

To the best of our knowledge, the study of Vigne *et al.* (2004b) is the first and only safety assessment study of recombination with transgenic grapevines expressing a virus-derived gene under field conditions of heavy disease pressure but low, if any, selection pressure against recombinant viruses. The likelihood of recombination within transgenic plants expressing virus-derived genes should be limited if the plants resist viral infection. Conversely, the probability of recombination occurring may increase if transgenic plants expressing virus-derived genes are susceptible to virus infection. The majority of the 18 transgenic grapevine lines tested in the study was susceptible to GFLV infection in vineyard, and though the risk assessment studies of viral recombination need to be followed up over the lifetime of the vineyard, the results obtained by Vigne *et al.* (2004b) under these conditions confirm earlier predictions by Falk and Bruening (1994) who hypothesized that the "potential benefits of engineered resistance genes far outweigh the vanishing small risk of creating new and harmful viruses in significant excess over those being created by natural processes."

3.2.2.5 Impact of GM grapevines on biodiversity

When considering the impact of transgenic plants on biodiversity, it is worthwhile distinguishing

between the biodiversity at large and the biodiversity associated with agricultural practice, which is generally referred to as “agrobiodiversity”. In current-day agriculture, agrobiodiversity is the result of a long process of plant domestication, the establishment of landraces and the deliberate breeding of new cultivars. In viticulture, natural calamities during the 18th and 19th centuries and economic or commercial constraints and regulations during the 20th century (see Section 1.5) have considerably narrowed the varietal basis of world wine production (Bouquet and Boursiquot, 1999). In the future, planting of transgenic cultivars with improved cultural or technological qualities may further reduce the number of available cultivars and thus contribute to worsen the narrowing of the genetic diversity of the cultivated grapevine. Transgenic cultivars would also reduce the level of clonal variability, since in most cases only a single clone will be transformed. But this situation will not be different from that of new cultivars obtained by conventional breeding, since these are typically monoclonal. A solution might be found in the use of somaclonal variation (Larkin and Scowcroft, 1981; Karp, 1991; Torregrosa *et al.*, 2001) that is currently being investigated in France to restore, amplify, or induce clonal variability in traditional or new cultivars (Torregrosa *et al.*, 2001).

3.3 Expected Technologies

The standard procedure for grapevine transformation at the present time is to first initiate embryogenic cultures and then transform these either by *Agrobacterium* or biolistics with plant regeneration occurring by somatic embryogenesis. The process is laborious and months are required to first establish embryogenic cultures followed by many more months after transformation before the first plants are regenerated (Iocco *et al.*, 2001; Bouquet *et al.*, 2006). While transgenic plantlets can be obtained in as little as 18 weeks post transformation (Iocco *et al.*, 2001), it usually requires a year or more to obtain greater than 100 transgenic plants from independent transformation events. Additional years are then required to evaluate genetic modifications affecting flowering or fruit due to the reproductive biology of the grapevine. Some

cultivars, such as Sultana, do not flower under glasshouse conditions and even more amenable cultivars such as Chardonnay still have flowering problems under glasshouse conditions. In most cases this necessitates field plantings of transgenic vines to properly evaluate flowering, yield, fruit development and quality. A typical experiment evaluating yield and fruit from 100 transgenic plants might require a minimum of 2 years from embryogenic culture initiation to regenerated plants followed by a 5-year field trial to collect data over a number of seasons. In practice, the process is actually longer and can require 8 or more years as harvests over three seasons are required and not all plants flower after 2 years in the field (M. Thomas, personal communication).

New technologies and plant material that can reduce the time from genetic modification to evaluation of the modified trait will be of greatest benefit to grapevine transgenic research. The use of *A. rhizogenes* for producing transgenic root cultures appears to show some promise for evaluating modified root traits (Lupo *et al.*, 1994; Torregrosa and Bouquet, 1997; Franks *et al.*, 2006). The use of *Agrobacterium* infiltration techniques for tobacco leaves (Schob *et al.*, 1997) and tomato fruit (Orzaez *et al.*, 2006) might have application for grapevine but there are no published reports for grapevine. The same situation is true for VIGS methods (Baulcombe, 1999) that use virus vectors introduced by transformation or viral infection with no reports of success with grapevine. The use of rapid flowering grapevine genotypes (Boss and Thomas, 2002) for stable transformation studies might also find application in reducing the time from plant regeneration to fruit evaluation. New stable transformation and regeneration methods that avoid the use of embryogenic cultures would also be an advantage but as discussed above (Section 2.2.1.2) the successful report by Mezetti *et al.* (2002b) using adventitious budding tissue has certain limitations.

It is also expected that in the future new selectable markers and vectors more acceptable to regulators of GM plants and products will be developed that benefit all plant transgenic research. The gene encoding the streptavidin protein from *S. avidinii*, placed under the control of an embryo specific promoter could possibly be used as a selection system for grape (see Perl *et al.*,

2003). The public availability of the assembled grapevine genome sequence in 2007–2008 will also make it easier for researchers to identify and use grapevine promoters that have specific spatial and temporal specificity. Grapevine microarray data and other HTP gene expression assays will also provide a wealth of information on key genes involved in specific processes and traits, providing the basis for transgenic experiments to confirm gene function.

3.4 Current Perceptions

The area of intellectual property (IP) rights covering plant transgenic technologies, genes, promoters, and new genotypes and phenotypes is complex. However, the CAMBIA organization (<http://www.cambia.org>) has made available a variety of free tools and information on patents and IP that greatly assist the public scientist in determining their “freedom to operate” status. The CAMBIA site also hosts free patent searching through the Patent Lens portal and grapevine specific patents can be found by using this resource. At the present time, patents involving grapevine are reasonably specific and not of a nature that will inhibit GM research.

The global grape and wine industry is a consumer-driven industry and any negative media attention can very quickly affect company sales and marketability of a product. Negative media reports in 1999 and concerns by both consumers and some winemakers resulted in the champagne producer Moët & Chandon quickly ending a field trial of GM vines. Because of public concern about GM plants, especially in the major European market, the industry in all parts of the world has taken a wait and see approach. In Australia, many in the industry are aware of the potential benefits of GM technology and while supporting the research have also publicly stated: “No genetically modified grapes or yeasts are used in the production of Australian wines. And there will be no commercial use of genetically modified organisms until it is clear they are safe, of high quality, and beneficial to consumers.” This industry stance has now been adopted in many other countries. As outlined above, and in Tables 1 and 2, research is actively continuing into genetic modification as a means of vine improvement. Genetic modification of yeast

is also a very active area of research for improving wine quality (Pretorius, 2000; Schuller and Casal, 2005) and some modified strains have recently been given “Generally Regarded As Safe” status by the Food and Drug Administration (FDA) in the United States and commercialized in both Moldova and the United States (Husnik *et al.*, 2006). It is expected that general acceptance of GM yeasts in commercial wine production by both the wine industry and consumers will help pave the way for similar acceptance of GM vines.

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Olive

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Palynological, anthropological, and archeological evidences demonstrated the presence of some forms of olive during the last glaciation in the western and eastern Mediterranean regions 18 000 years BC (Carrion and Dupre, 1996; Watts *et al.*, 1996). Numerous studies confirmed that olives have been present for several thousands of years before its domestication in the Mediterranean basin, particularly in the Middle East. Olive was probably domesticated in the Jordan River valley ca. 5700–5500 BC (Zohary and Spiegel-Roy, 1975) and more precisely according to Liphshitz *et al.* (1991) domestication occurred during the bronze period (5200 BC). The spread of the cultivated olive to other countries of the Mediterranean region accompanied migrations of different civilizations (Egyptian, Phoenician, Greek, Etruscan, Roman, and Arab). Wild and domesticated olives grow in the same areas and show some morphological differences with cultivars, i.e., smaller fruit size and lower oil content in the mesocarp (Terral and Arnold-Simard, 1996). Two distinct wild olives have been recognized: *oleaster* and feral forms. *Oleaster* occupies primary niches in undisturbed areas as a constituent of evergreen plant associations. Wild and cultivated varieties, representing two genetically separated complexes, have been interconnected as a result of occasional hybridizations that could have allowed the introgression of genes from the wild forms

into the cultivated varieties. Due to their long life, there has been relatively little selection and the cultivated olives are assumed to be very similar to their progenitors (Liphshitz *et al.*, 1991).

1.2 Botanical Description

There are 30–40 *Olea* species distributed in Oceania, Asia, Africa, and the Mediterranean region (Johnson, 1957), which are included within the Oleideae subfamily, and in the Oleaceae tribe and are considered to be a diploid species ($2n = 2x = 46$). The 1C DNA content has been estimated for some cultivars to be approximately 2.2 pg (picogram) (Rugini *et al.*, 1996) corresponding to a genome size of 2200 Mb.

The genus is divided into two subgenera, *Olea* and *Paniculatae*; subgenus *Olea* is divided into section *Olea* (cultivated olive and wild relatives) and section *Ligustroides*. The section *Olea* includes the complex of *Olea europaea* L., the Mediterranean olive tree (Green and Wickens, 1989). These authors distinguished four subspecies according to their morphology and their geographical distribution: *O. europaea* ssp. *europaea*, of the Mediterranean basin; *O. europaea* ssp. *laperrinei* (Batt. and Trab.) Ciferri of the Sahara Massifs; *O. europaea* ssp. *cerasiformis* (Webb. and Berth.) Kunk. and Sund. of the Canary Islands and Madeira; *O. europaea* ssp. *cuspidata* (Wall. Ciferri) of Asia (China, India, Pakistan, Iran, South Arabia) and southeast Africa. *O. europaea* ssp. *europaea* includes the cultivated

varieties (var. *europaea*) and wild plants (var. *sylvestris* = Oleaster) of the Mediterranean basin. Green and Wickens (1989) and Zohary (1994) hypothesized that the different wild forms of the *O. europaea* complex have contributed to the evolution of cultivated olive.

The great diversity of germplasm in the Mediterranean region represents an important source for future breeding. To exploit this resource, more information is needed, including the level and distribution of variability within cultivars, the genetic relationships among cultivars and with wild olives, the markers linked to the main traits under selection, characterization of genes, their regulation and expression at different developmental phases in different tissues. Until few years ago, germplasm of *O. europaea* has been identified by morphological descriptions. Then, biochemical and molecular analysis have been developed by using isozymes and randomly amplified polymorphic DNA (RAPD) primarily for cultivar identification, study the taxonomy within the genus and the relationships between cultivated and wild olives, for determining the origin of cultivars, and to map and to identify the markers linked to the most important agronomic traits. Molecular biology allows the evaluation of the genetic variability in a precise manner across technologies that emphasize any genetic changes by means of biochemical analysis (isoenzymes and allozymes) or DNA marker analysis using restriction fragment length polymorphism (RFLP), RAPD, inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP). Isozymes were first used for cultivar identification (Pontikis *et al.*, 1980) and subsequently cultivar identification has been based on DNA markers, i.e., RAPDs (Bogani *et al.*, 1994; Fabbri *et al.*, 1995; Cresti *et al.*, 1996; Wiesman *et al.*, 1998; Bartolini *et al.*, 2006a), using different protocols (Belaj *et al.*, 1999, 2001, 2002; Mekuria *et al.*, 1999; Barranco *et al.*, 2000; Gemas *et al.*, 2000; Besnard *et al.*, 2001b). More than 200 cultivars in the World Olive Germplasm Bank (Cordoba, Spain) have been characterized using relatively few primers. Cultivars with common and distant areas of origin show high similarity, which supports the hypothesis of a multilocal selection for most cultivars (Besnard *et al.*, 2001a). AFLP data are available on the cultivars of most of the Italian

regions (Baldoni *et al.*, 2000; Bracci *et al.*, 2006) and some cultivars from Tunisia (Grati Kamoun *et al.*, 2006). For cultivar characterization, SSRs (Rallo *et al.*, 2000; Sefc *et al.*, 2000; Carriero *et al.*, 2002; Cipriani *et al.*, 2002; Marra *et al.*, 2006; Reale *et al.*, 2006), ISSR (Pasqualone *et al.*, 2001; Vargas and Kadereit, 2001), and sequence-characterized amplified region (SCAR) markers have also been attempted from RAPDs (Hernandez *et al.*, 2001b).

The two different forms of wild olives (oleasters and feral forms) have been distinguished by isozymes (Lumaret *et al.*, 1997) and AFLP (Angiolillo *et al.*, 1999). RFLP markers have been identified from mitochondrial (mtDNA), chloroplast (cpDNA), and nuclear DNA, and together with isozyme markers, they provide evidence for the survival of indigenous oleaster populations, particularly in the western part of the Mediterranean region. The domesticated olive represents a subset of the genetic variation in genuinely wild olive populations that persist today (Lumaret and Ouazzani, 2001). The phylogenetic relationships within the *Olea* genus have been assessed by nucleotide variation at a noncoding cpDNA region in some *Olea* species and four groups could be distinguished: *Olea capensis* and *Olea lancea*, both belonging to subgenus *Ligustroides*, the *Olea* forms from southeast Africa, those from Asia, and the taxa of northwest Africa and the Mediterranean region, which include the cultivated olive (Baldoni *et al.*, 2002). These relationships are consistent with those previously reported with cpDNA-RFLPs (Lumaret *et al.*, 2000).

Five distinct chlorotypes were distinguished on the basis of cpDNA restriction patterns, and wild olives were more variable than cultivars (Amane *et al.*, 1999). With mtDNA-RFLPs, different mitotypes have been identified. Within wild populations, a clear distinction exists between the eastern and western Mediterranean regions. For cultivars, a more complex mitotype distribution exists, probably due to exchanges of plant material during the long history of olive cultivation (Besnard and Berville, 2000; Bronzini de Caraffa *et al.*, 2002a, b). The colonization history of *O. europaea* has been analyzed based on internal transcribed spacer 1 (ITS-1) sequences, RAPD, and ISSR markers in Macronesia (Hess *et al.*, 2000). *O. europaea* retroelements have also been identified (Hernandez *et al.*, 2001a) and the

total copy number in the genome has been estimated (Stergiou *et al.*, 2002). The cpDNA-RFLP, performed on 15 *Olea* taxa, has resulted in the clear separation of species in Section *Olea* from those of Section *Ligustroides*; the latter group of species appears to possess ancestral variants of cpDNA (Lumaret *et al.*, 2000). AFLP analysis has been used to distinguish *Olea* species from the Indian Ocean and Oceania from the species of East Africa and Asia, which clustered together. *Olea* spp. from northwest Africa, e.g., *O. laperrinei* and *O. maroccana*, showed a higher level of similarity with cultivated and wild samples of *O. europaea* (Angiolillo *et al.*, 1999). Analysis performed on ribosomal DNA (rDNA), cpDNA, and mtDNA polymorphisms has confirmed the ancient separation of the genus *Olea* into two subgenera: Asia-Australia (subgenus *Paniculatae*) and Africa (subgenus *Olea*). Three distinct main phyla were characterized: *O. cuspidata* – *O. chrysophylla* (= *cuspidata* phylum) of Asia, *O. africana* (= *africana* phylum) of East and South Africa, and *O. europaea* – *O. laperrinei* – *O. maroccana* – *O. cerasiformis* (= *europaea* phylum) of the Mediterranean region, Sahara, and northwest Africa (Besnard and Bervillé, 2002; Besnard *et al.*, 2002a, b). According to cpDNA and mtDNA analysis, *O. europaea* ssp. *maroccana* represents a well-differentiated and relict taxon, belonging to the same subtaxon as *O. europaea* ssp. *guanchica* (Medail *et al.*, 2001).

Two tandemly repeated sequences (81 bp and pOS218) were isolated by Katsiotis *et al.* (1998) and were localized by *in situ* hybridization on the chromosomes, showing a telomeric or interstitial location. These elements are also present in oleasters, *O. chrysophylla* and *O. africana*, but are absent in other genera of the Oleaceae, i.e., *Phyllirea*, *Forsythia*, *Ligustrum*, *Parasyringa*, and *Jasminum*. The amount and organization of heterochromatin and the frequency of other tandemly repeated sequences (*Oe*Taq80, *Oe*Taq178, *Oe*GEM86) indicate that DNA content is positively correlated with the copy number of DNA repeats in the genomes of different *Olea* forms (Bitonti *et al.*, 1999; Lorite *et al.*, 2001; Contento *et al.*, 2002). Cytological hybridization of these tandem repeats has made it possible to distinguish most of the olive chromosomes and to reveal structural heterozygosity in three chromosome pairs (Minelli *et al.*, 2000).

1.2.1 Use of molecular markers in genome analysis and improvement of olive

The first RAPD analysis was carried out on 11 cultivars by Bogani *et al.* (1994) by using five arbitrary primers. Only 17 primers produced reproducible polymorphic markers among all 40 primers used by Fabbri *et al.* (1995) that were able to distinguish 17 different cultivars, but no apparent relationship between olive cultivars and their geographic origin was shown. Many other researchers used RAPD to distinguish among both cultivars of *O. europaea* and diverse species of *Olea* (Roselli *et al.*, 1995, 2002; Cresti *et al.*, 1996; Vergari *et al.*, 1996; Perri *et al.*, 1997; Cavalieri *et al.*, 1998; Wiesman *et al.*, 1998). A high number of fragments per primer in RAPD analysis were found by Mekuria *et al.* (1999). The same authors found a high degree of genetic similarity among the cultivars Manzanillo, Kalamata, Nevadillo, Picual, Correggiolo, while for small genetic variations somatic mutations might have occurred. As far as the other analyzed cultivars were concerned, a high degree of heterogeneity was found and the authors suggested that misnaming has occurred on several occasions. Recently AFLP has been used for marker fingerprinting and has produced a sufficient number of polymorphic fragments to separate the different species of *Olea* and to group together the genotypes within the cultivar of *O. europaea* (Angiolillo *et al.*, 1999; Baldoni *et al.*, 2000). By this technique, genotypes from different geographical origin were grouped together using five AFLP primers, which produced about 290 polymorphic fragments. A combination of three RAPD primers was proposed for the identification of the cultivars (Besnard *et al.*, 2001b). Chloroplast genomes have been a primary source of molecular data for genetic variability analyses in *Olea* as they are maternally inherited. Despite the variability of nuclear markers, RFLPs of chloroplast DNA showed a low level of variation among cultivars (Amane *et al.*, 1999). The relatively rapidly evolving chloroplast spacers and introns are widely used in systematic studies at low taxonomical ranks in *Olea* (Baldoni *et al.*, 2002; Besnard *et al.*, 2002a, b). Besnard *et al.* (2000) used RFLPs to analyze mtDNA and cpDNA of a progeny derived from the cross of male sterile “Olivière” and “Arbequina”, and established that male sterility is maternally

inherited and is associated with the chlorotype CCK and mitotype MCK.

SSRs or microsatellites are of great interest as they are co-dominant, robust, and highly informative and reproducible. The advantages of SSRs compared with other molecular markers in the olive have been demonstrated by Belaj *et al.* (2003). Many SSRs have been isolated in the olive, (Rallo *et al.*, 2000; Sefc *et al.*, 2000; Carriero *et al.*, 2002; Cipriani *et al.*, 2002; De La Rosa *et al.*, 2003) a large number of genotypes have been used by these authors and others for genotyping olive germplasm resources (Belaj *et al.*, 2004; Lopes *et al.*, 2004; La Mantia *et al.*, 2005; Montemurro *et al.*, 2005; Chessa *et al.*, 2006; Diaz *et al.*, 2006). Intravarietal allelic differences at SSR loci have been identified (Cipriani *et al.*, 2002; Lopes *et al.*, 2004; La Mantia *et al.*, 2005) that could be explained as somatic mutations, occurring in long-living trees that reproduce by vegetative means (Cipriani *et al.*, 2002; La Mantia *et al.*, 2005).

The SNPs represent the next generation of markers in plants because by using this technique it is expected to obtain a better understanding of the genetic basis for complex characters in plants including production, development, strategies of adaptation to abiotic and biotic stresses, and to realize the potential of genetic improvement programs. Genes of known sequence that putatively influence the traits of interest can be selected and mapped, and functional maps can be constructed (Schneider *et al.*, 2002). A new SNPs technology was reported (Muleo *et al.*, 2006), high-resolution DNA melting analysis (HRMA), for either mutation scanning of polymerase chain reaction (PCR) products or genotyping with unlabelled probes, which have a very high sensitivity for scanning procedures, for detecting substitution and indel mutations, and for detecting heterozygous SNPs within a large PCR product.

1.2.2 Genome map

Normally, for observing the characters expressed at the mature phase such as, fruit characteristics, oil content and quality, tree architecture and size, it is necessary to attend 15–18 years of the life of a olive tree; however, it is possible to force seedling growth in order to obtain early flowering and first production at 3–4 years after germination (Santos Antunes *et al.*, 1999). Nonetheless, for

certain traits it would be more efficient to select the plants at the seedling stage, if molecular markers linked to these traits were available. Those markers co-segregating with the trait of interest are the tools for marker-assisted selection (MAS). The first attempts to construct a linkage genome map for olive have been reported (Baldoni *et al.*, 1999; De la Rosa *et al.*, 2003) using the progeny derived from the cross between Leccino and Dolce Agogia. The use of an F₁ progeny, allowing the use of markers in the condition of pseudo-testcross, limits the number of useful markers and results in the construction of two distinct maps; one for each parental line. This can be overcome by the use of markers that produce a very high number of scorable markers in each reaction, i.e., AFLPs and by the use of co-dominant markers, i.e., microsatellites, to anchor the different maps in a general one for the species. In the “Leccino” map, 249 markers (110 RAPDs, 127 AFLPs, 8 RFLPs, and 3 SSRs) were linked. This resulted in 22 major linkage groups and 17 minor groups with less than 4 markers. In the “Dolce Agogia” map, 236 markers (93 RAPDs, 133 AFLPs, 6 RFLPs, and 4 SSRs) were linked. In this case, 27 major linkage groups and 3 minor groups were obtained.

Another integrated molecular linkage map was constructed, based on RAPD, SCAR, and SSR markers, on a population of 104 individuals generated from F₁ full-sib family of a cross between “Frantoio” and “Kalamata” (Wu *et al.*, 2004). Twenty-three linkage groups were mapped for “Kalamata”, covering 759 cM of the genome with 89 loci and an average distance between loci of 11.5 cM. Twenty-seven linkage groups were mapped for “Frantoio”, covering 798 cM of the genome with 92 loci and an average distance between loci of 12.3 cM. For the integrated map, 15 linkage groups covered 879 cM of the genome with 101 loci and an average distance between loci of 10.2 cM. The size of the genomic DNA was estimated to be around 3000 cM. The maps are the starting point for studies on the structure, evolution, and function of the olive genome.

1.2.3 Gene cloning

Genes, codifying proteins involved in essential biochemical processes, were isolated in olive. The first one was the stearyl-acyl carrier protein desaturase (Baldoni *et al.*, 1996), the enzyme key

Table 1 Number of sequences in olive deposited in the GeneBank up to September 30, 2006

Nuclear sequences	109
Ribosomal	90
cpDNA + mtDNA	136
EST	26
sequence-related amplified polymorphism (SRAP) markers	44
Retrotransposon	16

in the trial of the fatty acids insaturation of the membranes and of the accumulation lipids. It determines the formation of the double connection that transforms the stearic acid into oleic acid, the main constituent of olive oil. Different genes codifying for a family of proteins that induce allergic symptoms in humans were also isolated. These proteins differed among themselves in length but all of them are expressed during the formation of the pollen (Lambardero *et al.*, 1994; Villalba *et al.*, 1994; Asturias *et al.*, 1997; Batanero *et al.*, 1997; Ledesma *et al.*, 1998).

There are 109 nuclear gene sequences deposited in the GenBank up to September 30, 2006. Most of them are the sequences of the cytoplasmatic genome, while the number of expressed sequence tags (ESTs) is reduced (Table 1). Many genes encoding key enzymes for fatty acid biosynthesis and modification, triacylglycerol synthesis and storage have been isolated. These are enoyl-acyl carrier protein reductase (*ear*), stearyl-ACP desaturase, $\omega 6$ plastidial desaturase (*fad6*), $\omega 3$ plastidial desaturase (*fad7*), cytochrome b5 (*cyt b5*), $\omega 6$ cytoplasmic desaturase (*fad2*), $\omega 3$ cytoplasmic desaturase (*fad3*), acyl Co-A diacylglycerol acyltransferase (DGAT), and oleosin (Giannoulia *et al.*, 2007). Stearyl-ACP desaturase (Baldoni *et al.*, 1996) is the key enzyme for the conversion of saturated stearic acid (C18:0) to mono-unsaturated oleic acid (C18:1), the main component of olive oil and responsible for its high dietary value. A complementary DNA (cDNA) has been isolated encoding a fatty acid desaturase, which is responsible for biosynthesis of a trienoic acid, linolenic acid, a major component of chloroplast membranes and a precursor of the oxylipins, i.e., methyl jasmonate. The latter is important in signal transduction pathways relating to plant development and responses to stress, and have been studied in leaves, anthers, and embryos (Poghosyan *et al.*, 1999). Two cytochrome

b₅ genes and their spatial and temporal patterns of expression have been characterized during floral and fruit development (Martsinkovskaya *et al.*, 1999). The expression of oleosin gene is strongly embryonic-stage dependent and the transcript reaches maximum levels at midtorpedo stage and thereafter declines, coinciding the stages of most oil accumulation in embryo tissues, while in mesocarp tissues oleosin gene is not expressed (Giannoulia *et al.*, 2007). A triterpene synthase cDNA, cloned from olive leaves by PCR amplification using primers designed from oxidosqualene cyclases, codes for the lupeol synthase protein (Shibuya *et al.*, 1999). The differential expression of DGAT genes has been evaluated in different olive tissues (Giannoulia *et al.*, 2000). The sequence of a partial cDNA clone (1402 bp) of the chloroplast ribulose 1,5-bisphosphate carboxylase large subunit (*rbcL*) gene of olive has been compared with that of other genera in order to establish the systematic position of the Oleaceae family (Wallander and Albert, 2000). Similarly, the chloroplast *ndhF* gene has been sequenced for phylogenetic studies (Olmstead *et al.*, 2000).

1.3 Economic Importance

Olive is one of the most important tree crop species of the Mediterranean area and its global importance is rapidly increasing. The traditional area of olive cultivation is the Mediterranean basin, which accounts for 95% of the olive groves of the world. Spain, Italy, and Greece produce almost 99% of the world's olive oil and more than 80% of table olives (IOOC, 2005). In 2005, world cultivation was approximately 7.5 million ha, on 60% of which represents the main crop, with a production of approximately 14 442 metric tons destined to both oil and fresh consumption (FAOSTAT, 2006). The world production and consumption trend of olive oil in the last 30 years have increased significantly and will continue to increase considering the recent introduction of its cultivation in Japan, Australia, China, South America, and South Africa. It has been estimated that in the year 2008 olive production will rise by about 3 million tons, as a result of an increase of olive cultivation of 120 000 ha per year.

The traditional Mediterranean diet has demonstrated to be the most beneficial for maintaining good health due to the high quantity of

antioxidants present in olive oil, fruit, and vegetables. Among the antioxidants, α -tocopherol is the main tocopherol found in olive with concentrations varying from 98 to 370 mg kg⁻¹ depending on the olive cultivar and the environmental conditions (Psomiadou *et al.*, 2000; Rotondi *et al.*, 2004). Furthermore, olive has a characteristic-free acid content and composition as well as total polyphenol concentration that makes olive oil one of the healthiest vegetable oils, and efforts to improve other oil-producing crops have attempted to imitate its composition (Cahoon and Shanklin, 2000; Bruner *et al.*, 2001; Rahman *et al.*, 2001). Oil accumulation occurs in olive fruit mesocarp and to a lesser extent in the olive seed (Harwood and Sanchez, 2000). The low percentage of oil (which rarely exceeds 25% of fresh weight), the peculiar composition of oleic acid (a C18:1 mono-unsaturated fatty acid), which is almost 75% of the total triacylglycerides while linoleic acid is only 10%; however, the fatty acid composition could change according to variety and environmental conditions, and the parameters of quality in native olive oils make the genetics of oil metabolism of special interest for the clarification of gene regulation involved in oil accumulation.

Although the production of oil and table olives is by far the most important reason for olive cultivation, the species serves other important functions. In several countries of the Mediterranean basin the olive has been closely associated from ancient times with the traditional landscape, and its presence, together with cypress, is typical of religious sites. In these areas, it is one of the few species with characteristics of high drought resistance, and it is suitable for use in calcareous, rocky, and sloping soils. Finally, its wood is of great value due to its natural durability and beauty for furniture and parquet; hence, in some areas of south of Italy, it represents a secondary source of profit from olive groves.

1.4 Traditional Breeding: Objectives; Tools and Strategies; Achievements and Limitations

More than 1000 cultivars under cultivation originated mostly from selections made by growers over many centuries, and often they have not been replaced by new genotypes suitable for intensive

cultivation. Plants for intensive cultivation should be of reduced size, reduced apical dominance, and constant and high productivity in terms of fruit and oil. Selection of constant self-fertile genotypes having fruit with a suitable composition of fatty acids with high polyphenol content and with a correct pattern of fruit ripening in terms of intensity of the degradation of phenolic and pectic compounds and tolerance to frost, salts, drought, diseases, and pests should be attempted. No cultivated species contain desirable traits such as, biotic stress resistance, adaptation to extreme environmental conditions, different plant vigor, and canopy architecture that could be introgressed into cultivars by conventional breeding or by gene transfer. Those plants are conserved distant from the center of origin.

Breeding in olive is difficult to carry out due to the very long juvenile period (>10 years) and different flower fertility: full male sterility (Tombesi, 1978; Fontanazza, 1993; Bartolini and Guerriero, 1995), self-fertility (Fontanazza *et al.*, 1990), and partial self-fertility. In the last two cases, flower emasculation is necessary; however considering the much reduced size of the flower and the low percentage of setting (1–2%) this operation is tedious and time consuming. Some genes controlling flower initiation in *Arabidopsis* have been identified (Yanotsky, 1995; Simpson *et al.*, 1999). Two of them, *LEAFY* (*LFY*) and *APETALA1* (*API*), have been used to transform *Citrus* (Pena *et al.*, 2001) and results showed flower initiation in 1-year-old plantlets.

At present, the juvenile period can be shortened by an accurate method of plant training by continuous illumination, although the length of juvenility is genotype dependent. In addition, the choice of right cultivars as pollinators, such as Leccino and Verdale, can reduce the juvenile period of offspring, and also cv. Leccino when used as mother plant, contrary to the progeny of cv. Coratina, Picholine, and Tanche (Bellini, 1990; Fontanazza and Bartolozzi, 1998). Up to now, only a few cultivars have been obtained by breeding programs, producing interesting plants at F₂ generation. Two cultivars, one for table and one for oil extraction; the last one being also resistant to peacock spot, have been selected. It seems that some characters as vigor, leaf size, fruit shape are dominant in F₁ progenies (Rugini and Lavee, 1992; Bellini *et al.*, 1995). Other characters

such as carpological traits of fruit, blossoming intensity, fruit set, period of fruit ripening and yield have been considered (Bellini, 1993; Parlati *et al.*, 1994; Bellini *et al.*, 1995). Recently, the embryo rescue technique has been applied with successful results in hybrid plants between the cultivars Mary and an Iranian local variety because many plants were obtained that grew normally in pots (Hossein Ava and Hajnajari, 2006). In Spain, agronomic characterization of selections from crossings between the main Spanish cultivars by using different methodologies for the reduction of the juvenile period of seedlings revealed interesting characteristics of the offspring regarding oil content and yield efficiency (Leon *et al.*, 2006). In Italy, field evaluation of selections derived from a breeding program between several local varieties showed a different vegetative and productive behavior among the genotypes (Bartolini *et al.*, 2006b; Pannelli *et al.*, 2006; Ripa *et al.*, 2006).

Other attempts of genetic improvement, such as clonal selection and mutation through γ -irradiation, did not give good results. Protoplast technology, haploid culture, somaclonal variation, changes in ploidy level, and somatic hybridation have also been tried with rare success due to the absence of efficient methods of regeneration from *in vitro* grown tissues.

The selection of new clones has not been completely explored, so it is important to search new accessions to solve the present lack of suitable cultivars (Morettini, 1972; Berenguer, 1978; Fontanazza, 1987). More recently, clonal rootstocks have been selected with high rooting ability and ability to control scion vigor (Baldoni and Fontanazza, 1990) and many accessions, particularly for small tree size are under observation (Pannelli *et al.*, 1993). The criteria for the selection of rootstocks include good rooting potential, control of scion vigor, resistance to drought, saline and heavy soils, resistance to root diseases, and graft compatibility with the scion. Very few studies have addressed the selection of clonal rootstocks. Preliminary work was concerned with the influence of rootstocks on scion performance. Some rootstocks are able to control scion vigor and confer cold tolerance (Pannelli *et al.*, 2002).

Stable mutation rarely occurs in olive, while chimeric plants of cv. Frantoio (Roselli, 1982) and

Canino (Parlati *et al.*, 1994) have been observed. On the contrary dwarf plants from artificially irradiated rooted cuttings of cv. Ascolana Tenera have been obtained by Roselli and Donini (1982) and they are used as ornamentals. Other compact mixoploid mutants have been obtained following irradiation of “Frantoio” and “Leccino” plantlets resulting in the selection of dwarf “Leccino”, which is self-sterile and late blooming and seems to be a promising rootstock (Pannelli *et al.*, 1990). The mixoploid mutants allowed us to select triploid and tetraploids plants (Rugini *et al.*, 1996).

1.4.1 Protoplast technology

Protoplast fusion is useful to produce hybrids from cross-incompatible varieties and species. Furthermore, protoplasts allow genetic modifications by introducing exogenous DNA by liposome or cellular organelles. Viable protoplasts from hypocotyls, cotyledons, and leaves of micropropagated shoots were isolated and cultured, and in some cases microcalli have been achieved (Rugini, 1986; Cañas *et al.*, 1987; Mencuccini, 1991; Perri *et al.*, 1994a). This technology cannot be applied at this moment in olive because regeneration is not possible yet from derived protoplast callus. Although protoplast technology may be useful in genetic improvement research in olive, one should not expect good results, since somaclonal variation and chromosome rearrangement could heavily change the expected results substantially.

1.4.2 Haploids and homozygotic plants

Considering the self-sterility characteristic of olive plants and the long juvenile phase, the achievement of homozygous plants by self-fertilization seems very improbable. Homozygous plants would be of great interest for isolation of mutants and recessive traits. Anthers and ovary or pollen and ovule cultures should be explored with the aim to produce dihaploid plants by doubling the chromosome number. Many efforts have been paid in the past to recover haploids of olive but with little success (Mulè *et al.*, 1992; Perri *et al.*, 1994b). Recently, promising results were obtained employing a new method of isolated microspore culture, which led to cell division and pro-embryos

formation in the cultivar Arbequina (Bueno *et al.*, 2006).

1.4.3 Somaclonal variation

For increasing the induction of genetic variation, the selection of spontaneous mutants that could occur during the regeneration phase, a common and efficient technique consists in the co-cultivation of callus, tissues, or organs in a medium containing a selective agent in order to create a selective pressure conditions that induce changes or allow the selection of already changing cells. The selective agent can be of biotic or abiotic origin such as, ionizing radiation, fungal filtrate, purified toxin of some pathogens, high osmotic pressure, etc. Reports on somaclonal variation in olive have not been reported yet following plant production through the three methods of plant production. Some phenotypic variations of *in vitro* grown shoots or whole plants have been observed but they were only of temporary effect of juvenility induced by the *in vitro* culture. Today, this technique can be exploited by using the potent cyclic somatic embryogenesis of some cultivars as Canino.

1.4.4 Changes in ploidy level

Rugini *et al.* (1996) described the procedure by which triploid and tetraploid plants of the cultivar Leccino have been produced. Mixoploid plants, obtained by γ -irradiation of cuttings, produced some bigger fruits than others, which resulted in triploid seedlings. Tetraploids plants were obtained by *in vitro* culture of axillary bud stimulation of those mixoploid plants. During *in vitro* culture the mixoploid shoots split spontaneously in two kinds of shoots; they were recognizable by a wider, longer, and thicker leaves than diploid ones. Isolation of stable tetraploids by organogenesis has so far been unsuccessful. In field trial $4n$ plants were smaller than $2n$ plants. These plants are characterized by more flexible branches and the absence of a juvenile phase and by having larger leaves and fruits than plants with normal ploidy level while the intensity of blossom were scarce (Figure 1). When tested as rootstocks, they seemed to reduce the growth of the scions (Figure 2).



Figure 1 Phenotypic characteristics of branches, leaves, and fruits of diploid and tetraploid plants of cultivar Leccino

2. DEVELOPMENT OF TRANSGENIC OLIVES

Very few reports have been presented on biotechnological approaches of improving olive trees. Genetic improvement in olive has targeted to change some traits including self-fertility, oil content and composition, parthenocarpy, abiotic stress tolerance, plant habit, fruit ripening, regular cropping, and resistance to pathogens and pests; however, the low efficiency of both conventional and modern techniques for genetic improvement suggests that genetic transformation by alien gene transfer could be a promising technique to speed up the development of new cultivars. Several genes of different origins that govern these characters have been identified; however, development of efficient regeneration methods from mature tissue of elite selections remains the limiting factor for using this technology. In olive, two procedures have been used to accomplish gene transfer into plant cells: *Agrobacterium*-mediated gene transfer and microprojectiles coated with DNA.

Most of the reports on transformation and isolation of transgenic plants of olive have involved *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. These bacteria transfer a segment of the transfer DNA (T-DNA) known as Ti or ri plasmid into the nuclear genome of the plants (Chilton *et al.*, 1982). The efficiency of transformation depends on several factors such as, bacterial strains, plant cell competence, transgenic



Figure 2 Tetraploid plant (a) and diploid plants (b) of cultivar Leccino used as rootstocks in the experimental field at the Università della Tuscia, Viterbo

cell selection, and plant regeneration methods. In olive, the best strain of *A. rhizogenes* resulted is 1855 (E. Rugini, personal communication).

Biolistic has emerged recently as a simple and efficient technique for stable genetic transformation of recalcitrant species, and for studying gene expression and regulation. Most recently, transient expression in various tissues of woody species have been achieved by using microprojectile-mediated DNA delivery (Ellis *et al.*, 1991; Goldfarb *et al.*, 1991; Charest *et al.*, 1993; Duchesne *et al.*, 1993; Hay *et al.*, 1994); however, genetic transformation requires the optimization of several factors, including the *in vitro* regeneration systems, the methodologies for DNA delivery, and a high expression of the introduced gene in the plant tissue. The only DNA delivery system being used in olive up till now is DNA delivery by microprojectile bombardment (Lambardi *et al.*, 1999).

In olive, genetic transformation experiments have been realized aiming at the induction of

modified growth habit and/or disease resistance (Rugini *et al.*, 2000).

2.1 Explants Used

The recovery of transgenic plants depends on the number of transformed cells and the possibility to differentiate into shoots or embryos. The most common explants experienced in olive for transformation were leaf discs, leaf petioles, zygotic and somatic embryos, and whole shoots to induce rooting by *A. rhizogenes*. Immature zygotic embryos, capable of inducing somatic embryogenesis, were also used with some success (Rugini and Fedeli, 1990; Mencuccini *et al.*, 1997). Attempts to produce transgenic whole plants from leaf discs or petioles of cultivars Moraiolo and Dolce Agogia with the aim that the transgenic cells for *rol* genes, which are involved in hormone sensitivity, could lead to regeneration, was unsuccessful but only transgenic callus

was recovered (Mencuccini, 1994; Mencuccini *et al.*, 1999). At present, the best explants used for transformation, which allowed recovery of transgenic plants by using *Agrobacterium*, are somatic embryos of the cv. Canino, which are able to produce continuous cycles of cycling embryogenesis from epidermal cells (Rugini *et al.*, 1999).

2.2 Selective Marker

The *neomycin phosphotransferase II* (*nptII*) gene, encoding resistance to the antibiotic kanamycin, is very commonly used in transformation experiments. This gene inactivates other aminoglycoside antibiotics such as paromomycin, neomycin, and geneticin (Yoshikura, 1989). Efficient selection of the transformed cells is usually carried out with the selective antibiotic kanamycin at a concentration 50–100 mg l⁻¹ according to the explant. The critical concentration of kanamycin and the time of application of the selective pressure must be previously determined for each type of explant. The *in vitro*-grown apical olive shoots stop growing even at 30–40 mg l⁻¹ of kanamycin; while 20–25 mg l⁻¹ kanamycin in liquid medium is sufficient to inhibit the greening of single white embryos, grown in the dark, after exposure to light. The selection of transformed embryos, based on visual observations, attached to morphogenic masses (in the solid medium) take a long time even with high concentration of kanamycin, greater than 100 mg l⁻¹, probably due to the scarce mobility in the tissue. Considering this fact, selection of transgenic embryos should be carried out on embryos or embryogenic masses using a shaken liquid medium, initially for 2 days in the dark to avoid kanamycin degradation, then in the light (Rugini *et al.*, 2000).

2.3 Strategies for Regenerating Whole Plants

At present, besides zygotic explant, regeneration of shoots or embryos from mature explants of cultivars can be carried out only by using *in vitro*-grown shoots, thus an efficient method of micropropagation is essential.

2.3.1 Axillary bud stimulation

The first efficient protocols for *in vitro* propagation of the olive cultivars by axillary bud stimulation were reported by Rugini (1984) who formulated a medium specifically for this specie (Olive Medium or OM, or Rugini Olive Medium from DUCHEFA). The medium was formulated by analyzing the mineral element contained in the mature seeds. This medium is currently used for propagation of more than 50 olive cultivars in commercial plant production in Italy (Zuccherelli and Zuccherelli, 2000).

The axillary bud stimulation allows the rapid growth, better performance, and higher qualitative characteristics of the *in vitro*-grown plants than plants derived from propagation by stem cutting. Furthermore, *in vitro* propagation permits a high percentage of rooting of the explants even in those cultivars considered recalcitrant to propagation by cutting (Briccoli Bati and Lombardo, 1995). Exogenous treatment with putrescine is normally essential for rooting induction and for improving root system quality and plant growth (Rugini and Wang, 1986; Rugini, 1992; Rugini *et al.*, 1997). The marked effect of putrescine on rooting is presumably due to a general low content of endogenous polyamines in olive shoots at the end of proliferation, in comparison with other woody species (e.g., walnut and chestnut), which are not affected by putrescine treatment (Rugini, 1992; Rugini *et al.*, 1993). It was proved that putrescine affects early root differentiation similar to exogenous treatments with H₂O₂, a product of polyamine catabolism. These observations led to the hypothesis that putrescine acts during the root induction phase by means of an increase in peroxidase activity through H₂O₂. In fact, a drastic reduction of polyamines was observed at the basal part of able-to-root microcuttings, within 48 h after shoot preparation, even when they were cultured in putrescine-containing medium (Rugini *et al.*, 1997).

In the experimental field, the micropropagated plants behave in the same manner as plants derived from traditional propagation by cutting if the initial explants are collected from mature twigs (Figure 2). On the contrary, if initial explants come from suckers, the plants start blossom 2–3 years later. Neither phenotypic variations nor genotypic alterations of *in vitro* propagated plants till now

were observed by PCR-RAPD analysis (Rugini *et al.*, 1996; Briccoli Bati *et al.*, 2000; Garcia-Fèrriz *et al.*, 2000; Leva *et al.*, 2000).

2.3.2 Shoot organogenesis

Regeneration by organogenesis system should be very suitable because it occurs from single cell avoiding thus the chimerism in whole plant; however, the frequency is low in both zygotic and particularly in mature tissues of the cultivars. Adventitious shoots and roots were obtained for the first time from seedling explants by Gilad and Lavee (1974). Subsequently, Wang *et al.* (1979) described two histologically different meristematic tissues originating from olive callus, namely, cellular masses and nodules, which evolved, respectively, into adventitious roots and buds. Direct shoot regeneration was induced on section of hypocotyls of seed (Bao *et al.*, 1980), on cotyledons of mature seeds (Rugini, 1986), on calli derived from cotyledon segments proximal to the embryo axes of the cultivars, Tanche and Picual. These results suggested that a gradient of regeneration potential existed from the proximal to the distal region of olive cotyledons (Cañas and Benbadis, 1988).

Mencuccini and Rugini (1993) obtained adventitious buds in petioles from *in vitro*-grown shoots of cultivars Moraiolo, Dolce Agogia, and Chalkidikis but the efficiency of shoot organogenesis does not exceed 40% and it is heavily dependent on the cultivar, the position of petiole along the shoot (apical nodes better than basal ones), the medium/hormone combination, type of tissue, and the maintenance of darkness during culture. Regeneration has never been obtained from leaf discs and midribs, neither from petioles excised from field or potted plants. The regenerated plantlets did not show any morphological differences compared with the micropropagated donor plantlets.

2.3.3 Somatic embryogenesis

The first reports on somatic embryogenesis both from immature zygotic embryos and from mature tissues of various cultivars were by Rugini (1988) and Rugini and Caricato (1995), respectively.

Somatic embryogenesis occurred from zygotic embryos in high percentage in cv. Dolce Agogia, Leccino, Frantoio, and Moraiolo when the fruits were harvested at around 75 days after full bloom, while before or after this period a drastic reduction of frequency with high callus production was observed. The high embryogenic capacity could be maintained for at least 2 months, by storing at 14–15 °C the two and a half-month-old fruits, in which the small embryos continued to develop without losing their embryogenic capacity (Rugini, 1995). These results were confirmed by Leva *et al.* (1995) in cvs. Picholine, Frangivento, and Frantoio, while Pritsa, and Voyiatzis (1999) reported somatic embryogenesis also from 126-day-old zygotic embryos of the cv. Chalkidikis. Orinos and Mitrakos (1991) observed the highest embryogenic capacity in calli derived from radicle explants of mature zygotic embryos of wild olive (*O. europaea* var. *sylvestris*) after 21 days of culture on inductive medium with high concentration of auxin. Dissimilar morphogenetic expression of calli from different parts of mature embryo was observed also by Mitrakos *et al.* (1992) in the cv. Koroneiki, in Moroccan genotypes (Abousalim *et al.*, 2006), and cv. S. Agostino (Rugini and Tarini, 1986) and Nabali (Shibli *et al.*, 2001).

Rugini and Caricato (1995) developed a novel strategy named as “double regeneration system” that allowed somatic embryogenesis in two cultivars Canino and Moraiolo. It consists of regenerating first the adventitious buds from leaf petioles of *in vitro*-grown shoots and then subculturing their small leaflets, with the aid of a microscope, in a proper medium, until pro-embryo masses appear. Numerous somatic embryos easily develop with the ability to produce cyclic somatic embryogenesis from the epidermis or from subepidermal layer. The cyclic somatic embryogenesis (represented by normal and abnormal embryos) can be maintained indefinitely even in hormone-free medium, and some can also germinate (Figure 3) (Rugini and Caricato, 1995; Lambardi *et al.*, 1999; Benelli *et al.*, 2000; Rugini *et al.*, 2005). The conversion of normal embryos into plants is difficult; however, they in the field show juvenility with a retardation of blossom 3–4 years later than micropropagated plants by axillary bud stimulation. The continuous production of embryos from epidermal cells could



Figure 3 Cyclic somatic embryogenesis of cultivar Canino

be a great advantage in regenerating plants from transgenic cells avoiding callus formation, which could be a wide source of undesirable genetic variability. Somatic embryo germination can be achieved after their isolation into shaken liquid medium containing zeatin (Rugini and Caricato, 1995).

2.4 Alien Gene Transfer

Both an efficient *in vitro* regeneration method and the availability of suitable genes are essential factors. Up to now most of the transformation experiments have been carried out by using somatic embryos of cultivars because an efficient protocol for regeneration via cyclic somatic embryogenesis is available (Table 2).

2.4.1 Chimeric plants for increasing rooting ability

The rooting efficiency of *in vitro* grown shoots was increased by inoculating in the middle or in the basal part of the stem of the *in vitro* grown shoots with a scalpel infected with *A. rhizogenes* (Rugini, 1986, 1992). Roots that emerged from both the inoculation point and the basal part of shoots rarely resulted transformation with the *rol* genes (Rugini and Fedeli, 1990; Rugini, 1992; Rugini and Mariotti, 1992). Putrescine added to the hormone-free medium promoted early rooting and then the root system originated from shoots treated with *A. rhizogenes*, when transformed, produced numerous secondary roots during the first months on hormone-free medium. There were no results from the modification of the aerial parts in the field grown plants; however, according to our experience on plum trees, a drastic reduction of growth of the whole plant has been observed. Similar results have been obtained in cherry when it was grafted on a transgenic rootstock for *ri*-T-DNA (Biasi *et al.*, 2003; Gutiérrez-Pesce *et al.*, 2005).

2.4.2 Modifications of vegetative habits

Dwarf and semi-dwarf new varieties with changes in the canopy architecture regarding short and numerous shoots are more suitable for high-density olive groves. For other species, the use of the *rol* genes for plant improvement have demonstrated production of drastic effects on the plant architecture (Rugini *et al.*, 1991; van der Salm *et al.*, 1997, 1998; Perez-Molphe-Balch and Ochoa-Alejo 1998; Mercuri *et al.*, 2001; Zhu *et al.*, 2001), but scarce is the knowledge on the interaction of these genes with the genome of the

Table 2 Genetic transformation in olive cultivars

Goals	Cultivar and explant	Genes	Promoter	References
Chimeric plants Growth habit	Dolce Agogia, shoots	<i>ri T-DNA</i>	Natural	Rugini, 1986, 1992
	Moraiolo, Z. embryos	<i>rolABC</i>	Natural	Rugini and Fedeli, 1990
	Canino, S. embryos	<i>rolABC</i>	Natural	Rugini, 1995; Rugini <i>et al.</i> , 1999
	Dolce Agogia, petioles	<i>rolABC</i>	Natural	Mencuccini <i>et al.</i> , 1999
Disease resistance	Canino, S. embryos	<i>Osmotin</i>	35S	Rugini <i>et al.</i> , 1999
Reporter gene	Canino, S. embryos	<i>gus</i>	35S	Lambardi <i>et al.</i> , 1999

plant. Moreover, the development of plants with an extensive root system and/or with reduced water consumption could be an advantage in areas with scarce water availability. The *rolABC* genes of *A. rhizogenes* and the marker gene *nptII* were transferred to somatic embryos of the cultivar Canino by using the *A. tumefaciens* strain LBA4404 (Rugini and Caricato, 1995; Rugini *et al.*, 1999). Transgenic somatic embryos selected in liquid medium under light conditions converted into plantlets, which were transferred to the experimental field.

2.4.3 Increasing resistance to biotic and abiotic stress

The challenge of using biotechnological techniques is to generate broad resistance mechanisms, which are difficult to achieve by classical plant breeding approaches. *Osmotin* is a gene present in plants and belongs to the family related to proteins for pathogenesis, which are expressed under stress conditions, including pathogenic attacks. The tobacco *osmotin* gene has been employed for plant transformation since it is involved in the strategies developed by plants to resist to biotic and abiotic stresses. In fact, the role of *osmotin* as a defense protein in fungal disease is already known in grape (Salzman *et al.*, 1998) and potato (Liu *et al.*, 1994; Zhu *et al.*, 1995), although its accumulation has been proved to be induced also by abiotic stress conditions, such as osmotic or cold stresses (Zhu *et al.*, 1993; Grillo *et al.*, 1995). *Osmotin* gene is normally unexpressed in plant tissues, but 4 days of exposure to *Phytophthora infestans* induces the accumulation of both messenger RNA (mRNA) and protein (Zhu *et al.*, 1995). A longer exposure seems to be necessary to induce the response when low temperature or osmotic stress (NaCl) is used as elicitors. In fact, 7 days are necessary to reach a full response, i.e., an osmotin accumulation of 12% of the total cellular protein content (Grillo *et al.*, 1995). Moreover, in transgenic potato and tobacco plants osmotin overexpression is able to delay the development of *Phytophthora* disease symptoms (Liu *et al.*, 1994). However, its mRNA induction did not always lead to protein accumulation (Grillo *et al.*, 1995), suggesting transcription and post-translation controls.

In olive, transgenic plants for the *osmotin* gene under cauliflower mosaic virus (CaMV)

35S promoter, have been recovered from somatic embryos derived from petioles of cultivar Canino (Rugini *et al.*, 1999). The resistant clones were grafted on nontransgenic rootstock to speed up growth in order to evaluate their level of resistance to *Spilotea oleagina* (D'Angeli *et al.*, 2001). Other experiments are in progress with three genes from tobacco (*osmotin* + *chitinase* + *PRI*) in one construct with the CaMV 35S promoter. Transformed somatic embryos have been selected and plants have been established in the greenhouse (Rugini *et al.*, 2000).

Against olive fly (*Bactrocera oleae*), a group of researchers have developed a Minos-based transposon vector carrying a self-activating cassette that overexpresses the enhanced green fluorescent protein (EGFP). The self-activating gene construct combined with transposase mRNA present a system with potential for transgenesis in very diverse species (Koukidou *et al.*, 2006).

2.5 Evaluation of Transgenic Tissues and Plants

Transgenic plants for *rolABC* and *osmotin* produced in our laboratories were planted in the experimental field of the University of Tuscia, Viterbo, in 1999 under the authorization of the Environmental Minister for further evaluations.

2.5.1 Transgenic plants for *rolABC* genes

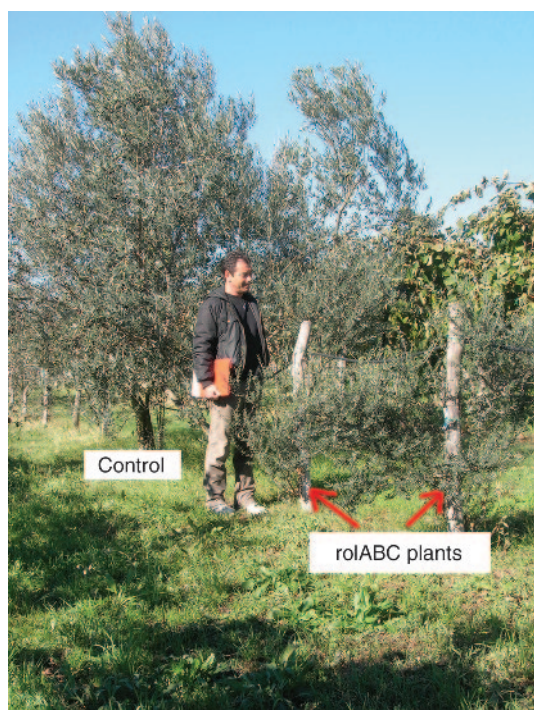
Reverse transcriptase PCR (RT-PCR) analysis of the putative transgenic clones confirmed the stable integration of the *rol* genes in the genome of the plants and their correct transcription.

Canino plants transgenic with the *rol* genes showed different expression of each gene in the organs studied although they are under the same promoter. The alien genes had influence on plant architecture; leaves had a wider angle of insertion on the stem and they showed changes in leaf blade shape with an increase number of internodes per stem due to the reduced internodes length. Furthermore, leaves of plantlets of transgenic clone-5 are enriched in chlorophyll content (Table 3). The transgenic clones showed different plant development and growth as demonstrated by the significant increase of the number of

Table 3 Some characteristics between transgenic plants of Canino for *rolABC* and the wild type

	Canino <i>rolABC</i>	Canino wild type
Chlorophyll A (mg g ⁻¹ fw)	18.42 ± 1.2	11.69 ± 1.2
Chlorophyll B (mg g ⁻¹ fw)	8.05 ± 1.5	4.14 ± 0.4
Total chlorophyll (mg g ⁻¹ fw)	26.47 ± 3.5	15.82 ± 1.6
Leaf area (mm)	36.3 ± 7.1	57.7 ± 12.2
LAM	17.7 ± 1.6	20.8 ± 2.1
LAMin	3 ± 0.4	4.3 ± 0.4
Number of internodes	10.6 ± 2.5	7.8 ± 1.1
Number of lateral shoots	6 ± 1.22	0.2 ± 1.4

internodes, wider angle of insertion of the leaves with a reduced area and less apical dominance as demonstrated by the outgrowth of lateral shoots giving them their bushy structure (Figure 4). Molecular analysis showed an overexpression of glycosyl-transferase elongation factor, *psbB*, *psbC*, and *psbD* genes in the transgenic clones compared to control plants (Figure 5). RT-PCR analysis showed different expression of *rol* genes in leaves

**Figure 4** Reduced size and bushy aspect of 8-year-old transgenic plants for *rolABC* and control of cultivar Canino

and stem from the *in vitro* grown clone-5, and from roots, leaves, and stems of the *in vivo* clone-5. Furthermore, molecular analysis showed that not all the transcript of the *rol* genes is polyTail. The levels of polyTail transcript of all *rol* genes are different in the diverse tissue of plantlets. The less amount of transcript, independently of the *rol* gene has been detected in leaf tissue. This behavior is not related with the culture conditions because the same trend has been found both *in vitro* and *in vivo*. Total amount of transcript did not differ among the tissue, as seen by *rolA* transcript, but it is different in *rolB* and *rolC* (Miano *et al.*, 2004). Grafting combinations with the transgenic clones as rootstocks were carried out with a nontransgenic cultivar and are under evaluation.

2.5.2 Transgenic plants for *osmotin* gene

After three growing seasons, the transgenic plants for the *osmotin* gene showed a compact phenotype with short internodes and reduced size of the leaves, exhibiting less vigor (Rugini *et al.*, 2000). The variation in the phenotypic characteristics was much more evident in the field-grown plants (Figure 6 and Table 4), probably due to the longer maintenance of the juvenility traits. In fact, in the greenhouse conditions, the low light intensity, responsible for a higher shoot elongation, and the constriction of the root system in the pot, may allow the plants to overcome the juvenility phase faster.

To check the possible acquired resistance to fungal pathogens, some transgenic plants were grown in pots under high humidity conditions together with plants affected by heavy symptom of *S. oleagina* (Cast). Hugh. The *in situ* localization of *osmotin* was performed by using a polyclonal primary antibody against osmotin jointed with a secondary antibody with alkaline phosphatase (AP) activity. The AP reaction in presence of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (NBT/BCIP) substrate caused a purple color into the plant tissues. In transections of 1-year-old stems, coming from the apical internodes of field-grown plants, osmotin labeling was observed in epidermal and subepidermal tissues and, rarely, also in the most superficial layers of the cortical parenchyma. At

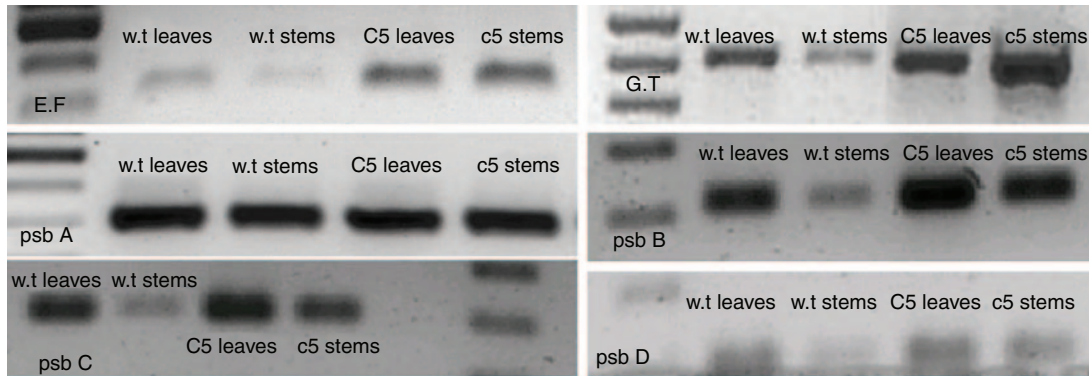


Figure 5 Semi-quantitative expression levels of Elongation Factor (E.F.), Glycosyl transferase (G.T.), and psb A, B, C, D between Canino wild type (w.t.) and Canino clone-5 (c5) from leaves and stems

cellular level, the protein was mainly localized around the vacuole (Figure 7). The signal was also present in the phloem and, mainly, in the cambium, but to a lesser extent than in the superficial tissues. In some cases, immature deutoxylem cells resulted were also labeled (D'Angeli *et al.*, 2001).

Inoculation tests for the presence of *S. oleagina* spots showed that one transgenic genotype seems to possess higher susceptibility than control plants (micropropagated and regenerated by somatic embryogenesis), whereas the other one shows a lower susceptibility. However, we cannot draw any conclusion since in these first phases of fungal infection the differences may be not completely defined (Table 5).

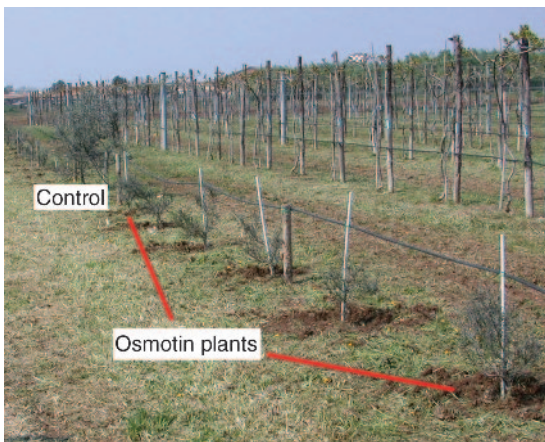


Figure 6 Transgenic plants of cultivar Canino for the *osmotin* gene showed a very compact phenotype and low vigor after three growing seasons

In transections of the apical third internodes of 1-year-old twigs from unstressed plants grown in the field, osmotin labeling was observed in the epidermal and subepidermal tissues and rarely, also in the most superficial layers (A–B) of the cortical parenchyma. At cellular level, the protein was mainly localized around the vacuole (C). The signal was also present in the phloem and, mainly, in the cambium (not shown), but at lower levels than in the superficial tissues. In some cases, immature deutoxylem cells were also labeled. No signal was ever observed in the unstressed leaves. Our results confirm the organ specificity of the protein accumulation controlled by a post-translation pathway able to negatively interfere with the gene constitutive expression of 35S promoter.

Table 4 Leaf and stem characteristics of the transgenic olive plants, cv. Canino, for the *osmotin* gene

	Osm 1	Osm 2	Control
Palisade/spongy	0.62	0.63	0.58
Stomata density (stomata mm ⁻²)	0.6	0.37	0.41
Cutin thickness (mm)	4.6 ^(a)	4.3 ^(a)	5.1
Thickness (mm)	414	397 ^(b)	405
Area (cm ²)	0.38 ^(b)	0.35 ^(b)	3.08
Internodes length (cm)	1.5 ^(b)	1.09 ^(b)	2.77
Xylem + phloem height (mm)	672 ^(a)	704	773
Vessel area (mm ²)	233 ^(b)	239 ^(b)	689
Cortex/stem	0.23	0.32	0.35

^(a)Low significance $P < 0.05$

^(b)High significance $P < 0.01$

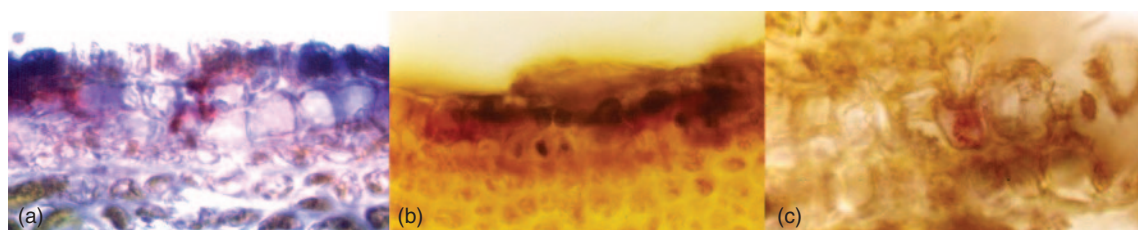


Figure 7 Osmotin labeling was observed in the epidermal and subepidermal tissues and, rarely, also in the most superficial layers (a and b) of the cortical parenchyma. At cellular level, the protein was mainly localized around the vacuole (c). In some cases, immature deuterioylem cells were also labeled. (The labeling is in purple). No signal was ever observed in the unstressed leaves

Table 5 *S. oleagina* infection detected on control and transformed leaves

	Total leaves	Healthy leaves	Leaves with spots	% Infection
Control Mz ^(a)	48	40	8	16.7
Control Esy ^(b)	50	36	14	28.0
Osm 1	46	40	6	13.0
Osm 2	46	26	20	43.5

^(a)Control plants coming from micropropagation

^(b)Control plants coming from somatic embryogenesis, the same as osm 1 and osm 2 clones

3. FUTURE ROAD MAP

3.1 Potential Applications of Gene Transfer for the Genetic Improvement in Olive

Genetic transformation programs in this genus are focused on solving the main agronomic and commercial problems by transfer of one or more foreign genes or to limit the expression of the endogenous ones. Large availability of genes is essential to start a good program of genetic improvement by this technology, both from olive species and from other species or genera. About 400 sequences have been deposited in the GeneBank up to now (Table 1).

3.1.1 Fruit ripening and oil quality

The regulation of fruit ripening and the increase of oil content and quality are of much interest, since many cultivars ripen early and have fruits with high water content and early fruit drop. This pattern may be corrected by generating transgenic plants with a reduced ethylene biosynthesis, by using an antisense gene. The introduction of antisense genes for blocking ethylene synthesis (Oeller *et al.*, 1991)

or for reducing polygalacturonase activity (Smith *et al.*, 1988) could be useful.

With regard to the oil quality, two molecular strategies can be used to modify the oil composition and content: the alteration of the major fatty acid levels by suppressing or expressing a specific key enzyme in lipid biosynthesis and/or the creation of an unusual fatty acid. Stearoyl-acyl carrier protein denaturase is the key enzyme for the conversion of saturated stearic acid (C18:0) to mono-unsaturated oleic acid (C18:1), the main component of olive oil and responsible for its high dietary value (Baldoni *et al.*, 1996). Temporal and transient expression of the S-ACP denaturase gene has been studied during fruit development (Haralampidis *et al.*, 1998), and its expression is developmentally regulated with earlier expression in embryos than in mesocarp. By antisense suppression or co-suppression of oleate denaturase it was possible to increase oleic acid (C18:1) from 24% to 80% in the transgenic soybean (Kinney, 1995, 1996a, b, 1997; Yadav, 1996) and in *Brassica* seed by the antisense expression of a stearoyl-ACP denaturase gene (Knutson *et al.*, 1992). The same strategy was adopted to increase stearate acid (C18:0) by up to 30% both in canola and rapeseed (Auld *et al.*, 1992; Falco *et al.*, 1995). Unusual fatty acids can be produced in one plant by transferring a gene encoding the specific biosynthetic enzyme. An example can be seen in canola, which naturally does not produce laurate (C12:0), while a new transgenic genotype does contain laurate. The oil content of some nut crops used for cosmetics, such as almond, could be increased or their composition could be modified by these techniques. The oxidative stability of the oleic acid in soybean oil can be also improved as reported by Ellis *et al.* (1996).

3.1.2 Parthenocarpy

A major limiting factor for olive productivity is the alternate bearing and often the lack of pollination that causes this problem in areas with less intensive olive cultivation. Olive shows some tendency to natural parthenocarpy under some environmental conditions, although the fruits remain very small because they do not develop normally. Genes inducing parthenocarpy would permit the development of parthenocarpic fruits with regular size, and would overcome the problem related to self-sterility among olive cultivars. Self-sterility is under the control of the placental-ovule-specific *defh9* gene regulator sequence, expressed during early flower development. The introduction of the parthenocarpy gene of *Arabidopsis* may allow fruit development and subsequently boost the yield, and may help to overcome the pollination problem as already successfully tried in eggplant (Rotino *et al.*, 1997). In addition, the reduction of flower numbers observed in kiwi may contribute to solve the waste of energy and to reduce the alternate bearing (Rugini *et al.*, 1997).

3.1.3 Abiotic stress tolerance

Cold hardiness is a very important objective for olive improvement (Bartolozzi and Fontanazza, 1999). Transformation could involve the antifreeze protein (Hightower *et al.*, 1991), the overexpression of the superoxide dismutase gene, which permits the repair of frost-damaged cells (McKersie *et al.*, 1993; Van Camp *et al.*, 1994), and the overexpression of *Arabidopsis CBF1* gene enhances freezing tolerance by inducing genes associated with cold acclimatization, and could also be useful in olive too (Jaglo-Ottosen *et al.*, 1993).

3.1.4 Plant architecture

Another possibility offered by biotechnology for modifying the growth and reproductive behavior is the modification of plant receptors in order to change the light perception. The transformation with phytochrome genes (*phyA*, and/or *phyB*, sense or antisense), which together with other photoreceptors control plant development (circa-

dian rhythms, apical dominance, blossoms, growth and fruit ripening, photosynthesis products distribution, development of photosynthetic systems, transpiration control, and hormone synthesis), may contribute to develop plants with high agronomic value and suitable for very high density planting (Tucker, 1976; Vince-Prue and Canham, 1983; Baraldi *et al.*, 1992; Muleo and Thomas, 1993, 1997).

3.1.5 Resistance to pathogens and pests

The challenge of using biotechnologies in this area is to generate broad resistance mechanisms that have been difficult to achieve with classical breeding approaches. The use of antifungal genes or a pool of genes may be effective for improving resistance to fungal diseases, particularly *Verticillium* wilt and *S. oleagina*. *Osmotin* gene, *protein inactivating ribosomes* genes (Longmann *et al.*, 1992), *stilbene synthase* gene (Hain *et al.*, 1993), hydrolytic enzymes such as *chitinase* and *glucanase* (Broglie *et al.*, 1991; Yoshikawa *et al.*, 1993), *glucose oxidase* gene (Wu *et al.*, 1995), and polygalacturonase (*PGIPs*) that inhibit the activity of endo-polygalacturonases released by fungi on invasion of the plant cell wall could improve the olive defense system.

Regarding bacterial diseases, it is not so easy to introduce this kind of resistance into plants, because the product of the gene should act in the intercellular space; however, some species with enhanced resistance to bacteria have been obtained by introducing genes encoding bactericidal polypeptide, i.e., *thionin* (Carmona *et al.*, 1993), *attacin E* (Norelli *et al.*, 1994), the *synthetic analogue MB39* of cecropine (Mills *et al.*, 1994), and *cecropine* (Huang *et al.*, 1997). Particular attention should be paid to human lysozyme, which confers resistance to both fungi and to bacterium *P. syringae* in tobacco plants (Nakajima *et al.*, 1997).

The isolation of homologous genes from olive cultivars with low susceptibility to the fruit fly (*Bactrocera oleae*) is a potential way to achieve resistance to this pest in susceptible cultivars. Although, in the battle against insect attack, the *Bt* gene from *Bacillus thuringiensis* (Vaecck *et al.*, 1987) has successfully been introduced in other species with encouraging results, and could be an

alternative way to prevent olive fly damage. In addition, gene or genes isolation from resistant genotype, such as Bianca of Tirana should be attempted.

3.2 Expected Technologies

For olive transformation, the critical areas include the development of efficient regeneration protocols for elite cultivars, and the identification and evaluation of genes and specific promoters for useful traits.

Many potentially useful genes have already been isolated from several species, and could be introduced into olive separately or as multiples. The transformation selection procedure should be improved to increase public acceptance, possibly by replacing traditional selection markers (*nptII*) with other markers (*gfp*, *lecI*, *ipt*, *pmi*, *xyla*), although the percentage of escapes is quite high (>40%) in fruit crops (May *et al.*, 1995; Pena *et al.*, 1995, 1997; Mourgues *et al.*, 1998; Perl and Eshdat, 1998). Endo *et al.* (2001) suggested that the *ipt* gene from *A. tumefaciens* might be a suitable selectable gene. A multi-auto-transformation (MAT) vector, which combines the use of genes that stimulate growth and morphogenesis for positive selection of transformed cells with an excision mechanism to remove the markers and allow recovery of plants with normal phenotypes, could be very useful. The vector, which includes *ipt* and maize transposable element *Ac* for removing the *ipt* gene, seems to be promising (Ebinuma *et al.*, 1997). Other approaches might involve *xyla* (xylose isomerase) (Haldrup *et al.*, 1998) and *pmi* (phosphomannose isomerase) genes, both of which confer the capacity to use nonmetabolizable substrates. Finally, for highly regenerative cultures, it may be possible to eliminate the marker gene and select on the basis of physiological or morphological parameters, e.g., the response to toxins, culture filtrate, salt/drought resistance, etc. (Graham *et al.*, 1996).

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Almond

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Almond, *Prunus dulcis* (Mill) D.A. Webb, is a stone fruit crop originated from one or more of the many wild species that evolved in the deserts and lower mountains slopes of Central and Southwest Asia, presently China and Iran. Today, we can still find many natural populations along the mountains from Tianshan in Kurdistan, extending across Turkistan, Afghanistan, and Iran (Felipe, 2000).

In spite of the ancient origin of almond culture, its wide distribution and economic importance, still not much is known about its origin, ancestors, or domestication place (Browicz and Zohary, 1996; Ladizinsky, 1999). It is believed that cultivated almond originated from spontaneous crosses among wild species, such as *Prunus fenziiana*, *Prunus bucharica*, and *Prunus kuramica*, among others, occurring in their local habitats where they coexisted, as suggested by several Russian botanists (Grasselly and Crossa-Raynaud, 1980). Another possible origin, however, could be the domestication of populations of *Prunus communis* L., with which *P. dulcis* shares numerous morphological characteristics (Kester *et al.*, 1991). In some regions, it is also possible that another ancestor, *Prunus webbii*, may have contributed, which is found wild in various regions in Balkans, Sicilian, and other Mediterranean areas (Godini,

1979; Socias i Company, 1998), including Spain (Felipe, 2000).

Independent of almond's origin, mankind played a major role in its breeding and dissemination. Almond populations growing close to civilization centers facilitated dissemination since its edible part happens to be its propagation unit. In this way, almond could easily spread from Central Asia to Persia, Mesopotamia and, profiting from the commercial routes, reach all the primitive civilizations (Kester *et al.*, 1991). The introduction in the Mediterranean area is thought to have occurred approximately 4000 years ago by the Phoenicians, followed by the Hebrews and the Greeks. Around 450 BC, almond culture spread from the Greek to the Mediterranean coast, and several important centers appeared in Portugal, Spain, France, Italy, Morocco, Tunisia, Greece, and Turkey (Kester and Ross, 1996).

Nowadays almond grows in countries bordering the Mediterranean sea, as well as in California where it was probably introduced in 1700 AD, in the Northwest of Mexico, in Chile, Argentina, South Africa, Australia, and western China (in Xinjiang province).

Until the 19th century, almond culture has not evolved much and the plants were propagated almost exclusively by seed, which led to the emergence of many different cultivars. Grafting is a practice with only some 150 years, which, however,

permitted to expand many different local cultivars selected by the farmers.

All around the world one can find several thousands of almond varieties being cultivated, with a high number in each country, where almond is a traditional culture. It is the case of Spain, with 200, Italy with 600, Turkistan with 2000, and even a small country like Portugal with over 150 cultivars (Monteiro *et al.*, 2003). In western China more than 40 almond cultivars have been described and used in almond production (Zhu, 1983), and so far over 100 genotypes have been found in Xinjiang province, where in old times the Silk Route connected China to the West (Socias i Company, 1998; Xu *et al.*, 2004).

1.2 Botanical Description

Cultivated almond appears in many references, even recent ones, with different denominations: *P. dulcis* (Miller) D.A. Webb., *Prunus amygdalus* Batsch., *P. communis* (L.) Archangeli, or even *Amygdalus communis* L. In 1768, the nomenclature *P. dulcis* L. was used for the first time for the cultivated almond producing sweet fruits, however, in 1964, because of the discrepancies occurring along the years concerning nomenclature, the General Committee for Botanical Nomenclature proposed the scientific name *P. dulcis* (Miller) D.A. Webb, accepting as synonym to those in almost all previously used classifications. According to *Flora Europaea* (1978), the botanical classification of almond is the following: family Rosaceae, subfamily Prunoideae, genus *Prunus* L., subgenus *Amygdalus* (L.) Focke, and species *dulcis* (Miller) D.A. Webb (*A. communis* L., *Amygdalus dulcis* Miller, *P. communis* (L.) Archangeli, *P. amygdalus* Batsch.) and *P. webbii* (Spach) Vierh.

Almond and peach (*Prunus persica* (L.) Batsch) are very close and hybridize easily. Often interspecific hybrids are found in places where both species grow (Felipe, 2000). Some of these hybrids are used as rootstocks in orchards, as it is the case of the clone GF-677 selected during the 1950s (Grasselly and Duval, 1997).

Almond tree is about 5–12 m high, has a strong root system with a plumbed root, white or pink flowers, and entomophilous pollination. Leaves are narrow, long, sharp-pointed, and denticulated.

In the northern hemisphere (examples given hereafter), flower initiation occurs around July and August, although this varies from year to year, not only among cultivars but also within the same cultivar, indicating that the process is under both endogenous and environmental cues (Lamp *et al.*, 2001). The flower buds are borne laterally in leaf axils on long shoots and shoot spurs. Flower buds enclose a single terminal flower and no leaves. One to several flower buds can form on a single spur (Lamp *et al.*, 2001). Almond flowers are hermaphrodite and have five fused sepals, five petals, a variable number of stamens (20–40), and one carpel with two ovules.

After floral induction, flower buds initiate floral organ differentiation, which is arrested usually around October when buds enter a dormancy period in preparation for autumn/winter. Promotion of dormancy seems to be independent of environmental signals. Budburst occurs mostly during winter and occasionally in spring (between January and March) depending on the varieties and locations as a consequence of dormancy breaking and usually before vegetative growth. Break of dormancy happens only after the chilling requirements, characteristic of each cultivar, have been fully satisfied. A recent study indicates that blooming time is a consequence of differences in chilling requirements among cultivars with only a small contribution from the heat requirements (Egea *et al.*, 2003).

Microsporogenesis occurs from November to January, depending on the cultivar, and ovary maturation occurs immediately after bloom (Rugini, 1986). When a pollen grain lands on a receptive stigma surface, it germinates and grows a tube that enters the style to ultimately reach the ovule. Gametophytic competition will favor genotypes with higher competitive abilities and result in the vigor of the subsequent sporophytic generation (Hormaza and Herrero, 1994).

As it happens in other Rosaceae and in the Solanaceae species, almond displays the gametophytic self-incompatibility system. Although not much appreciated by farmers, self-incompatibility is an important feature in fruit trees, ensuring outcrossing as the predominant mechanism for breeding, thus maintaining hybrid vigor. Self-incompatibility (SI) is controlled by a single multiallelic *S*-locus (De Nettancourt, 1977) coding

for RNase linked to S-locus (S-RNases) expressed in the pistil and for F-box proteins (SLF/SFB) expressed in the pollen (Ushijima *et al.*, 2003). The F-box proteins function as adaptors, interacting with self- and nonself-S-RNases differently, so that only the latter are driven to degradation via the ubiquitin/proteasome pathway (Kao and Tsukamoto, 2004). Therefore, S-RNases in self-(incompatible)-crosses remain active and able to degrade the pollen tube RNA, thus blocking its further growth.

Fertilization depends not only on the male gametophyte but also on the female gametophyte, the embryonic sac. In peach as in almond, despite the presence of two ovules, only one seed is usually produced. In peach, this is due to the fact that while one ovule grows following anthesis, the development of the other one is arrested (Arbeloa and Herrero, 1991; Rodrigo and Herrero, 1998). This situation was also suggested for almond since one ovule was consistently observed as much smaller than the other and both were still immature 1–2 days after pollination (Cortal *et al.*, 1999). Nevertheless, double kernels also occur, which is considered as a disadvantage for breeding. Double kernel formation is under genotypic and environmental control, with low temperatures at bloom being a major reason and justifying that earlier blooming flowers produce the higher number of double kernels (Spiegel-Roy and Kochba, 1981; Socias i Company and Felipe, 1994).

The almond fruit is a drupe, with a nonedible and green velvet skin covering the fleshy hull (exocarp and mesocarp, respectively). The shell (endocarp) can be from very hard (hard shell) to soft (paper shell) and, as already mentioned, contains one or two edible nuts. Twin seeds (developed within the same seed coat) may also form, and sometimes originate weak seedlings that were found to be haploids (Gulcan, 1975). Further studies have shown that low vigor twin seedlings are aneuploids (Martínez-Gómez and Gradziel, 2003).

Almond culture requires a hot and dry climate during summer, resisting well to drought when the rootstock is appropriate. Because of a high sensitivity to root anoxia, a good drainage is desirable.

The fruits are collected mechanically or manually by shaking the tree and the fruits with

a rod. The hull should be removed shortly after collection.

Almond trees have a juvenile phase of 3–4 years but in terms of production they are considered nonbearing until the fifth growing season, or 4 years after planting. An orchard can be productive for over 30 years.

Due to the repeated multiplication through seeds occurring under environmental conditions and natural selection, almond evolved in a way similar to forest species. Man also contributed to this evolution by successively selecting the best and larger seeds for growth. The conjugation of these two factors originated characteristic populations (ecotypes) corresponding to defined geographic regions (Felipe, 2000). Nowadays almond propagation is done by grafting on various rootstocks (mainly bitter almond, peach, and hybrids that usually provide higher vigor and yields). The plants for grafting are prepared in nurseries and planted during dormancy after 1 or 2 years.

Having a low chromosome number ($2n = 2x = 16$) almond has a relatively simple genome structure, with one of the smaller DNA contents per genome (0.54–0.55 pg/2C), similar to its close relative peach (Arumuganathan and Earle, 1991), which makes it an ideal species for molecular and genetic studies. For peach, Baird *et al.* (1994) reported a genome size of 290 Mbp (mega base pair) per haploid genome. The low chromosome number and the small genome size are features, in general, applicable to various *Prunus*, reason why they have been recommended as good models for woody species (Messeguer and Messeguer, 1998).

1.3 Economic Importance

Almond tree is grown for its edible fruits (seeds) that are industrially exploited in various countries with Mediterranean climate (Kester and Asay, 1975).

In the United States (California), almond orchards occupy an area that approaches 180 000 ha, reaching mean yields of 1800 kg nut ha⁻¹ in large irrigated farms, completely mechanized and mostly with a single producing cultivar. With much less almond growth area as compared to Spain (607 000 ha) or Tunisia (169 000 ha), California ensures much higher production (Kaska,

2001). According to the Food and Agriculture Organization (FAO, 2005), the top five almond producers in 2005 were the United States with 666 600 metric tons of nut (42% of the world's production), followed by Spain (204 500 t, 13%), Syria (130 000 t, 8%), Italy (105 312 t, 6%), and Iran (80 000 t, 5%). Although California's high yields (2378 kg ha⁻¹) are not as high as those of Syria (3174 kg ha⁻¹, with a total area of 21 000 ha) or France (2647 kg ha⁻¹ with an area of 1700 ha) (Kaska, 2001), the growth area and the productive conditions of California can offer a very homogeneous and attractive product associated with an efficient commercial organization strongly dedicated to the international market. These factors combined with a good stock management and capacity to regularly supply the consuming market in the presence of world prices that depend on the American currency, justify the Californian supremacy, even in years of reduced cropping (Monteiro *et al.*, 2003).

Almond world kernel production has reached 500 000 t/year in 2004, with United States being the main producer (1.3–1.5 t ha⁻¹), followed by Spain (1.0–1.2 t ha⁻¹). The other European countries are, by order of importance, Italy, Greece, Portugal, and France, representing a very low production level (FAO, 2005). The fact that United States produces soft shell almonds strongly contributes to the high shelling percentage (30–50% shelling for semi-soft and 50–70% for paper shell) as compared to countries where hard shell varieties are grown (10–30% shelling) (Socias i Company *et al.*, 2007).

In 2005, Australia contributed with 16 500 t of kernel (2390 t ha⁻¹ of bearing trees). However, the Australian Almond Board expects to double this value by 2008 since from the total planting area, approaching 13 759 ha, about half was younger than 3 years and still nonbearing (Almond Board of Australia, 2006). Profiting from an optimized irrigation drip system and nutritional program, yields up to 3900 and 4602 kg ha⁻¹ were obtained with “Nonpareil” and “Carmel”, respectively (Kennedy, 2006).

In contrast to Australia and California, where almond is grown in intensive and irrigated system, most production in Spain (90%) and other Mediterranean countries is still largely traditional and unirrigated, although in certain areas there are newly established and high producing orchards (Cordeiro and Monteiro,

2002). Concerted efforts in several European countries supported launching almond culture in competitive conditions. For this purpose, organizations such as GREMPA (“Group de Recherches Méditerranéennes pour le Pistachier et l’Amandier”, former “Group de Recherches et d’Études Méditerranéennes pour l’Amandier”), FAO-CIHEAM dry fruits network (FAO-CIHEAM Inter-Regional Cooperative Research Network on Nuts), and also EU/Research Programs, have played a crucial role.

While Germany and Spain are the major markets of shelled almond (with 66 758 and 61 457 t, respectively), India is the largest global market for in-shell almonds, importing 25 785 t (corresponding to 94% of its consumption) (FAO, 2004). Most Indian imports are made from the United States but the preferred and more costly almonds are obtained from Iran and Afghanistan (Anonymous, 2004). Pakistan, China, and Lebanon are also big markets for in-shell almonds.

In European countries, almost all almonds are collected when they are completely mature (dry), which happens 6–8 months after full bloom. In most cases, only the kernel is used for consumption, but there is the exception of Turkey, Sicily, France, and a few countries in the Middle East and Scandinavia where the whole fruit is consumed collected a few weeks after blooming (Schirra, 1997; Felipe, 2000). The fruit hull has become commercially important as an animal feed, although initially it was only a by-product (Aguilar *et al.*, 1984).

Although the nutritional quality of almonds shows strong variations among cultivars (Socias i Company *et al.*, 2007), there are a number of characteristics that point to their high nutritional value for human consumption. Almonds have a high content of lipids, proteins, mineral salts, and vitamins (Souty *et al.*, 1971; Kester *et al.*, 1991; García-López *et al.*, 1996; Cordeiro *et al.*, 2001). Almonds have also been marketed as a good source of vitamin E, dietary fiber, and monounsaturated fat, the latter of which has been associated with decreased risk of heart disease (Spiller *et al.*, 1998). A good coverage of almond quality characterization and breeding for quality traits can be found in Socias i Company *et al.* (2007).

The almond kernel may be consumed raw or roasted, and when processed, it can be prepared

in many ways including as almond flour, salted, sliced, diced, in marzipan, nougat, ice cream, or almond milk, being used in snacks, cereals, confectionery, and desserts. The bitter or sweet oils extracted from almond kernel are often used in pharmaceutical industry and cosmetics. For direct consumption, usually only sweet almonds are used, as the bitter ones are toxic due to amygdalin, a cyanogenic glycoside they accumulate. The kernel also contains the enzyme amygdalase, which, by cell lysis and contact with amygdalin, processes it into benzylaldehyde and cyanide, which is poisonous (Schirra, 1997). The oil of bitter almonds, however, is much appreciated in food processing and in the pharmaceutical industry.

The wide variety of almond uses that evolved in different regions and cultures naturally entail different quality requirements (Socias i Company *et al.*, 2007).

1.4 Traditional Breeding

Almond is highly heterozygous and is one of the most polymorphic fruit species, due to its high SI and method of traditional culture using open-pollinated seedlings (Kester *et al.*, 1991; Socias i Company and Felipe, 1992; Socias i Company, 1998). Its broad genetic pool can be seen as an advantage for breeding, but it has also complicated the study of the species.

Up to now, the most important traits for breeding have been late flowering and self-compatibility as the more relevant for higher yield. These traits have required expensive and time-consuming progeny analyses, but in recent years they could have the support of biotechnology tools, specifically molecular markers, to assist in selection for earlier growth stages (for a recent review on almond genomics, see Martínez-Gómez *et al.*, 2006).

Grasselly and Olivier carried out crosses to obtain self-compatible descendants (Grasselly and Crossa-Raynaud, 1980). However, the first study on the transmission of self-compatibility in almond progenies was that of Socias i Company and Felipe (1977), who pointed out the heterozygosity of the self-compatible progenitor and the dominance of self-compatibility. Dicenta and García (1993b), by fluorescence microscopy, and Rovira *et al.* (1997), by fruit set, determined that SI is a monogenic trait with a multi-

allelic *S* series, the self-compatible allele (*Sf*) being dominant over the others. The SI-associated S-RNase genes represent an extreme example of allele polymorphism. Extensive studies have been conducted in almond for characterization of S-phenotypes by analysis of stylar S-RNases using nonequilibrium pH gradient electrophoresis (NEpHGE) of proteins produced in pistils collected prior to blooming (Basković *et al.*, 1997; Ballester *et al.*, 1998) and S-genotypes (DNA extracted from young leaves to analyze S-RNase genes) as well as for identification of a number of new alleles (Ma and Oliveira, 2001a, b, c, 2002; Certal *et al.*, 2002) (for an update see Martínez-Gómez *et al.*, 2006). Also, the transmission of *S*-alleles to the offspring was studied by NEpHGE and correlation of the S-proteins with the almond SI alleles (Batlle *et al.*, 1997; Basković *et al.*, 1997, 1998, 1999). Breeding strategies capable of ensuring self-compatibility in the offspring have been supported by different methods of analysis, namely, fluorescence microscopy, stylar ribonuclease assay, and *S*-allele PCR (polymerase chain reaction) (Bošković *et al.*, 1999; Tamura *et al.*, 2000; Ortega and Dicenta, 2003).

In most breeding programs aiming to introduce self-compatibility, the almond breeders have used Tuono, a self-compatible almond variety (Batlle *et al.*, 1997; Egea *et al.*, 2000; Duval *et al.*, 2001; Socias i Company *et al.*, 2004). Tuono is originally from Italy, from the region of Apulia where other *Prunus* such as *P. webbii* coexist. Because *P. webbii* is reported as self-compatible (SC) species, it has been assumed that SC trait was introgressed in almond from this wild relative (Godini, 1979, 2000), probably through *Sf* (Socias i Company, 2002), however, this has not yet been proved. Moreover, some data have shown that *P. webbii* carries S-RNase genes, and that only some accessions carry the *Sf* gene (Sánchez and Oliveira, 2005). Tuono has the *Sf* genotype, where *Sf* presumably confers self-fertility. The *Sf* allele has been identified in Tuono and this allele segregates with self-compatibility, with no RNase activity linked to *Sf* (Bošković *et al.*, 1999; Ortega and Dicenta, 2003). The *Sf* gene sequence was isolated from Tuono (Channuntapipat *et al.*, 2001; Ma and Oliveira, 2001c), however, the actual causes of SC remain unknown. According to Vargas *et al.* (1998) there are around 30 self-fertile almond cultivars worldwide, although none is widely cultivated.

Blooming time is another very important trait in almond, since this species has shown the earliest blooming time among all the fruit tree species. Early bloom restricted almond growing to regions with a low risk of winter/spring frosts. However, over centuries of almond growing, its culture has expanded into inland regions where the occurrence of winter/spring frosts is common. Thus, late blooming has become an important trait in almond breeding programs that attempt to develop later blooming cultivars to avoid frost damage. Breeding for late blooming has been conducted by numerous researchers (Kester, 1965; Grasselly, 1972; Vargas *et al.*, 1984; Socias i Company *et al.*, 1998a), and the transmission of this character in progenies has been studied (Kester *et al.*, 1973, 1977b; Dicenta *et al.*, 1993a). Blooming time is considered to be inherited quantitatively in most fruit species and most results confirm this type of transmission in almond. However, Kester (1965) suggested that in some progenies of the late-blooming budsport “Tardy Nonpareil”, a single dominant gene could be involved in determining the blooming date, since a bimodal distribution of blooming dates was observed among the seedlings obtained. A selection derived from “Tardy Nonpareil” was used to follow the transmission of the late-blooming allele for several generations and similar segregation patterns were found, confirming the dominance of the mutated allele (Grasselly, 1978). Thus, blooming time in almond seems to be determined by a major gene *LATEBLOOMING* (*Lb*), with late bloom dominant over early bloom, and by modifier genes inherited quantitatively.

The *Lb* gene was mapped using the progeny from a cross between “D-3-5” (a *Lb/lb* heterozygous genotype) and “Bertina” (a Spanish cultivar with unknown origin) (Ballester *et al.*, 2001) and was found to be located on the *Prunus* linkage group 4 (G4). Furthermore, blooming time has been studied as a quantitative character in an intraspecific peach F₂ population by Dirlewanger *et al.* (1999), in an interspecific (*P. persica* × *Prunus ferganensis*) backcross by Verde *et al.* (2002) and in the F₂ of the interspecific cross between the almond variety “Texas” and the peach variety “Earlygold”, by Joobeur (1998). Two candidate genes putatively involved in almond flowering have been assigned in the *Prunus* genome to linkage groups corresponding to the regions where two

quantitative trait loci (QTLs) for blooming time were detected (Silva, 2005; Silva *et al.*, 2005). However, to date, both the sequence and the specific function of *Lb* genes responsible for the blooming time QTLs remain unknown. One additional flowering time candidate gene was co-located with the position of the *Evergrowing* gene, which determines a nondeciduous behavior in peach (Silva *et al.*, 2005).

Other breeding goals have focused on tree behavior and on kernel quality. Breeding for a high fruit set apparently depends on a high number of factors, such as efficient pollination, flower fertility, and the maximum number of fruits that a tree can bear (Socias i Company *et al.*, 2004, 2005), although it has been suggested that in general, blooming density is a reliable indicator of the productivity (Sánchez-Pérez *et al.*, 2007b). Blooming density and productivity have been studied by Grasselly (1972), Kester and Asay (1975), Grasselly and Crossa-Raynaud (1980), Vargas *et al.* (1984), Dicenta *et al.* (1993a), and Socias i Company *et al.* (2004). However, only few studies have been performed regarding another important character, ripening time (Grasselly, 1972; Kester and Asay, 1975; Dicenta *et al.*, 1993b, 2005). On the other hand, there are many studies regarding the transmission of kernel traits including kernel weight, shell hardness, kernel percentage, double kernels, and kernel bitterness (Grasselly, 1972; Spiegel-Roy and Kochba, 1974, 1981; Kester *et al.*, 1977a; Vargas *et al.*, 1984; Dicenta and García, 1993a; Dicenta *et al.*, 1993b; Socias i Company and Felipe, 1994; Martínez-Gómez *et al.*, 2006). Bitterness was characterized as a monogenic trait, the bitter genotype being recessive (Heppner, 1923, 1926; Dicenta and García, 1993a; Vargas *et al.*, 2001; Martínez-Gómez *et al.*, 2006). Kernel bitterness is independent of the fertilizing pollen, instead being the result of the mother tree genotype and of the transportation of precursors from the leaves to the seed (Socias i Company *et al.*, 2007). In Australia, to understand control and improvement of the flavor of almond kernels, markers are being searched to identify the bitter or marzipan characters in breeding progenies (Wirthensohn, 2006).

As reported by Socias i Company *et al.* (2007), the present tendency is to improve almond varieties having specific quality traits for various applied

purposes. These authors have gone in detail through the different quality goals that breeders may target, from physical traits (kernel size, kernel shape, seed coat, double and twin kernels, and fruit hull) to biochemical characteristics (cyanoglucosides, phenolics, vitamins, and minerals, lipids, proteins, carbohydrates, fibers, and allergens). In most cases, the quality characterization of different varieties studied at regional level has not been conducted using uniform evaluation methods and therefore, is inconsistent (Socias i Company *et al.*, 2007). The resulting meager knowledge about the genetic control of the existing variability creates an obvious breeding difficulty. Moreover, to have a breeding strategy directed toward special product applications means that different varieties may be selected for different uses and that a separation must be ensured in collection, storage, processing, and commercialization routes.

Another target for breeding includes disease resistance, although almond is usually considered as challenging for a variety of diseases. Viruses from the Bromoviridae family, such as calico (infectious bud failure) caused by *Prunus* necrotic ringspot virus, or Prune dwarf virus cause systemic diseases in almond and other *Prunus* species. Viral diseases are, in most cases, insufficiently characterized, and no resistant varieties have been reported. Bacterial, phytoplasma and fungal diseases are also damaging in almond orchards. This is the case of bacterial spot (caused by *Xanthomonas campestris* pv. *pruni*) or brown rot, a common disease in fruit species (caused by *Monilia laxa*), leading to the decline of young branches especially in a wet spring. Anthracnose (caused by *Colletotrichum acutatum*) infecting blossoms, leaves, fruits, and small branches, was recently reported as a problem in the new almond orchards in Australia (Hall *et al.*, 1998) and also reported since 1995 as infecting numerous varieties in United States including “Nonpareil” (Adaskaveg *et al.*, 1999). Among the fungi affecting almond roots *Phytophthora* (particularly in clay soils), *Verticillium dahliae* and *Armillaria* are the most damaging. The use of appropriate rootstocks, with reduced susceptibility to these fungi, has been a good strategy to prevent negative effects in the scion (Grasselly and Duval, 1997). Almond scab, a disease caused by *Venturia carpophila* (*Fusicladium carpophilum* as imperfect form) and also affecting peach, plum, and apricot, can be very serious in

some regions leading to complete necrosis of the smaller branches.

The fungus *Phomopsis amygdali* (Del.) Tuset and Portilla (*Fusicoccum amygdali* Delacr. as imperfect form) is responsible for *Fusicoccum* canker or constriction canker, a disease responsible for important economic losses in almond orchards around the Mediterranean region (Cabrita *et al.*, 2004). *Fusicoccum* affects mainly twigs of the lower part of the trees, causing canker on shoots and necrosis on leaves (Barbé, 1993). This disease is favored mainly by high nitrogen levels in the soil and by wet weather, since it is transmitted just by rain. The diffusion of this disease is very slow but its eradication from an orchard is very difficult and expensive (Romero and Vargas, 1981). Because it takes several years for the plant to develop symptoms, the development of molecular markers linked to the tolerance/sensitivity phenotype could be of high value for breeding purposes and some studies have been conducted to meet this goal (Martins *et al.*, 2001, 2005; Cabrita *et al.*, personal communication). Additionally, the identification of specific alleles of resistance genes (R genes) from very tolerant varieties, may allow the development of markers exploitable in marker-assisted selection (Martins and Oliveira, 2005).

There are several almond breeding programs presently running worldwide. In Spain, three programs are being developed, in Reus, Zaragoza, and Murcia. The Reus program, conducted at IRTA, Mas Bové has focused on early selection performed during the 2nd to 5th growth years. Characters like leafing date (related to flowering time in early medium blooming varieties), vigor, growth, and branching are analyzed in the 2nd year, while blooming date, early cropping production, nut characteristics, self-fertility, and some disease sensibilities, are assessed in between the 3rd and 5th years (Vargas *et al.*, 2005). The breeding program of Zaragoza was focused on obtaining self-compatible and late-blooming cultivars (Socias i Company, 1990; Felipe, 2000). However, this program is continuing to obtain better performing cultivars, characterizing selections in relation to some flower traits and evaluating their self-fruitfulness based on the efficiency of their pollen tube growth and on the stigma/anthers position (Socias i Company *et al.*, 1998b). Main focus at present is directed to improve nut quality, based on multivariate

analyses of chemical components, assuming that a similar biochemical composition will result in similar commercial, industrial, sensorial, and nutritional qualities (Kodad, 2006; Socias i Company *et al.*, 2007). In Murcia, the main aim is to obtain self-fertility and late flowering to allow almond production in the inner and colder regions of Spain. The breeding program in Murcia is being assisted by molecular markers to fasten discarding self-incompatible varieties (Sánchez-Pérez *et al.*, 2004). The molecular identification of almond bitterness will also be implemented for regular application within a couple of years (F. Dicenta, 2006, personal communication).

The American almond breeding program has been directed to introduce self-fertility, improved growth habits, drought resistance, and kernel quality, using germplasm improvement, propagation techniques, and molecular markers (Gradziel *et al.*, 2001; Martínez-Gómez and Gradziel, 2002).

In Australia the breeding program started in 1997, aiming to produce superior scion and rootstocks adapted to local conditions (Sedgley *et al.*, 2002). Classical hybridization for improved nut and quality ranking is being combined with molecular breeding (for self-fertility, genetic fingerprinting, genome mapping, and transformation) and with pathological analyses for viruses and anthracnose (Sedgley *et al.*, 2002).

The availability of good molecular markers can make it easier to plan crosses in a breeding program, maximizing genotypic differences that often are not noticed at phenotypic level. Markers also make it possible to organize the germplasm in genetic pools, thus facilitating choices and considerably reducing the number of crosses requiring evaluation, and advance hybrid combinations to perform.

As a useful tool for breeding projects, a saturated linkage map (*Prunus* reference map) of 246 markers was constructed for an almond (cv. "Texas") × peach (cv. "Earlygold") F₂ progeny within a European project (Joobeur *et al.*, 1998). New markers have been added to this map (Aranzana *et al.*, 2003; Dirlewanger *et al.*, 2004; Howard *et al.*, 2005) and it now has 826 markers including 449 simple sequence repeats (SSRs), 361 restriction fragment length polymorphisms (RFLPs), 11 isozymes, and 5 sequence tagged sites (STSs) (P.

Arús, personal communication). These markers span a total distance of 524 cm with an average density of 0.63 cm/marker (Howard *et al.*, 2005) and a maximum interval of 7 cm (P. Arús, personal communication). The existence of the T×E map has been very useful for the *Prunus* research community, providing a highly polymorphic population for linkage studies and establishing a common terminology for linkage groups. The T×E map provides a set of transferable markers (anchor markers) of known map position that have facilitated the development of framework maps in others crosses. It has also allowed the location of different major genes and QTLs in a unique map, the search for markers to saturate specific genome regions or the establishment of map comparisons with other *Prunus* species. In fact, a recent study on comparative mapping, led to the conclusion that, at the genome level, the *Prunus* genus can be treated as a single genetic entity (Dirlewanger *et al.*, 2004).

Other maps have been constructed, namely using a cross between Nonpareil and Lauranne (Gregory *et al.*, 2005) or R1000 (a French cultivar) and Desmayo Langueta (Sánchez-Pérez *et al.*, 2007a).

The availability of expressed sequence tag (EST) collections is another feature that strongly facilitates the genetic and molecular analyses, especially in perennial trees. A growing collection of peach and almond ESTs has been released to public databases. The collection includes 9984 ESTs from a peach mesocarp complementary DNA (cDNA) library and 2794 ESTs from an almond developing seed cDNA library. A Rosaceae database has been recently created (including for instance genetic and physical maps, ESTs, bacterial artificial chromosomes (BACs), and markers) assembling a wide amount of genetic and molecular information and making it available to researchers working with this family (Genome Database for Rosaceae, GDR; <http://www.bioinfo.wsu.edu/gdr>) (Jung *et al.*, 2004).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Viruses are among the most difficult to eliminate disease agents. Although a virus disease damaging

to other Prunoideae as plum pox (responsible for sharka disease) is not known for almond, the establishment of new orchards with healthy certified trees does not guarantee virus eradication for long periods. Viruses from the Bromoviridae family (*Ilarvirus* genus), including Prune dwarf virus (PDV) and *Prunus* necrotic ringspot (PNRSV) are among those that spread rapidly in an orchard, even with good orchard management practices. PDV and PNRSV are transported through pollen, profiting from a living host and from a special delivery system—the pollinator insects, making cross-pollination (obligatory for seed set) a common source of infection. As recently observed by *in situ* reverse transcriptional-PCR, PDV aggregates inside the pollen grain forming a ring surrounding the vegetative and the germinative nuclei (Silva *et al.*, 2003). In spite of the infection, the pollen grain is able to germinate on a receptive stigma (Nyeki and Vertesy, 1974) and this allows the virus to colonize the pollinated tree. It is known that these viruses are able to infect a new orchard in less than 3 years causing dwarfism and various unquantified damages. Control of virus propagation through genetic engineering is at present one of the most efficient strategies achievable in most cases by the use of viral genes, such as coat protein genes or genes coding for movement proteins (for a revision on strategies to block virus proliferation see for instance Yadav *et al.*, 2005).

The fact that damage by virus infections has not yet been quantified in almond orchards, leads to the supposition that either the damage is not too severe, or perhaps it has not yet been possible to maintain clean orchards as control. Nevertheless, the strategy of genetic engineering for virus resistance (PDV) has been studied to understand possible mechanisms to interfere with the virus replication (see Section 2.1).

An important breeding goal eventually achievable through genetic engineering is self-compatibility for elite cultivars. With this strategy it would be possible to retain the nut quality and characters already accepted in the market while profiting from a minor modification that would allow the farmers to spare pollinator bees, pollenizer trees, and associated problems. Efficient pollination is crucial for seed set and for instance in California, shortage of honeybee colonies

due to mite (*Varroa destructor*) infestations has been a major constraint in almond production (Holden, 2006). Over 1 million honeybee colonies are needed to pollinate the 180 000 ha of almond orchards. In parallel to the programs attempting to obtain self-compatible cultures through conventional breeding, the use of genetic engineering may be important when production relies on one or few cultivars. For instance, California production is mainly ensured by a few cultivars, “Nonpareil” (38%), “Carmel” (17%), and “Butte” (11%) (data from 2004) (Boriss and Brunke, 2005). Although with a much smaller growth area, the situation in Australia is similar, with “Nonpareil” ensuring 51% of the production (Australian Almond Planting Report, 2006). Breaking down SI in these varieties would be possible by a transgenic approach using antisense technology or RNA interference, to block the expression of one of the S-RNases. Such a strategy would allow the farmers to avoid the use of pollenizer trees, thus having the chance of dedicating the whole farm area to the variety of their choice. However, considering what happens in orchards with self-compatible cultivars, it is not clear if pollinating bees would not still be needed. If honeybees could be avoided, a better control of virus diseases might be attainable.

2. DEVELOPMENT OF TRANSGENIC ALMONDS

2.1 Donor Gene and Transgene Construction

There are very few reports on almond transformation, and with the exception of the use of *rol* genes, all of them only mention the use of marker genes. Other genes of interest have not yet been introduced in almond. As reporter genes, the β -glucuronidase gene (*gusA*) under the control of CaMV 35S signals (promoter and terminator) was used (p35SGUSint plasmid) by a number of authors (Archillecti *et al.*, 1995; Miguel and Oliveira, 1999; Ainsley *et al.*, 2001a; Costa *et al.*, 2006). The luciferase (*luc*) gene under the control of 35S promoter and the Nos terminator was also employed in plasmid pAL23 (Miguel, 1998). Recently, a green fluorescent protein gene, with modified codon usage and designed to be

targeted to the endoplasmic reticulum (*mgfp-5-ER*) (Haseloff *et al.*, 1997), was also used with the 35S promoter and the Nos terminator (plasmid pBI121mgfp-5-ER) (Ramesh *et al.*, 2006).

As selectable marker genes, neomycin phosphotransferase (*nptII*) (with the Nos promoter and terminator) was used to achieve tolerance to kanamycin (Archilletti *et al.*, 1995; Ainsley *et al.*, 2001a). The herbicide R gene phosphinotricine acetyltransferase (*bar*) was used together with hygromycin phosphotransferase (*hpt*) both under the control of 35S promoter and Nos terminator sequences (plasmid pWRG2426) (Miguel, 1998). The use of phosphomannose isomerase gene (*pmi*) under the control of CPMS promoter (cestrum yellow leaf curling virus promoter—short version) and Nos terminator has also been reported (plasmid pNOV2819 *manA*) (Ramesh *et al.*, 2006).

Production of transgenic roots has been obtained by infection of microcuttings with *Agrobacterium rhizogenes* 1855 NCPPB and the transfer of *rol* genes was confirmed by Southern blot analysis (Damiano *et al.*, 1995).

The different transformation experiments conducted in almond are summarized in Table 1.

Studies aiming to identify good constructs for almond engineering for disease control, however, have also been conducted. In this case, virus diseases have been targeted, specifically the *Ilarvirus* (Bromoviridae) PDV (Raquel *et al.*, 2005). The gene constructs, based on the coat protein gene sequence of a PDV strain isolated from infected almond plants, were prepared from the viral RNA4, cloned in intermediate vectors (pCRII, through TA-cloning) and inserted in pGREEN vectors (JIC) (Hellens *et al.*, 2000) for plant transformation. Until present the gene constructs, driven by the CaMV 35S promoter, were only tested in the transformation of a model host plant (*Nicotiana benthamiana*) (see Section 3.1).

2.2 Transformation Methods Employed in Almond

For successful genetic transformation, the transforming nucleic acids have to travel across the cell wall (except when protoplasts are used), the plasma membrane, and the nuclear envelope, without

compromising cell viability. Although several transformation methods are available for plants (Oliveira *et al.*, 1996), *Agrobacterium*-mediated gene transfer, biolistic DNA transfer and, to a less extent protoplast-based direct gene transfer, are the major techniques used in virtually all transformation work aimed at direct production of improved cultivars (Siemens and Schieder, 1996; Birch, 1997).

2.2.1 *Agrobacterium*-mediated transformation

Almond tissues have been shown to be susceptible to infection by *Agrobacterium* (De Cleene and De Ley, 1976) and *Agrobacterium*-mediated transformation has been the method of choice for transformation experiments in almond.

Agrobacterium tumefaciens infection of almond leaf explants has been performed following procedures such as wounding the leaves with a scalpel previously dipped in bacterial suspension (Miguel and Oliveira, 1999; Costa *et al.*, 2006) (Figure 1a–d) or immersion (a few seconds to 1 h) of wounded leaves (Miguel, 1998) or leaf pieces (Archilletti *et al.*, 1995; Ainsley *et al.*, 2001a; Ramesh *et al.*, 2006) in the bacterial suspension. In the case of immersion of wounded leaves, leaf explants were wounded either by transversal cuts across the midrib or by particle bombardment (BIORAD PDS-1000 He) with 1.6 μ m uncoated gold particles and 1300 psi of helium pressure (Miguel, 1998). The type of infection method has been shown to affect mainly the leaf regeneration ability and in the case of “Boa Casta” seedlings, the infection method involving the wounding of leaves with a scalpel dipped in the bacterial suspension was chosen (Miguel, 1998). In this method, the leaves are incompletely sectioned (Figure 1a), and the bacteria released in the wound area are observed contacting the epidermal and mesophyll cells (Figure 1b) and penetrating through the close-by stomata (Figure 1c). The *Agrobacterium* virulence status is also confirmed by the formation of the typical cellulose fibrils (Figure 1b). This infection method has the advantage of preventing full contact of the explant with the bacterial suspension. Instead, only transformation-competent areas, consisting of the cells surrounding the wounds, are infected by

Table 1 Transformation studies in almond

Cultivar	Explant	Transformation method/strain	Genes transferred	Regenerated tissues	Transgenic status (analysis)	References
"MN51" "Supernova" (micropropagated shoot cultures)	Leaves	<i>A. tumefaciens</i> LBA 4404	<i>GusA</i> (35S pro, ter) <i>npII</i> (nos pro, ter)	Transgenic callus	GUS + Southern +	Archilletti <i>et al.</i> , 1995
"Boa Casta" seedlings (micropropagated shoot cultures)	Leaves	Biolistics (ACCELL™ 12–22 kv or Handgun 300–400 psi)	<i>luc</i> , <i>bar</i> , and <i>hpt</i> (35S pro, nos ter)	Callus, nontransgenic shoots	LUC and GUS transient expression, PCR –	Miguel, 1998
"Boa Casta" seedlings (micropropagated shoot cultures)	Leaves	<i>A. tumefaciens</i> LBA 4404, EHA105	<i>GusA</i> (35S pro, ter) <i>npII</i> (nos pro, ter)	Transgenic shoots (when using EHA105)	GUS + PCR + Southern +	Miguel and Oliveira, 1999; Costa <i>et al.</i> , 2006
"Ne Plus Ultra" "Nonpareil" (micropropagated shoot cultures)	Leaves	<i>A. tumefaciens</i> LBA4404, EHA105	<i>gusA</i> (35S pro, ter) <i>npII</i> (nos pro, ter)	Transgenic callus (when using EHA105)	GUS + PCR + Southern +	Ainsley <i>et al.</i> , 2001a
"Ne Plus Ultra" (micropropagated shoot cultures)	Leaves	<i>A. tumefaciens</i> EHA105 (with pBI12imgfp-5-ER) AGL1 (with pNOV2819manA)	<i>mgfp-5-ER</i> (35S pro, nos ter) <i>pmi</i> (CPMS pro, nos ter)	Transgenic plants (using both EHA105 and AGL1)	GFP + PCR + Southern +	Ramesh <i>et al.</i> , 2006
"Supernova"	Microcuttings	<i>A. rhizogenes</i> 1855 NCPB	<i>rol</i> genes of wild strain	Transgenic roots	Phenotypic confirmation Southern +	Damiano <i>et al.</i> , 1995

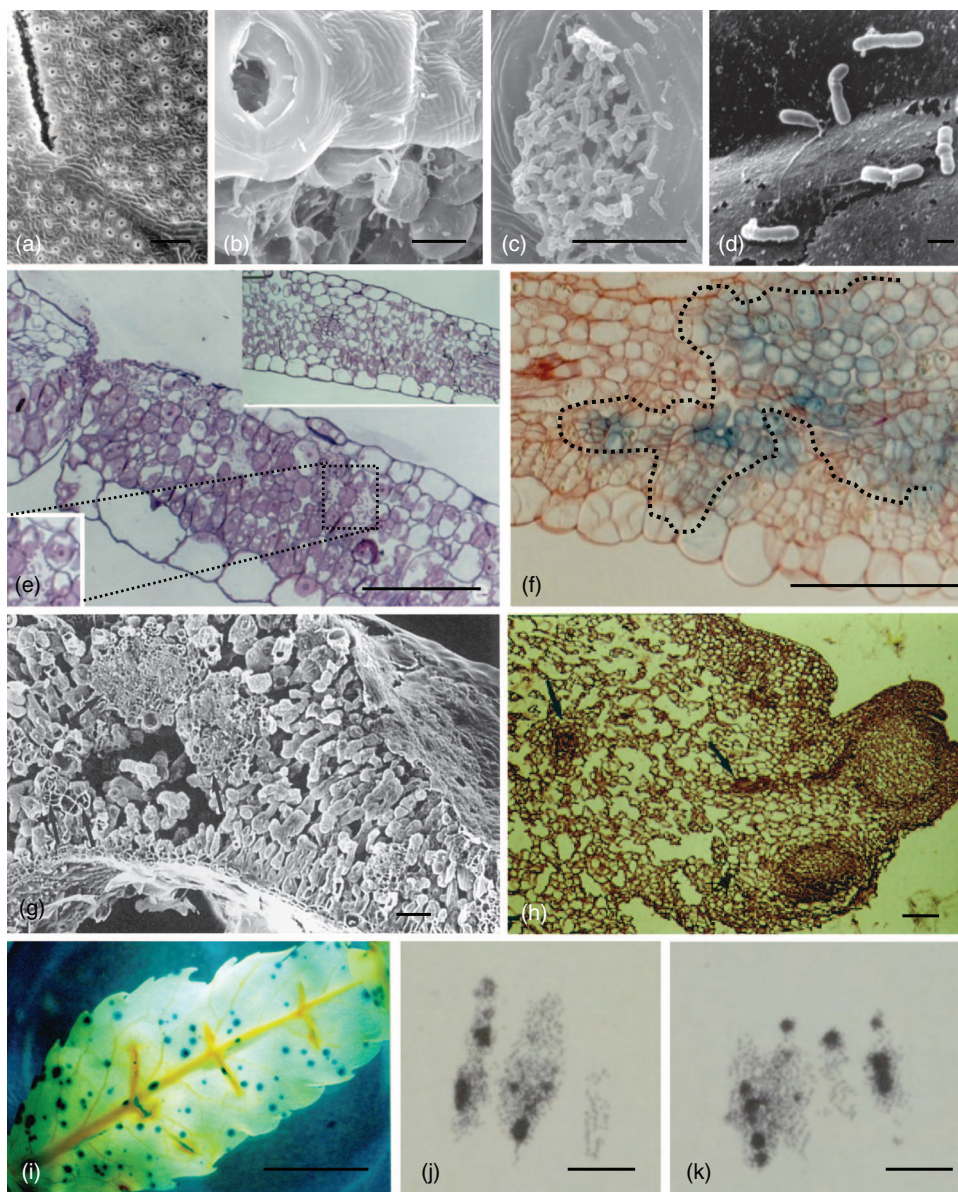


Figure 1 Early stages in the transformation of almond leaves. (a–d) Scanning electron microscopy (SEM) aspects of: (a) the abaxial surface of a leaf wounded by scalpel (*bar*: 100 μ m), (b) detail of *Agrobacterium* EHA105 attached to the plant cells and with the typical cellulose fibrils, (c, d) aspects of bacteria at the wound and also entering through the stomata. (e) Light microscopy image of leaf sections to reveal the wound site and the area reached by *Agrobacterium* (detail in bottom insert) 3 days after co-cultivation, and a control leaf (top insert) with profuse intercellular spaces. Leaf orientation is displayed as in the culture medium (abaxial side up). (f) GUS assay performed 1 week after co-cultivation and showing that GUS positive cells occur all across the leaf (indicated by a dotted line). (g) SEM image showing the proliferation of meristematic centers (at various stages) in the dedifferentiating leaf (*bar*: 100 μ m). (h) Emergence of two buds initials and aspect of the vascular connection linking the more developed bud to mother tissue vessel (arrows). (i–k) Transient expression analyses assessed 24 h after particle bombardment, to monitor the transfer of the GUS gene (i), or the luciferase (j, k). Bars: 1 μ m: (b), 10 μ m: (c) and (d), 100 μ m: (a), (e)–(h), 5 mm: (i)–(k)

Agrobacterium (Figure 1e–f). Since the regeneration of adventitious shoots also occurs preferentially from these areas (Figure 1g, h), no additional stress needs to be imposed on the remaining explant cells.

In general, woody species are difficult to transform and some strategies have been designed to improve transformation efficiency, such as the addition of acetosyringone (AS) during co-cultivation. AS has been applied, for instance in apple, kiwifruit, and citrange (James *et al.*, 1993; Janssen and Gardner, 1993; Cervera *et al.*, 1998) with the aim of stimulating transcription of *Agrobacterium* virulence genes. In almond, AS was used in the *Agrobacterium* growth medium prior to infection (Miguel, 1998; Miguel and Oliveira, 1999; Ramesh *et al.*, 2006). However, in the experiments of Costa *et al.* (2006) AS was employed in a different way, being added to the plant culture medium at a concentration of 150 μ M during the whole preculture, co-cultivation, and induction period (3, 3, and 21 days, respectively), with a clear positive effect. Actually the combination of AS treatment with a modified selection scheme allowed increasing the transformation efficiency by a 100-fold (Costa *et al.*, 2006).

In *Agrobacterium*-mediated transformation different strains have been used, namely EHA105, LBA4404, and AGL1 but regeneration of transgenic plants has been reported only with EHA105 and AGL1 (Miguel and Oliveira, 1999; Costa *et al.*, 2006; Ramesh *et al.*, 2006). Both EHA105 and AGL1 are of the succinamopine type, supervirulent, derived from C58 strain and carry the virulence plasmid pTiBo542, while LBA4404 is an octopine strain carrying pLBA4404. The high transformation ability of pTiBo542 has been previously demonstrated for a number of plants and correlated with a higher expression of the *vir* genes, especially *virG* (Liu *et al.*, 1992; Ghorbel *et al.*, 2001 and references therein).

When comparing the strains EHA105 and LBA4404 at least a threefold increase in the mean number of β -glucuronidase (GUS)-expressing spots per leaf was observed with EHA105 (Miguel and Oliveira, 1999). For the induction of rooting, *A. rhizogenes* 1855 NCPPB was successfully used on almond cuttings as confirmed by the hairy root phenotype observed and positive results in Southern blot analysis (Damiano *et al.*, 1995).

2.2.2 Transformation by particle bombardment

Microprojectile bombardment of almond leaf tissues has been tested as an alternative transformation method using the electric discharge particle acceleration device ACCELLTM technology (Agracetus, Middleton, USA) (McCabe and Christou, 1993) or a handgun device (John Innes Centre, Norwich, UK) (Miguel, 1998). It has been stated that the ability to deliver foreign DNA into regenerable cells, tissues, or organs, appears to provide the best method for achieving truly genotype-independent transformation in agronomic crops, bypassing *Agrobacterium* host-specificity (Christou, 1995). Due to the physical nature of this process, there is no biological constraint to the actual DNA delivery. However, in particular cases, in which recovery of transgenic plants has not been reported, the main problem appears to be the absence of a favorable regeneration response and not a problem in the DNA delivery method (Christou, 1995).

For transformation of almond leaf explants, specific bombardment parameters were selected after a series of transient expression assays for *gusA* and *luc* reporter genes (Figure 1i–k). Due to the importance of targeting the appropriate cells that are simultaneously able to be regenerated and transformed, the depth of penetration becomes one of the most important variables. Particle size and, to a less extent, the impact of the acceleration force that determines particle velocity (Bilang *et al.*, 1993), are determinant factors for the depth of tissue penetration, but the number of viable cells as a result of injury also has to be considered. Since adventitious shoot initials in almond leaves, seem to develop from the inner cell layers (Figure 1f–h) (Miguel, 1998), the bombardment parameters used were those allowing the deeper penetration of tissues without causing too much damage, specifically using 14 kv with ACCELLTM equipment and 350 or 400 psi with the handgun, with gold particle sizes of 0.95 or 1.3 μ m.

Transient expression was observed after bombardment (Figure 1i–k) but no stable transformants were obtained even though the regeneration ability of leaf explants seemed not to be affected by the bombardment process as evaluated by the control explants (bombarded with uncoated

particles) (Miguel, 1998). This may indicate that the *Agrobacterium* functions of transfer DNA (T-DNA) targeting to the nuclear genome may be important for the success of almond transformation. However, the fact that in biolistics transformation the transforming DNA is spread through the whole leaf and not specifically targeted to the organogenic regions may also justify the inability of this method to yield transgenic plants.

2.3 Selection of Transformed Tissue

For selection of transformed tissue, several selective agents have been reported including kanamycin, hygromycin, and phosphinothricin. Also, a positive selection strategy using mannose, to select for transformants carrying the *pmi* gene (phosphomannose isomerase structural gene), has been successfully used.

Kanamycin has been shown to have a dramatic effect on callus formation and proliferation from almond leaves and on adventitious shoot regeneration. Kanamycin sensitivity tests performed on untransformed leaf pieces have shown that concentrations above 68 μM induced severe explant necrosis completely inhibiting callus proliferation (Archilletti *et al.*, 1995; Ainsley *et al.*, 2001a). Thirty-four micromolar kanamycin initially allowed some callus formation but the explants gradually turned brown and no buds were regenerated (Miguel and Oliveira, 1999; Ainsley *et al.*, 2001a). On media containing 17 and 26 μM kanamycin, a few buds could be regenerated (less than 15% of regenerating leaves) but these remained small and white and did not develop further (Miguel and Oliveira, 1999). For callus formation, in order to obtain a higher percentage of transformed explants Archilletti *et al.* (1995) found it also important to start selection only after 3 or 6 days (depending on the genotype) on kanamycin-free medium, before transfer to the selective medium with 68 μM . On the other hand, Ainsley *et al.* (2001a) opted to start selection right after co-cultivation but using a lower level of kanamycin (25 μM), which was maintained through the whole regeneration process. Whilst low levels of kanamycin have the potential to give rise to escapes, higher concentrations compromise explant viability to the

extent that even if transformation occurred, cells are unlikely to survive the selection procedure.

In the case where transformed plants were recovered, Miguel and Oliveira (1999) report the application of kanamycin selection pressure immediately after co-cultivation with *Agrobacterium* but with a low concentration (17 or 26 μM), which was maintained during the induction period, but increased to 86 μM in the elongation phase. The surviving shoots were then propagated in selective conditions with 51 μM for two subcultures, and afterwards transferred to kanamycin-free micropropagation medium. In the report of Costa *et al.* (2006), selection was also applied immediately after co-cultivation with 26 μM kanamycin, which was maintained during shoot elongation and only increased in micropropagation, first to 51 μM for two cycles and to 86 μM thereafter (Costa *et al.*, 2006). This latter strategy allowed a 40-fold increase in transformation efficiency (from 0.1% to up to 4%) when compared to the previously reported work of Miguel and Oliveira (1999). Indeed, all shoots surviving five subcultures on micropropagation medium (3 subcultures at 86 μM kanamycin) were confirmed as transformed by Southern blotting (Costa *et al.*, 2006). Moreover, the transgenic shoots resisted an experiment to which they were subject 3 years after having been transformed: they were transferred to medium containing 172, 344, or 516 μM kanamycin, for four subcultures, and were found to resist these high concentrations and even grew axillary buds.

In the work of Ramesh *et al.* (2006), the regeneration of *nptII* PCR-positive transgenic plants was achieved in the absence of selection pressure, which was applied 70 days after co-cultivation with 15 μM kanamycin. According to these authors, initiation of selection 3 days after co-cultivation (with 15 μM kanamycin) completely blocked shoot initiation, and selection applied 21 days after co-cultivation (with 15 or 20 μM kanamycin) resulted in the formation of a small number of buds, which developed into small shoots that remained stunted and did not grow further. The delayed selection strategy allowed the recovery of transgenic shoots with an efficiency of 5.6%, based on PCR screens, and the authors report that six PCR-positive transgenic shoots selected for testing in Southern analyses confirmed transgene integration.

The effect on almond adventitious regeneration from leaves of other negative selection agents, as alternatives to kanamycin, has also been tested on explants subjected to microprojectile bombardment (Miguel, 1998). Hygromycin at 10 or 25 mg l⁻¹ and phosphinothricin at 1 or 2 mg l⁻¹ were tested for selection applied 5 or 21 days after bombardment and revealed extremely deleterious for control explants (nonbombarded leaves) in any test concentration. These, when cultured in the presence of hygromycin or phosphinothricin, regenerated only a few adventitious shoots if the selective agent was applied 3 weeks after culture initiation, but severe browning of shoots and eventual necrosis occurred within 3–4 weeks. Bombarded control leaves not subjected to selective pressure showed a regeneration rate of 57%, which is already within the range documented for untransformed leaves (Miguel, 1998).

Recently, Ramesh *et al.* (2006) have reported the use of a positive selection strategy based on the inhibition of growth of nontransformed tissue through carbohydrate starvation. The phosphomannose isomerase structural gene (*pmi*) has been used as selection marker. This gene gives the ability to transgenic tissues to survive by utilizing mannose as a carbohydrate source that would otherwise accumulate in plant cells as mannose-6-phosphate blocking glycolysis and preventing further growth (Goldsworthy and Street, 1965). In tissue culture, almond can readily utilize sucrose, fructose, or glucose as carbon source but grows poorly on mannose, making the mannose/PMI (phosphomannose isomerase) selection protocol an attractive selection option. At 20 g l⁻¹ mannose, no callus formation was observed from almond leaf explants but mannose concentrations above 2.5 g l⁻¹ severely impacted callus growth and shoot regeneration (Ramesh *et al.*, 2006). The authors reported the regeneration of transgenic shoots from the cultivar Ne Plus Ultra on medium containing 2.5 g l⁻¹ mannose together with 15 g l⁻¹ sucrose but selection pressure was initiated 21 days after transformation.

2.4 Regeneration of Whole Plant

One of the prerequisites for successful plant transformation, the ability to induce adventitious

shoot regeneration, has been demonstrated in almond from juvenile tissues (Mehra and Mehra, 1974; Seirlis *et al.*, 1979; Bimal and Jha, 1985; Rugini, 1986; Miguel, 1998; Ainsley *et al.*, 2001b) and from adult tissues (Miguel *et al.*, 1996; Ainsley *et al.*, 2000). Regeneration efficiencies are usually low, particularly when working with mature explants. Because biotechnology will ultimately allow the discrete modification of existing elite cultivars in one or more traits, regeneration pathways involving explants of mature tree origin are of major importance. However, the difficulties found in almond regeneration have limited the use of genetic transformation strategies for the improvement of cultivars of this species. Up to now, regeneration of whole plants from transformed almond tissues has been reported from leaf explants of both juvenile and adult material through adventitious shoot organogenesis (Miguel and Oliveira, 1999; Costa *et al.*, 2006; Ramesh *et al.*, 2006).

The infection and co-cultivation of almond leaf explants with *Agrobacterium*, as compared to the control leaves (untransformed leaves induced on cefotaxime-containing medium) has lowered the efficiency of adventitious shoot regeneration, in which average regeneration rates of 75% were observed (Miguel, 1998). There was a general decrease in regeneration capacity after co-cultivation even in the absence of selective agent.

The use of a preculture period of leaves for 3 days on shoot induction medium before wounding and *Agrobacterium* infection was reported as resulting in higher regeneration rates and leading to almost a sevenfold increase in the percentage of GUS positive explants. The effect on transformation efficiency of explant preculture prior to infection with *Agrobacterium* is not very clear. It has been reported that extended preculture may be deleterious for transformation in some species such as apple and kiwifruit (Janssen and Gardner, 1993; De Bondt *et al.*, 1994). In other cases, the preculture period may be considered important for tissue recovery and cell division induction after the stress imposed by wounding. In almond, preculture is usually applied before further wounding the excised leaves, therefore factors other than tissue recovery after wounding stress may be involved. It is possible that the previous exposure of leaf cells to a hormone-containing shoot induction medium brings them to a receptive

state, so that when wounding is performed cell division starts more rapidly. Since T-DNA integration occurs when cells are dividing, the higher number of GUS-expressing units when preculture is applied could be explained by the existence of more cells in active division during the co-cultivation period, and therefore a higher number of receptive cells.

Ramesh *et al.* (2006) also report on the use of a leaf preculture period of 3 days in liquid medium prior to wounding and transformation. Nevertheless, and in spite of the several improvements introduced in the available transformation protocols, the rate of transgenic plant recovery is still limiting.

From the study of the distribution of GUS-expressing cells and observation of leaf explants by light microscopy and scanning electron microscopy following co-cultivation with *Agrobacterium*, it is clear that neither *Agrobacterium* interaction nor T-DNA transfer appear to be the limiting step in transgenic almond recovery. Bacteria have access to all cell types within the explant (Figure 1e) and therefore, accessibility of the transformation vector to the regeneration-competent cells, should not impose constraints on transformation. Extensive bacterial proliferation was observed in the intercellular spaces near the wounds (Figure 1e, detail in bottom insert) and surrounding the dividing cells. A second early step in infection is the attachment of bacteria to the target plant cell, possibly through specific recognition sites. Fibrils that are produced by *Agrobacterium* for attachment to the plant cell wall were also observed (Figure 1d), but were much more frequent in leaves co-cultivated with EHA105 strain than with LBA4404 (Miguel, 1998).

In spite of the readily access of bacteria to all cell types within the almond leaf explants, mainly in wound surrounding areas, it is known from studies in other plant species that not all cell types show susceptibility to *Agrobacterium* infection. Precise localization at the microscopic level of the cells showing expression of the *gusA* transgene also was therefore attempted. Most GUS-expressing cells were found in the mesophyll, in several cell layers between the adaxial and abaxial epidermis, and also in the vascular tissues (Figure 1f). The great majority of these cells were located in the proximity of wounded tissues.

The investigation of the cellular origins and timing of events leading to shoot induction from leaf explants (Miguel, 1998) suggested that shoots had a multicellular origin and revealed vascular connections between the adventitious shoots and the vascular bundles of the original explant (Figure 1g, h). Both these situations are detrimental to the development of transformed shoots. Although nutrient supply from the main body of the explant is essential for the developing shoots, the effects of antibiotic selection on the untransformed explant tissues can be extremely detrimental to the transformed shoots. The problems underlying the difficulties in regenerating transgenic almond plants are probably more related to the shoot regeneration pattern than to the transformation efficiency. To overcome this recalcitrance, alternative regeneration systems based on different developmental patterns should be investigated. In other species such as *Linum*, *Vitis*, and *Dendranthema*, it has been reported that recalcitrance to transgenic shoot regeneration may result from differential competence of cells for transformation and regeneration (Jordan and McHughen, 1988; Colby *et al.*, 1991; Lowe *et al.*, 1993). In *Kohleria*, where a highly efficient regeneration system is available, transgenic plant recovery is inefficient because shoot regeneration occurs from a particular epidermal cell type, which proved hardly susceptible to *Agrobacterium* infection (Geier and Sangwan, 1996).

The regeneration patterns from different types of explants, such as roots and internodes, should be investigated. Adventitious shoots (Mehra and Mehra, 1974; Seirlis *et al.*, 1979; Bimal and Jha, 1985; Rugini, 1986; Miguel, 1998; Ainsley *et al.*, 2000) and structures resembling somatic embryos (Miguel, 1998) have been induced from cotyledons. Whilst this approach would not maintain clonal integrity, efficient regeneration from immature almond explants could provide information to improve the protocols used for regenerating adult tissues. Costa *et al.* (2006) have tested the effect of having 150 μ M AS in the induction medium used for adventitious regeneration from almond leaves. When using the modified selection scheme, these authors obtained a threefold increase in transformation efficiency (from slightly less than 4% to over 12%). It had been previously reported that this compound added to the plant culture medium

promoted organized growth that otherwise did not occur, in the aromatic plant *Levisticum officinale* transformed with *A. rhizogenes* (Santos *et al.*, 2005). Costa *et al.* (2006) suggest that prolonged leaf explant exposure to AS may have induced a favorable stress response leading to improved regeneration ability, besides its well-known effect as inducer of *Agrobacterium* virulence during co-cultivation. In such work, the presence of AS together with the use of a different selection strategy allowed to increase the transformation efficiency from 0.1% (Miguel and Oliveira, 1999) to 12.3%. This improvement was even higher considering the regeneration efficiency of control leaves

2.5 Testing of Activity and Stability of Inheritance of the Gene, Adverse Effects on Growth, Yield, and Quality

Almond adventitious roots obtained by *A. rhizogenes*-mediated transfer of *rol* genes, reported by Damiano *et al.* (1995), were found to display the typical plagiotropic emergence, but after transfer to soil they grew vigorously and the plants did not have any special requirements.

The first transgenic almond shoots, regenerated 9 years ago (Miguel and Oliveira, 1999) have in recent analyses proved to maintain the transgenic status. These plants have been maintained *in vitro* in the absence of selection conditions under regular subculture regime (every 2 months) and remained positive as assessed by Southern blot analyses performed in parallel on newly transformed shoots (Costa *et al.*, 2006). This transgenic material was never transferred to soil. However, in the work of Ramesh *et al.* (2006), where transgenic plants were rooted and transferred to soil, the authors report that in a contained glasshouse, and under natural light and temperature conditions over winter, the plants were able to flower in less than 18 months after planting (Ramesh *et al.*, 2006) and produced kernels (Almond Board of Australia, <http://www.aussiealmonds.com>). Ramesh *et al.* (2006) suggest that this fast flowering may be due to the *in vitro* culture that led to a reduction in the vegetative phase common to almond plants raised from seeds. However, if adult (cultivar) material was used in the transformation/regeneration experiments, this precocity might be suspected. No

data are yet available regarding negative effects on growth, yield, or quality of this material.

3. FUTURE ROAD MAP

3.1 Expected Products

One of the main expectations from almond genetic engineering is breaking down SI in specific cultivars by silencing the S-RNase genes, either by antisense technology or RNA interference. This is especially important when specific cultivars are identified as important producers, as it is the case of “Nonpareil” in California. This goal appears now attainable since the S-RNase gene sequences of various alleles are already obtained and since a transformation protocol for a cultivar (“Ne Plus Ultra”) is available since 2006 (see point 2.1, Table 1). From transformation experiments with “Nonpareil”, however, it was still only possible to regenerate transgenic callus (Ainsley *et al.*, 2001a).

Also important to obtain but still hard to reach through genetic engineering is late blooming. The complex system controlling flowering time still did not reveal a key gene sequence able to delay flowering in almond. However, from molecular studies conducted to identify genes involved in almond flowering, there is a candidate gene, *PrpFAR1*, that may be interesting to study further. This gene shares 52% homology to *far-red-impaired responsive protein gene* of *Arabidopsis thaliana*, and co-segregated (Silva *et al.*, 2005, 2007) with a QTL for late blooming identified by Dirlewanger *et al.* (1999) that mapped in the linkage group 7.

Diseases are potentially another target for transgenic breeding, especially when no control methods are available as it happens for viral diseases. Constructs aiming to block the proliferation of PDV were prepared carrying the original or modified PDV coat protein gene sequences (in sense or antisense orientation, mutated or without start codon), aiming to understand the mechanisms underlying PDV replication in host cells and to identify the most appropriate construct for almond transformation. The constructs prepared were constitutively expressed in *N. benthamiana* (an easy-to-transform model plant susceptible to PDV) and the transgenic plant behavior was studied in progenies upon controlled infection

using a different PDV strain (Raquel, 2005). After extensive molecular characterization and controlled infections performed on transformants and their progenies, PDV resistance achieved by the coat protein messenger RNA (mRNA) was observed in plants infected by a PDV isolate sharing only 91% homology with the isolate used for cpPDV cloning. When coat protein was expressed, resistance was only achieved if the construct was mutated in the active site responsible for activation of virus replication. This result points at a major role of the mutated sequence in the virus for its ability to replicate and proliferate. This work (Raquel *et al.*, 2008) demonstrates that PDV resistance can be achieved in plants expressing the PDV coat protein sequences, and provides a good clue of which construct could be the best for controlling PDV in almond orchards.

From other experiments, a number of genes isolated from almond could be selected for homologous and heterologous transformation assays for gene function assessment. It is the case, for instance, of genes putatively involved in adventitious regeneration, such as the almond homolog of *knotted-1*, named *PdKn1* (for *Prunus dulcis Knotted1-like* homeobox gene), and a number of genes identified from hybridization of two cDNA suppression subtractive libraries (SSH) and microarray analyses. The two libraries were prepared for two time frames of the adventitious regeneration process (the early stage of shoot induction, days 1–8, and the late stage, days 9–20) and some differentially expressed genes were selected for expression analyses by real-time quantitative PCR (Santos *et al.*, 2007).

3.2 Addressing Risks and Concerns

Like any other breeding strategy, transgenesis may yield high quality and high yielding varieties that may tend to disrupt traditional production schemes and replace older/worse performing cultivars. This, however, is not a new event in agriculture and, like other breeding systems, both the governments and the private sector should endeavor to protect farmers'/breeders' rights and also to preserve, for future generations and for a rapidly changing global climate, the existing germplasm diversity. In some countries (as in Portugal), new almond orchards are no

longer established using local varieties, but instead are established using improved higher yielding cultivars obtained from abroad, with obvious impact on the erosion of local genetic diversity, sometimes cultivars with higher nut quality.

Hardly, will any transformation event leading to a better producing almond plant, such as self-fertility, late blooming, or virus resistance through coat protein gene sequences, have any harmful effect in human health. However, as with other transgenic plant products, appropriate quality analyses should be performed to identify potential negative effects, eventually also including postmarket surveillance (Batista *et al.*, 2005).

A putative impact on the environment has been anticipated in the case of engineering viral resistance through coat protein gene sequences. The wardens of this vision argue that the transgene sequences may find ways to recombine with other viruses (Frischmuth and Stanley, 1998) leading to potentially new and particularly virulent viral forms. However, there is no scientific reason to suppose that the putative viral recombination will differ for transgenic and nontransgenic plants infected with two viruses, unless, for instance, viral recombination frequency is increased in transgenics. The transgenic plant carrying a virus-derived sequence presents an increase in risk compared to the nontransgenic plant only if the frequency with which viable recombinants are generated in transgenic is significantly higher.

However, any field releases of transgenic virus-resistant material should be monitored regarding potential environmental impacts.

3.3 Expected Technologies

The production of transgenic plants relies upon the selection and recovery of transformed cells. The use of either antibiotic or herbicide tolerance/R genes as selectable markers may raise concerns regarding safety and environmental impacts. The use of positive selection protocols such as the use of mannose/*pmi* selection reported for almond (Ramesh *et al.*, 2006) may circumvent these problems, although it may still be desirable to have an efficient system to remove selectable marker genes. This would allow retransforming transgenic plants using the same original marker and thereby repeatedly pyramid a number of

transgenes as more traits have to be incorporated into a transgenic crop. For removal of marker genes, several methods, such as co-transformation, transposition or site-specific recombination, have been successfully employed (for reviews, see Hare and Chua, 2002; Scutt *et al.*, 2002). The multiautotransformation (MAT) vector system represents a highly sophisticated approach for the removal of nuclear marker genes (Ebinuma *et al.*, 1997), in which a chosen trait transgene is placed adjacent to a multigenic element flanked by recombination sites. The obvious disadvantage of these strategies is the need for repeated and time-consuming transformation cycles, and the increased chances of somaclonal variation.

In association with these technologies, the precise control of the integration site could be important for optimizing the transformation technology, although much higher transformation efficiency would be required.

3.4 Intellectual Property Rights (IPR) and Public Perceptions

The use of transgenic breeding for almond improvement does not seem economically viable except for specific cases in which elite cultivars of vast utilization are targeted (as it could be the case for “Nonpareil”). This situation is due to the fact that most biotechnologies are patented and those that are no longer under the scope of the patents are conveniently regarded as old fashioned and potentially harmful to health or environment (such as the use of selectable markers for antibiotic resistance/tolerance). Patents not only have the role of rewarding innovation and encouraging the private sector to invest, but also are frequently viewed as necessary for attracting venture capital in a start up biotech company. However, transactions involving IPR are very expensive, and this makes it quite inevitable since at present transgenic breeding can only be handled by companies large enough, and prepared enough, to deal with, and handle, IPR processes. Small/starting companies are simply discouraged by the need to negotiate existing patent claims on important technologies.

Additionally, conflicts with farmers who prefer to use nontransgenic plants can be particularly hard for a plant with high SI such as almond that

requires cross-pollination (and pollinating bees) and from which the edible part is the fertilization-derived kernel. The kernel will thus contain the genes brought from the pollen-donor tree.

Regarding human health, environmental impacts and public perception, clearly the introduction of self-fertility would be less controversial than coat protein-mediated virus resistance. Moreover, the damage by Ilarviruses in almond is not comparable to damages by plum pox virus in other *Prunoideae* that makes it harder to defend the need for engineering virus resistance in almond.

Still, genetic engineering tools for almond are important and should be available for future needs, including for gene functional studies.

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Persian Walnut

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The Persian walnut (*Juglans regia* L.) is the horticulturally most developed and widely cultivated of all walnut species. It is mentioned in the Bible and was used for food and medicine in Greek and Roman times, and likely had even earlier horticultural origins in China. Millennia of trade, transport, and commerce have contributed to widespread naturalization of cultivated trees, greatly extending the original range, which is believed to encompass the lower altitudes of the mountainous regions of central Asia. Although “Persian” better describes the native range of this walnut species, its most common name in the United States is “English” walnut, apparently because nuts were originally shipped there in English ships.

J. regia is native to central Asia, where it grows as a wild or semicultivated tree in a wide area from southeastern Europe and the Caucasus to Turkey and Iran, through southern portions of the former Soviet Union into China and the eastern Himalayas. It has been cultivated for its nuts for at least several thousand years and was likely introduced into European commerce and agriculture by the ancient Greeks. It was prized by the Romans as *Jovis Glans* (“Jupiter’s nut”) and was utilized in medieval Europe as an herbal

medicine, particularly for brain and scalp ailments. Since its introduction into North America it has commonly been referred to as the English walnut to distinguish it from the American black walnut (Leslie and McGranahan, 1998).

1.2 Botanical Description

There are approximately 20 species of walnut worldwide, many of which are valued for their nut production and timber quality. *J. regia*, the Persian walnut, is the primary species used in commercial walnut production. It is currently cultivated in many temperate regions including much of the Mediterranean, central Asia, northern India, China, South Africa, Argentina, Chile, and Australia. In the United States, this crop is produced almost entirely in California, where scion cultivars are grafted onto rootstock of either the native California black walnut (*Juglans hindsii*) or *J. hindsii* × *J. regia* hybrids known as Paradox.

1.2.1 Taxonomy

The family Juglandaceae includes about 60 species of deciduous, monoecious trees or shrubs with alternate, pinnately compound leaves. The genus *Juglans* consists of four sections. Three of these, *Rhysocaryon* (black walnuts native to the North

America), *Cardiocaryon* (Japanese, Manchurian and Chinese walnuts, including selections known as heartnuts), and *Trachycaryon* (the butternut of eastern North America), exhibit thick shells and nondehiscent hulls. The fourth section, *Juglans*, is comprised of a single species, *J. regia* L., distinguished by a dehiscent hull, which separates from the shell at maturity (Manning, 1978).

1.2.2 Habit

Persian walnut trees are typically over 30 m tall, with a broad canopy of compound pinnate leaves (Prasad, 2003). The first branches are formed 2 to 4 years after germination, after which hierarchical branching continues from the main stem. Initial flowering is seen in 3- to 9-year-old trees. An unusual type in Asia flowers in the first year. After flowering begins, the main stem becomes forked and canopy architecture develops (Sabatier and Barthélémy, 2001).

There is a high degree of morphological variability among *J. regia* cultivars. Some bear their flowers (and thus, nuts) from terminal buds, while others bear on axial buds. Mid to late leafing and lateral fruit-bearing offspring populations predominate in California varieties and tend to be more precocious and have a higher yield earlier than those that bear their nuts terminally (McGranahan and Leslie, 1990).

J. regia trees are monoecious with male flowers borne in catkins and female flowers in pistillate spikes of mostly two, but sometimes as many as five, flowers at the tips of terminal or lateral shoots. Flowering is dichogamous with either the male (protandry) or female (protogyny) flowers maturing first. Pollen is wind borne and the dichogamy promotes outcrossing (Forde and Griggs, 1972). The walnut fruit is an ovoid drupe with an external fleshy pulp surrounding the nut. After ripening, the fruit dehisces and releases the nut, which is composed of a highly lignified shell surrounding the seed (Cannella and Dernini, 2006).

1.2.3 Habitat

In their native habitat, walnuts are most commonly found among the foothills and at temperate elevations, where they grow on disturbed forest

sites or on well-drained river bottom soils with a variety of coniferous and broad-leaved forest species. They are a component of wild fruit forests that include wild apricot, pear, almond, myrobalan plum, and grape. Most varieties of walnut require 600–1200 chilling hours (below 7 °C) for nut production. Cultivated walnuts do best in the Mediterranean climate in deep, well-drained, nonstratified soils (Ramos, 1998). In Europe, commercially cultivated trees can still be found in small orchards, but establishment of large orchards is now more common, similar to the practice in California.

1.3 Economic Importance

Worldwide, over 1.5 million metric tons of walnuts are produced annually valued at over \$2 billion. The top 10 walnut-producing countries are detailed in Table 1. Yield per hectare can be as high as 6 t with new cultivars, although a good yield is 2–3 t. Major walnut-producing regions of the world are China with 420 000 t, the United States with 322 000 t, and Europe with about 260 000 t. Within the United States, 100% of Persian walnut (*J. regia*) production occurs on over 95 000 ha in California, where it is one of the top 20 agricultural commodities, with an annual production value of more than \$500 million (California Department of Food and Agriculture, 2006; United States Department of Agriculture, 2006).

1.3.1 Utilities

While Persian walnuts are primarily a food crop, the timber is also greatly prized, particularly in Europe, where it is considered to be the most valuable wood for furniture manufacture. Harvesting of walnut wood has depleted some of the forests in its native range. In addition, shells are used as an abrasive in diverse applications including metal polishing and oil drilling.

1.3.2 Nutritional and/or other economic attributes

Walnuts are very high in nutritional content, containing 14–24% protein and 52–70% fat. Almost all of the oil is in the form of

Table 1 Leading walnut-producing countries in 2005^(a)

Country	Rank	Production (t)	Percentage of world production	Area harvested (ha)	Yield (t ha ⁻¹)
China	1	420 000	27.76	186 000	2.26
United States	2	322 050	21.29	95 000	3.39
Iran	3	150 000	9.92	65 000	2.31
Turkey	4	133 000	8.79	70 000	1.90
Ukraine	5	93 000	6.15	14 000	6.64
France	6	33 241	2.20	16 238	2.05
India	7	31 500	2.08	30 800	1.02
Egypt	8	27 000	1.78	5 000	5.40
Serbia and Montenegro	9	24 000	1.59	13 200	1.82
Spain	10	22 000	1.45	7 500	2.93
All others		257 791	16.99	132 987	1.93
Total		1 512 816	100.00	635 725	2.38

^(a) Source: FAOSTAT, Agricultural production data, crops primary, walnut

polyunsaturated fats, including α -linoleic acid. Among the protein constituents is the essential amino acid taurine, which is generally lacking in vegetarian diets. Walnuts also contain several nutritionally important vitamins and minerals, including vitamins A and E, niacin, riboflavin, thiamin, magnesium, phosphorus, and potassium (Prasad, 2003). Vitamin E is an antioxidant that may provide some protection against walnut rancidity. Phytosterols may lower serum cholesterol in consumers due to their ability to inhibit absorption in the intestine (Savage *et al.*, 2001).

Phenolic compounds are responsible for the slightly astringent flavor of walnuts. The pellicle, a thin skin surrounding the kernel, is rich in these antioxidant compounds, which naturally help protect the kernel against rancidity brought on by oxidation of fatty acids (Colaric *et al.*, 2005). When ingested, polyphenolics may inhibit oxidation of low-density lipoproteins, as indicated by the reduced incidence of cardiovascular disease in certain studies (Anderson *et al.*, 2001).

Over the past several years, a large body of evidence has accumulated from studies that point to health benefits derived from incorporating walnuts into a healthy diet. Diseases that appear to benefit from walnut consumption include cardiovascular and coronary heart disease (Zambon *et al.*, 2000; Morgan *et al.*, 2002; Zibaenezhad *et al.*, 2003; Ros *et al.*, 2004; Reiter *et al.*, 2005), type 2 diabetes (Fukuda *et al.*, 2004; Gillen *et al.*, 2005), Alzheimer's disease (Chauhan *et al.*, 2004), liver cytotoxicity (An *et al.*, 2005), tooth decay (Jagtap and Karkera, 2000), and acne

(Qadan *et al.*, 2005). Studies on the effects of walnuts and coronary heart disease have been reviewed by Feldman (2002). There is also evidence that compounds contained in walnut may be useful in reducing the toxicity of broad-spectrum chemotherapy drugs (Haque *et al.*, 2003).

The beneficial aspects of walnuts are derived from both their unique fatty acid composition and numerous products of secondary metabolism, including high levels of fiber, folate, polyphenolic compounds, tannins, and the amino acid arginine (Ros *et al.*, 2004). Although walnuts are high in total fat content, increased consumption of walnuts does not result in gains to body weight, because the resulting diet has a low proportion of saturated fat (Zambon *et al.*, 2000; Gillen *et al.*, 2005).

1.3.3 Industrial uses

Powdered walnut shells are used in abrasives, by the plastics industry for fillers, and by the oil industry in drilling mud. By-products of walnuts are also used as dyes, and in the processing of leather (tannins). Walnut oil is used in artists' paints, due to its fast drying time (Prasad, 2003).

1.4 Traditional Breeding

As is true for many seeded plants, the history of walnut breeding began through chance selection of superior nuts and seedlings. Walnuts have traditionally been grown from seed in most

parts of the world, but have been more recently been propagated by grafting. In California, a formal breeding program was initiated at the University of California at Davis in 1948 under the direction of Gene Serr and Harold Forde. After Gene Serr retired in 1965, the breeding program was continued under Harold Forde until 1978. This was a very successful partnership that resulted in the release of 15 cultivars (Serr and Forde, 1968; McGranahan *et al.*, 1990b; McGranahan *et al.*, 1992; Tulecke and McGranahan, 1994). The breeding program at UC Davis is ongoing with major breeding objectives to increase yield, quality, and range of harvest dates, while decreasing the amount of chemical input required to control pests and diseases. For a recent review of *J. regia* breeding and genetics, see Dandekar *et al.* (2005).

In Europe, older varieties are generally well adapted to local climatic conditions, but often lack important agronomic traits (Germain, 1992). For this reason, during the last 20 years significant work involving seedling selection has been carried out using local populations of *J. regia* throughout Europe. Selection of progeny from natural populations is critical to create, through hybridization, new cultivars with combinations of multiple valuable traits. Additionally, a goal of European selection and breeding is to separate the production of wood from the production of fruit, with some breeding programs designed to increase the production of high-quality wood and with particular attention to characters such as vigor, number of branches, and stem forms (Jay-Allemand *et al.*, 1996; Casal *et al.*, 2006; Fernández-López *et al.*, 2006; Hemery and Russell, 2006).

1.4.1 Rootstocks

Nearly all walnut production in the United States, and increasingly worldwide, is derived from grafted scion varieties on seedling rootstocks. This reflects the difficulty of rooting walnut cuttings, which precluded, until recently, substantial use of either rooted scions or improved clonal rootstocks. Employment of rootstocks is driven by the need to deal with soil, environment, disease, and pest problems (McGranahan and Catlin, 1987). In California, *J. hindsii* (Northern California black walnut) was preferred for much of the first half of

the 20th century as it is a native species adapted to several soil-related problems, including resistance to crown and root rot caused by *Armillaria mellea* (oak root fungus) and tolerance to water logging and drought (Smith *et al.*, 1912). Today, the most popular rootstocks are the interspecific hybrids, primarily “Paradox”, a hybrid between *J. hindsii* and *J. regia* described in 1893 by Burbank (Whitson *et al.*, 1914; Howard, 1945). However, because these hybrids are male sterile, traditional breeding between Paradox selections cannot be accomplished. However, crosses can be made using Paradox as the female parent.

The major objective for walnut rootstock breeding is vigor, in order to promote rapid growth of the scion under a variety of soil and environmental conditions and to establish rapidly a full-sized bearing canopy. Other objectives include resistance to diseases and pests, most notably *Phytophthora*, nematodes, and crown gall, as well as tolerance to cherry leafroll virus and tolerance of soil-related problems, including water logging and salt accumulation. There is interest in controlling tree size, but not at the cost of vigor.

1.4.2 Scions

The ideal walnut cultivar would be relatively late leafing to escape the rains that spread walnut blight (*Xanthomonas campestris* pv. *juglandis*), precocious (yielding more than 500 kg ha⁻¹ in the fourth year) and vegetatively vigorous with bearing on both terminal and lateral shoots. It would have a low incidence of pistillate flower abscission and fruit drop and would not be alternate bearing. It would have high production capacity with low chemical input required. The harvest season would end in early October. The nutshell would be relatively smooth, well sealed, and make up no more than the 50% of the nut weight. The nuts would fit the category of large or jumbo. The kernel would be plump and light colored, weighing about 7–8 g, and would be extracted easily in halves. The tree would be resistant to pests and diseases.

1.4.3 Tools and strategies

Traditional breeding activities incorporate the collection and evaluation of germplasm, controlled crosses, open-pollination between advanced

selections, evaluation of progeny, selection and field trials prior to release. The University of California walnut germplasm collection and the National Clonal Germplasm Repository walnut collection are both housed at UC Davis, and contain plants collected from the native Asian ranges of *Juglans*. This allows the introduction of genetic variation and desirable traits by crossing plants from the germplasm collections with existing elite cultivars (McGranahan and Leslie, 2006).

Clonal propagation of rootstocks is beginning to be commercialized in California and Europe. To evaluate the genetic diversity of commercially available Paradox rootstocks, a study was initiated in 1996. Evaluations included screens for resistance to root lesion nematode (*Pratylenchus vulnus*), crown gall, and *Phytophthora*. Some selections were found with tolerance to individual pathogens and are currently being micropropagated (McGranahan and Leslie, 2006).

Molecular markers can be used for traditional genetic and breeding strategies, including selection of desired progeny, linkage mapping, and map-based cloning. Mapping efforts in Persian walnuts have utilized random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellite markers (Aradhya *et al.*, 2001; Malvolti *et al.*, 2001). In Turkey, most *J. regia* are grown from seedlings, rather than grafted, representing an extensive germplasm. Some of these genotypes have been characterized using AFLP and SAMPL techniques (Kafkas *et al.*, 2005). Simple sequence repeat (SSR), intersimple sequence repeat (ISSR), and RAPD markers have also been used to characterize *J. regia* cultivars and to discriminate between intra- and interspecific hybrids and to estimate genetic diversity (Abuin *et al.*, 2002; Potter *et al.*, 2002a; Dangl *et al.*, 2005; Abuin *et al.*, 2006; Foroni *et al.*, 2006; Pollegioni *et al.*, 2006).

Isozyme markers have been used to identify the inheritance of important enzyme systems, including 6-phosphogluconate dehydrogenase, shikimate dehydrogenase, malate dehydrogenase, and phosphoglucomutase (Aleta *et al.*, 1993). The use of phenols as genetic markers in walnut has also been proposed to identify cultivars and to screen the progeny of interspecific crosses (Jay-Allemand *et al.*, 2001).

Although the term “Paradox” generally designates rootstock hybrids between *J. hindsii* and

J. regia, it can be applied to any hybrid between a species of black walnut and *J. regia*. In commercial nurseries, the trees are open pollinated and so the genotypes of the seedling progeny are unknown. A 1996 study used the ribosomal DNA intertranscribed (ITS) regions and noncoding spacer regions from the chloroplast genome to identify unique sequence markers for each species to develop molecular markers that now allow the parentage of individual Paradox seedlings to be determined. It was shown that several other black walnut species and their hybrids were used to produce Paradox seedlings (Potter *et al.*, 2002b).

Chloroplast and mitochondrial polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genetic markers have also been used to compare European and Asiatic genotypes of *J. regia*. The amount of diversity found between these genotypes has been used to support the hypothesis that some *J. regia* populations survived in Europe during the last glaciation. This hypothesis is in contrast to the generally held belief that *J. regia* became extinct in Europe during glacial periods, and was reintroduced from Asian populations after the climate had warmed (Fornari *et al.*, 2001).

1.5 Limitations of Conventional Breeding

Breeding and selection are actively used to improve scion cultivars. However, controlled pollinations are very labor intensive and produce relatively few seedlings per cross. Open pollination is also performed by blowing pollen from selected male parents into isolated orchards of the desired female parent. This is less labor intensive and produces thousands of seedlings (McGranahan and Leslie, 2006). But, because all walnut cultivars are very heterozygous in their genetic makeup, many years of evaluation are required before individual selections can be released. Therefore, to shorten the lengthy process, the use of techniques including embryo rescue and genetic transformation, combined with micropropagation, can provide the means to incorporate desired traits in existing elite cultivars and to pyramid disease and pest resistance in rootstocks.

Backcross breeding is currently being used to develop a rootstock that would incorporate the *J. regia* response to cherry leafroll virus (CLRV)

with the vigor of Paradox. To accomplish this, individual vigorous and tolerant seedlings are chosen from a backcross generation (*J. hindsii* × *J. regia*) × *J. regia*. However, this process takes many years and requires a great deal of resources to accomplish due to the long generation time of the trees: it has taken 17 years from the initial planting of 13 000 Paradox offspring seedlings through the selection process and clonal propagation to reach the stage where grower trials can begin (McGranahan and Leslie, 2006).

Phytophthora is the most significant root disease of walnut in California, and is easily spread by irrigation water and periodic winter flooding (Mircetich and Matheron, 1983). While it is possible to find individual healthy rootstocks in orchards decimated by this pathogen, it is very difficult to capture this potential resistance due to the variability in disease symptoms and the possibility of disease escapes. The difficulty in propagating such chance trees to conduct further testing and for subsequent commercialization is also a hindrance.

Wingnut (genus *Pterocarya*) is very resistant to *Phytophthora*, but when used as a rootstock it is graft incompatible with some cultivars of *J. regia*. Hybrids between walnut and wingnut could potentially be used as long as they are compatible and retain the resistant trait, but hybrids developed through controlled pollination and embryo rescue did not survive over the winter in the field (McGranahan *et al.*, 1986).

CLRV is the major viral disease of walnuts, and is found in California as well as in France, Italy, Hungary, and the United Kingdom. It is spread by pollen (Massalski and Cooper, 1984) and is systemic and virtually asymptomatic in *J. regia*. Most black and Paradox rootstocks are resistant to CLRV and prevent systemic spread by a hypersensitive response that is manifested as a black line at the graft union (Mircetich and Rowhani, 1984), which causes a lethal girdle resulting in the decline, dieback, and death of the scion (Mircetich *et al.*, 1980).

1.6 Micropropagation

Walnuts have traditionally been propagated by grafting onto seedling rootstock. Micropropagation has been investigated for propagation of

cultivars on their own roots, for production of selected rootstock clones, and for development of genetically engineered plants. Each walnut species and cultivar generally requires new optimization for successful tissue culture. Commercially, walnuts are micropropagated in only one lab in Spain (López, 2001) and one laboratory in California.

The first reports of micropropagation of Persian walnut are from the early 1980s (Chalupa, 1981; Somers *et al.*, 1982; Rodriguez, 1982a, 1982bb; Caruso, 1983; Cossio and Minolta, 1983; Driver and Kuniyuki, 1984). These techniques have been reviewed (McGranahan *et al.*, 1987; Preece *et al.*, 1989; Leslie and McGranahan, 1992). Micropropagation of mature cultivars (McGranahan *et al.*, 1988a) and better techniques for rooting and acclimatization (Jay-Allemand *et al.*, 1992; Ripetti *et al.*, 1994; Navatel and Bourrain, 2001; Vahdati *et al.*, 2004) have been described more recently.

Driver Kuniyuki walnut (DKW) medium (Driver and Kuniyuki, 1984) was developed specifically for walnut, but success has also been obtained on MS medium (Murashige and Skoog, 1962). A comparison of different media for *J. regia* conducted by Saadat and Hennerty (2002) found that DKW was optimal when 2.2 g l⁻¹ Phytigel was used as the gelling agent.

Walnuts are initiated into culture by introducing disinfested nodal segments of vigorous field or greenhouse-grown shoots. Multiplication occurs through axillary shoot proliferation. Rapid transfer (2–5 times per week) is essential after introduction into culture until discoloration of the medium is no longer evident. Once established, cultures need relatively frequent transfer (2 times per month) for optimum growth (Leslie *et al.*, 2006a).

Techniques for rooting are still under investigation and rooting ability is clone specific. The most promising rooting technique utilizes a two-phase system originally developed by Jay-Allemand *et al.* (1992) and subsequently modified by Navatel and Bourrain (2001) and Vahdati *et al.* (2004). Roots are induced by placing shoots on MS medium containing auxin and at least 40 g l⁻¹ sucrose for 6–8 days in the dark. Induced shoots are then transferred to a root development medium consisting of a mix of one-quarter strength basal DKW medium and vermiculite (to improve aeration) and maintained in the light for 3–4 weeks until roots are

visible. An alternate method is to treat unrooted microshoots with auxin, and root them in vermiculite in a fog chamber (Leslie *et al.*, 2006a). This technique results in a lower percentage of rooted shoots. However, those shoots that do root using this procedure have very little callus and produce roots that are more fibrous than microshoots rooted *in vitro* and show improved survival during acclimatization to greenhouse conditions. Recently, it has been demonstrated that the addition of 1 mM phloroglucinol to the multiplication media increases subsequent rooting (Leslie *et al.*, 2006a).

Rooted shoots are planted in a well-drained potting soil and are acclimated by growth in a fog chamber for 2 weeks followed by a week or two on a shaded greenhouse bench. However, the stress of acclimatization can lead to arrest of the apical meristem. Budbreak can be improved by application of 25 ml l⁻¹ Promalin® (a commercial product containing 1.8% gibberellic acid (a mixture of GA4 and GA7) and 1.8% benzylaminopurine (BAP, Valent Biosciences, Walnut Creek, CA)) as a foliar spray to stimulate growth (Vahdati *et al.*, 2004).

In a study comparing variations of this protocol on several different walnut cultivars, a correlation was found between the vigor of adult trees and their rooting ability. Although smaller microshoots can be produced more rapidly and more efficiently *in vitro*, longer microshoots appear to acclimatize better in the greenhouse, perhaps due to a greater internal reservoir of carbohydrates and a more lignified stem, which may lead to increased pathogen resistance (Vahdati *et al.*, 2004).

Bisbis *et al.* (2003) also found a link between shoot lignification and root development, concluding that signals from the roots, as well as auxin, enhance lignin formation. The lignin was only found in xylem cells, and was correlated positively with the number of developing roots, beginning immediately after treatment with exogenous auxin (Kevers *et al.*, 2004). This appears to trigger peroxidases that are involved in the process of building cell walls. Peroxidase activity had previously been shown to be a good marker for walnut rooting (Gaspar *et al.*, 1992; Ripetti *et al.*, 1994).

While the above techniques are generally effective, it is often found that media must be optimized for specific cultivars and clones. Recent

studies in which different root formation factors have been investigated include those described by Navatel and Bourrain (2001), Dolcet-Sanjuan *et al.* (2004), Caboni and Damiano (2006), Kaur *et al.* (2006), Leslie *et al.* (2006a), and Sánchez-Zamora *et al.* (2006).

1.7 Somatic Embryogenesis

Development of embryos from asexual tissues has been a very useful tool in genetic improvement, particularly because tissues (i.e., leaf discs, protoplasts) commonly used in other plants have not been successfully regenerated to plants in walnut. Somatic embryogenesis has been used to generate triploids (Tulecke and McGranahan, 1988), intergeneric hybrids (McGranahan *et al.*, 1986), and genetically transformed clones (McGranahan *et al.*, 1988b, 1990a; Dandekar *et al.*, 1989).

The techniques were developed for *J. regia* (Tulecke and McGranahan, 1985) but have been applied to other species (Neuman *et al.*, 1993). Immature cotyledonary explants harvested from developing nuts, cultured on conditioning medium for 2–4 weeks and then placed on basal DKW medium, will develop small white somatic embryos from single cells (Polito *et al.*, 1989) on the explants after 8–16 weeks. These new embryos are repetitively embryogenic and with monthly subculturing cultures can be maintained for years.

For initiation and multiplication, embryos are maintained at room temperature in the dark. In the light, embryos turn green and a certain percentage will germinate. Germination frequency can be increased following desiccation over a saturated salt solution (Zn₂SO₄, NH₄NH₃, or MgCl₂) until the embryos are white, with the consistency of popcorn, but not until they have turned brown. Embryos are then returned to DKW basal medium to germinate. Additional details of methods utilized in walnut embryogenesis are given in the reviews by Tulecke *et al.* (1995) and Preece *et al.* (1995).

Currently, a major challenge is to obtain embryogenesis or organogenesis from maternal tissue. This is important because embryos from zygotic tissue do not allow the exact genotype to be predicted, even if both parents are known, due to the high degree of heterozygosity in walnut.

Therefore, genetic transformants derived from zygotic embryos must be brought into the breeding program to combine desirable traits. Efforts to generate somatic embryos from nonzygotic tissues have been unsuccessful (Aly *et al.*, 1992; Vahdati *et al.*, 2006). Repetitively, embryogenic cultures have been obtained from immature anther tissue, but only from one cultivar ("Chandler"). Recently, a modified protocol allowed a somatic embryo line to be generated from immature anthers of a Paradox hybrid (Burbank) (McGranahan, unpublished). This may provide a source of elite rootstock tissue for genetic transformation.

1.8 Molecular Genetics and Genomics

Gene cloning is an important endeavor because tree crops are likely to possess many genes with unique functions that may not be discovered in other plants. However, little progress has been reported primarily due to the few walnut researchers worldwide and the lack of availability of resources to fund these endeavors. One of the useful sources of information on walnut genes is GenBank (NCBI), the public repository for DNA sequences. Currently, this database has over 18 000 entries for genetic sequences among *Juglans* species.

Genes specific to walnut currently being studied include those involved in tannin, naphthoquinone, unsaturated fatty acid, and flavonoid biosynthesis. Several of the genes involved in the biosynthetic pathway of flavonoids have been identified (Beritogoli *et al.*, 2002). Recently, homologs of two *Arabidopsis* genes controlling floral transition and flower differentiation were cloned from microshoots of early mature (EM) walnuts (Breton *et al.*, 2004). EM walnut trees can flower within 1 year of germination and develop into trees that are smaller, bushier, and more cold-hardy than other *J. regia* genotypes. These traits may be useful in transgenic plants and traditional breeding programs.

A key determinate of walnut kernel quality is the oil content, about 90% of which is polyunsaturated, and of that 25% is the ω -3 fatty acid α -linolenic acid (ALA). The presence of unsaturated fatty acids is an important factor in rancidification of walnuts, in which these acids are oxidized, reducing the shelf life of walnut kernels

(Greve *et al.*, 1992). The ω -3 fatty acids have been shown to play an important role in growth and development, nutrition, and disease prevention. Nutritional studies have demonstrated that walnut consumption can reduce the incidence of coronary heart disease. The genes encoding the various fatty acid desaturases involved in the synthesis of polyunsaturated fatty acids, including *fad 2* and *fad 3*, have been cloned from walnut (Dandekar, unpublished).

Oil biosynthesis in the embryo is a major metabolic pathway and some effort has been directed at functional characterization of two key steps in the biosynthesis of polyunsaturated fatty acids. Transgenic walnut embryos expressing antisense *fad 2* or sense *fad 3* have been developed (Dandekar, unpublished) and some of the lines show alterations in the profile and composition of fatty acids. It is hypothesized that expression of antisense *fad 2* will suppress the interconversion of oleic to linoleic acid, leading to an increase in the accumulation of the monounsaturated oleic acid. The expression of sense *fad 3* is aimed at overexpressing the enzyme involved in the conversion of linoleic acid to the ω -3 fatty acid linolenic acid. These studies will be useful in developing walnut lines with high oleic acid (monounsaturated fatty acid) content for stability and also to develop walnuts with increased ω -3 fatty acids.

Enzymes in the phenylpropanoid pathway from phenylalanine lead to the biosynthesis of a range of natural products including flavonoids. Genes for these enzymes, including the key enzyme chalcone synthase, have been investigated in walnuts. Walnuts expressing antisense chalcone synthase were found to be deficient in the accumulation of flavonoids but, interestingly, these deficient plants showed an increase in adventitious root formation (El Euch *et al.*, 1998). These results contrast with other root initiation studies using walnut cotyledons in which adventitious rooting was observed to occur when the appearance of the lateral root primordia coincided with the expression of chalcone synthase at the same location (Ermel *et al.*, 2000). The genes in the phenylpropanoid and flavonoid pathways were also studied to understand the accumulation of flavanols during heartwood formation in black walnut (Beritogoli *et al.*, 2002). The authors concluded that flavanol synthesis was due to the increased transcriptional activity of genes in

the phenylpropanoid pathway in black walnut sapwood cells that are undergoing the transition to heartwood.

Naphthoquinone metabolism has also been investigated and proteins involved in some of the steps have been identified. Naphthoquinones are important for plant defense and may also be involved in developmental processes (Duroux *et al.*, 1998).

2. WALNUT TRANSGENICS

Tree crops, such as walnut, are highly heterozygous and have very long generation times, which make traditional breeding difficult. A large investment in both time and land is needed to grow seedlings to maturity in order to determine nut quality. It is, therefore, advantageous to be able to introduce specific traits into existing elite cultivars. These traits may come from within the germplasm of the genus or from other organisms. Desirable traits for walnut include disease and pest resistance, as well as nut and timber quality. Herbicide tolerance would allow weeds to be controlled economically in nurseries and young orchards without damage to the trees.

2.1 Development of Transgenic Technology in Walnut

Walnut was one of the first woody plants for which *Agrobacterium*-mediated transformation and regeneration were demonstrated (Dandekar *et al.*, 1988; McGranahan *et al.*, 1988b). Somatic embryos, derived from immature cotyledons, are transformed and secondary embryos originating from single transformed cells proliferate as embryos without developing as callus (McGranahan *et al.*, 1988b; Polito *et al.*, 1989). These secondary embryos can be germinated to obtain transformed plants. Early research demonstrated that genes for selection with the antibiotic kanamycin and scorable marker β -glucuronidase (GUS) could be efficiently used to screen transformants (Dandekar *et al.*, 1989; McGranahan *et al.*, 1990a). Green fluorescent protein (GFP) has also been successfully used as a scorable marker (Escobar *et al.*, 2000). In the United States, field trials of transgenic walnut have been ongoing for

more than 15 years under Animal and Plant Health Inspection Service (APHIS) permits. These involve trees expressing genes for insect resistance, crown gall resistance, and differences in tree architecture (McGranahan and Leslie, 2006).

2.1.1 General protocol

Walnut is transformed by inserting genes into multiplying somatic embryo cultures. *Agrobacterium tumefaciens* readily infects young proliferating somatic embryos (McGranahan *et al.*, 1988b). Since new embryos develop from single epidermal cells (Polito *et al.*, 1989) transformed cells produce entirely transformed embryos and chimeras are eliminated. Embryos can be germinated to produce uniformly transformed plants. This method was used to produce trees that were field tested for the first time in 1989. Walnuts were the first transformed woody fruit or nut tree to be field tested. These trees bore nuts and the introduced genes were found to be both stably incorporated and inherited in a simple Mendelian fashion. Several independent transgenic lines can be obtained from a single embryo indicating multiple infection sites on the surface of the walnut embryo (McGranahan *et al.*, 1990a). This feature makes the walnut transformation system very efficient. Detailed protocols for the transformation of walnut have been published (Dandekar *et al.*, 1989; Leslie *et al.*, 2006b).

The *A. tumefaciens* strains that have been shown to work efficiently include the disarmed derivatives (e.g., EHA101) of the tumorigenic A281 strain that contains the Ti plasmid pTiBo542 and the nonpathogenic strain C58C1 that possesses a disarmed version of the tumorigenic Ti plasmid pTiC58 (Van Larebeke *et al.*, 1974; Hood *et al.*, 1986; Dandekar *et al.*, 1988). Transformation efficiency in walnut is approximately 20–25% (McGranahan *et al.*, 1990a; Dandekar *et al.*, 1998) with low rates of embryo germination. However, multiplication by micropropagation of transformed epicotyls is successfully used to increase the amount of transformed plant material (Reil *et al.*, 1998).

The binary vector plasmids used for walnut transformation are generally derivatives of those described by McBride and Summerfelt (1990) with genes under the control of the CaMV 35S

promoter. Generally, the neomycin phosphotransferase (*NPTII*) gene is used as a selectable marker and the *uidA* gene (coding for β -glucuronidase) is used as a scorable marker. Nontransformed embryos multiply poorly and appear yellowish on kanamycin-containing medium. Embryos that multiply well and appear healthy on kanamycin can be checked for GUS activity using X-glucuronide staining (Jefferson, 1987) or GFP fluorescence (Escobar *et al.*, 2000). Transformed embryos can be germinated following desiccation (Leslie *et al.*, 1997). This method has also been used to transform Eastern Black Walnut (Bosela *et al.*, 2004) and pecan (McGranahan *et al.*, 1993).

2.1.2 Regeneration

Because walnut somatic embryos are transformed without induction of a callus phase, and because the secondary somatic embryos derive from single cells (and thus from a single transformation event), existing tissue culture techniques can be used for the production of transgenic plantlets. As described above, somatic embryos are germinated and then can be micropropagated.

2.2 Applications of Transgenic Technology

2.2.1 Codling moth: Bt cry protein

Larvae of the codling moth (*Cydia pomonella*) are the key insect pests of walnut. Since there is little genetic resistance available in the germplasm, application of chemical pesticides is the main method of controlling this insect. To find a candidate gene to combat this pest, various insecticidal crystal proteins (ICP) from *Bacillus thuringiensis* were tested by incorporating the protein into insect diet (Vail *et al.*, 1991). Because these proteins are not toxic to mammals, they could be safely expressed in nuts. The *cryIA(c)* protein was found to be the most effective but transformation of walnut using the bacterial gene encoding this protein failed due to lack of expression (Dandekar *et al.*, 1994). This was a result of codon bias in the bacterial gene sequence, which resulted in very low levels of protein translation (Dandekar *et al.*, 1994; Leslie *et al.*, 1997). A synthetic gene correcting this problem

worked very well and high levels of codling moth mortality were observed when larvae were fed transgenic embryos (Dandekar *et al.*, 1998). Transgenic trees from these lines of the commercial cultivar "Chandler" are currently being tested in the field (Leslie *et al.*, 2001).

2.2.2 Chalcone synthase

The enzyme chalcone synthase catalyzes the first and rate-limiting step in the biosynthesis of flavonoid compounds in plants (Claudot *et al.*, 1997). It was hypothesized that flavonoids and flavonols might inhibit rooting and that, therefore, a reduction of these compounds through manipulation of this key enzyme could result in improved rooting of micropropagated plants (El Euch *et al.*, 1998). To achieve this, an antisense construct of the *chs* gene under the control of the CaMV 35S promoter was introduced into *J. regia* hybrid somatic embryos using *Agrobacterium*-mediated transformation. Lines that showed significant reduction in *chs* gene expression also had a concomitant decline in flavonoid compounds. Transformed microshoots were observed to have apical necrosis and large basal callus production. The rooting ability of the transformed microshoots was generally better than nontransgenic controls, but was seen to be variable (El Euch *et al.*, 1998).

2.2.3 Crown gall

Crown gall is a serious problem for many fruit, nut, and ornamental crops, greatly diminishing productivity. Walnuts are very susceptible to this disease. Losses are incurred from both contaminated nursery stock and infected orchard trees. Current prophylactic measures and mechanical removal of galls have not adequately controlled the problem.

Pathogenic *A. tumefaciens* transforms infected plants with the genes *iaaM* (tryptophan monooxygenase), *iaaH* (indole-3-acetamide hydrolase), and *ipt* (isopentenyl transferase). The *iaaM* and *iaaH* gene products convert tryptophan into indole-3-acetic acid, an auxin, while the *ipt* gene product catalyzes the production of isopentenyl AMP, which is converted to cytokinins by endogenous

plant enzymes. The resulting overproduction of auxin and cytokinins induces proliferation of callus tissue at the wound site, resulting in the development of large galls (Britton *et al.*, 2007).

To engineer resistance to this pathogen, a binary vector plasmid containing inverted repeats of portions of the *iaaM* and *ipt* genes was constructed and transformed into walnut using disarmed *A. tumefaciens* (Escobar *et al.*, 2002). Constitutive expression of this construct induces RNA interference mediated degradation of the *iaaM* and *ipt* transcripts, demonstrating for the first time the use of RNA interference to generate resistance to a major bacterial disease (Escobar *et al.*, 2001). Because the construct and the oncogenes do not need to have perfect homology for silencing to be effective, the resulting transgenic microshoots are resistant to a very wide range of *A. tumefaciens* strains, thus indicating a broad-spectrum durable resistance (Escobar *et al.*, 2002). Plants grown from these lines are expected to begin field trials in 2007.

2.2.4 Aflatoxin

Walnut is a tree crop whose nuts can be contaminated with aflatoxins, which are carcinogenic and teratogenic chemical compounds synthesized by members of the fungal genus *Aspergillus*. Many countries that import walnuts have set total aflatoxin action threshold levels at four ppb, significantly below the US Food and Drug Administration recommendation of 20 ppb. Because fungal infections often follow damage due to feeding of insects, such as codling moth larvae, an important objective of breeding and genetic engineering is to develop lines that demonstrate insect resistance (Campbell *et al.*, 2003). As discussed above, the engineering of walnuts to produce the insecticidal cryIA(c) protein shows promise to reduce codling moth damage and subsequent fungal infection.

It has also been discovered that kernels of the “Tulare” variety of Persian walnut are able to suppress the production of aflatoxin (Mahoney *et al.*, 2003). Evidence points to the high concentration of gallic acid in the pellicle (seed coat) as the factor that inhibits aflatoxin generation. The gene encoding shikimate dehydrogenase (SDH), the enzyme responsible

for gallic acid production, has been cloned. Somatic embryos of the cultivar “Chandler” were transformed to overexpress the *SDH* gene, resulting in the production of a high concentration of gallic acid and increased inhibition of aflatoxin production (Muir, 2005). Plants of these are in the greenhouse and will be tested when nut production commences.

2.2.5 Rhizogenes

It has been hypothesized that transformation with pathogenic *Agrobacterium rhizogenes*, or some of the genes contained in the *A. rhizogenes* Ri (root-inducing) plasmid, might improve the rootability of micropropagated walnut shoots. Transformation of *J. regia* with the wild-type strain 1855 NCPPB did induce rooting under certain media conditions, particularly in the presence of exogenous IBA (indolebutyric acid) (Caboni *et al.*, 1996; Falasca *et al.*, 2000).

The Ri plasmid genes *rolA*, *rolB*, and *rolC* (*rolABC* genes) have also been cloned from the Ri plasmid of *A. rhizogenes* strain A4, and placed in a binary vector under the control of their original promoters. This vector was introduced into *A. tumefaciens*, which was then used to transform somatic embryos of the *J. hindsii* × *J. regia* (Paradox) clone PX1. The resulting transformants were grown into stock plants. It was found that the presence of the *rolABC* genes altered canopy architecture, producing bushy canopies of dense foliage, with leaves that were wrinkled and slightly curled. However, there was no increase in rootability. When a *J. regia* scion cultivar (“Chandler”) was grafted onto the transgenic rootstock, there was no difference in growth, compared to scions grafted onto nontransformed rootstocks (Vahdati *et al.*, 2002).

3. FUTURE PROSPECTS

Currently, several lines of transgenic walnut rootstocks and scions, containing the genes described above, are in field trials in California. The commercial availability of transgenic rootstocks, however, is not expected for several years, after the completion of field trials. Acceptance of transformed rootstocks is expected due to grower

desire for traits such as disease resistance. By transforming only the rootstock, no transgenic pollen or nuts are created, eliminating the opportunity for the escape of introduced genes into the wild. It is unlikely that transgenic walnut scions will be commercialized unless there is consumer acceptance in many parts of the world (McGranahan and Leslie, 2006).

However, the techniques of nontransgenic biotechnology will continue to be used. These include genetic markers for detecting sensitivity to CLRV, which is being used to breed hypersensitive *J. regia* and the fingerprinting of all walnut cultivars to allow identification through DNA analysis, so that all the selections in the breeding program will have unique published fingerprints (Dangl *et al.*, 2005).

Improvements to walnut genomics are also on the near horizon, with thousands of expressed sequence tags being generated in current and future projects, both for *J. regia* cultivars and *J. hindsii* rootstocks. It is likely that, by 2010, a physical map of the *J. regia* chromosomes will be produced in preparation for eventual genome sequencing.

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Citrus

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The area of origin of *Citrus* is believed to be Southeastern Asia, including South China, the Indo-Chinese peninsula, Northeastern India, and Burma. This is a wide area, but attempts to localize more precisely the centers of origin of the most important *Citrus* types are still now controversial. It has become clear in recent times that only citron (*Citrus medica*), mandarin (*C. reticulata*), and pummelo (*C. grandis*) are true species within genus *Citrus*, being other important *Citrus* types, as sweet orange, sour orange, lemon, lime, grapefruit, and other mandarins originated from hybridization between these ancestral species. This view was convincingly supported by the phylogenetic study of Barrett and Rhodes (1976) who evaluated 146 morphological and biochemical tree, leaf, flower, and fruit characteristics, and it was later confirmed also using molecular markers (reviewed by Nicolosi, 2007). Therefore, Southeastern Asia would not only be the site of origin of most important *Citrus* types but also its major center of diversity. Domestication could have started in this area and expanded progressively in all directions. The genus *Citrus* is by far the most important among the Rutaceae family, but there are two other genera that have played a relevant role in citriculture, which are *Fortunella*, producing

edible fruits and commonly called kumquat, and *Poncirus*, sometimes vulgarized as trifoliate orange. All authors coincide in ascribing their origin to central China, since both genera are most cold-hardy than *Citrus* and are reported as growing wild in the Yellow river area in ancient Chinese literature.

The first historical documents mentioning the use of citrus come from China and India. Confucius describes prehistoric China in its classic “Book of History”, written ca. 500 BC, and in the “Tribute to Yu” states that the Chu and Yu were sent as annual tributes to the emperor Ta Yu (2205–2197 BC) from An Yang (north of the Yellow river) by the people of central and southern provinces, Chu and Yu being referred to *Citrus* types, most likely to mandarin and mandarin/pummelo natural hybrids, respectively. The Chu is mentioned in several Chinese classic books and also in the Bretschneider’s “Notes on Chinese Botany from Native and Western Sources” as cultivated along Yellow river central regions since 12th century BC. During the 2nd century BC, the Han emperor Wu Ti conquered and annexed the barbarian provinces of the south, and soon after, new *Citrus* types appeared in Chinese references. Kan possibly referred to sweet orange early types, Yu or Yau was then a pummelo type, and Chang was first used to describe sour orange types and later as a generic term for oranges, mainly sweet oranges. The first written reference to citrus fruits

appeared in India in the “Vajasaneyi Samhita”, a collection of sacred Brahma texts written in Sanskrit prior to 800 BC, where ancient lemon and citron types are called “jambila”.

The citron was the first *Citrus* type noticed by the Europeans and the only one known for centuries. Theophrastus describes the tree in his “Historia Plantarum” (around 300 BC) and its fruit is called “Persian citron” or “Median apple”, assuming that it was indigenous from that region. Although there are conflicting opinions on how citron arrived to Europe, most authorities agree in supporting that Alexander the Great brought it to Greece when returning from India (about 300 BC). According to a very famous Greek myth, one of the labors of Heracles was the theft of the golden apples of Hesperides, in which golden apples would be translated as citrons. *Citrus* were called Hesperides by Roman writers. Linnaeus gave the name Hesperideae to an order containing the genus *Citrus*. Nowadays, in botany the term hesperidium names a berry whose fleshy parts are divided into segments surrounded by a rind or hard shell, also in allusion to the golden apples of the Hesperides garden. Hebrews were also attracted by this “Persian tree” and it was adopted for worship during the feast of the Tabernacles, playing an important role in Jewish religious rituals. Latin writers as Vergil and Pliny later cited the citron and it was probably introduced to southern regions of Italy during the 1st century AD. Greeks and Romans held the citron in high esteem because of its delicate and penetrating fragrance. It was used both as a perfumant and moth repellent. It was not extended further in the continent until many years later (15th century) likely due to its cold sensitivity.

Although old mosaics indicate that orange and lemon were known by Romans, spread of sour orange and lemon in Europe through India, western Asia, and North Africa is due to the expansion of the Arab empire. The Crusaders further extended these and other *Citrus* types as limes and pummelos into Europe. It is not clear for historians when sweet orange first appeared in this continent, but it became widely spread only after the Portuguese established the commercial route with India and China in early 16th century. Before then, orange types were bitter and used mainly as condiments. Therefore, *Citrus* might be introduced into Europe many times at various periods of history by successive invaders and

traders, each being new introduction of increasing quality in terms of edibility and fragrance. There were attempts to establish *Citrus* trees in northern areas of Europe since the 1st century AD, but frost injury caused limiting problems. Because of this, *Citrus* types were cultured in special protected houses, first known as “stanzone per i cidri” and later as orangeries (14th century), which were the predecessors of greenhouses. Mandarin types, which were widely known and cultivated in Southeast Asia from ancient times, were not introduced to Europe from China until the 19th century.

There are many written references of *Citrus* cultivation in Japan since the 1st century AD, citrus being mostly referred to mandarin types. *Poncirus* was brought from China around the 8th century, but pummelo and sweet orange were introduced in Japan by Spanish and Portuguese travelers just in the 15th century. The origin of most popular satsuma mandarin from Japan is uncertain but it was not until the end of 19th century that it became expanded nationwide and commercialized (Mizutani, 2006).

Columbus took seeds of oranges, lemons, and citron to America on his second voyage, which arrived at Hispaniola (Dominican Republic and Haiti) in 1483. It was soon brought to other islands and continental America where citrus trees were fully adapted, spread widely, and become very abundant and even feral in some places of tropical/subtropical climate. Citrus were brought to Florida by the early Spanish explorers sometime between 1513 and 1565. About the same time, *Citrus* fruits were introduced into Brazil by the Portuguese. Portuguese travellers also introduced *Citrus* in West Africa while sweet oranges were the first *Citrus* introduced in South Africa by the Dutch colony in 1654. Citrus was first planted in Australia by the colonists of the First Fleet who brought oranges, limes, and lemons from Brazil. Oranges and lemons were first planted in California around 1769, after the settlement of Franciscan missions at San Diego area.

Due to their apomictic character, most *Citrus* varieties were propagated as seedlings during many centuries. In the case of monoembryonic genotypes, propagation by seeds led to generation of a lot of genetic variation and horticultural diversity, as it is exemplified by the high number of different mandarin types that have been grown

in China and Japan during many years. Although there are ancient Chinese references reporting the graft of mandarins onto *Poncirus trifoliata*, grafting only became a common practice in citriculture from the mid-19th century, after sweet orange seedlings grown in Europe were seriously affected by *Phytophthora* epidemics. Nowadays, the citrus industry relies on trees composed of two different genotypes: a mature fruit-producing *Citrus* scion grafted onto a highly apomictic juvenile rootstock.

Most of the information compiled in this section comes from Webber (1967) and Cooper (1982) where more detailed information on the origin and history of *Citrus* can be found.

1.2 Botanical Description

1.2.1 Taxonomy

The genus *Citrus* is one of the 33 genera in the subfamily Aurantoideae of the family Rutaceae. Within this subfamily, most taxonomists recognize that “true citrus fruit trees” belong to the tribe Citreae, subtribe Citrinae, with three genera of economic importance: *Poncirus*, *Fortunella*, and *Citrus*. The taxonomy of the genus *Citrus* is controversial. The system most commonly used comes from the classification of Swingle with modifications provided by the much more complex Tanaka’s classification. While Swingle recognizes 10 and 6 species, respectively, in the two subgenera *Citrus* and *Papeda* (Swingle and Reece, 1967), Tanaka identifies up to 157 species in different groups and subgroups (Tanaka, 1954).

From the 10 *Citrus* species designated by Swingle, 8 are of commercial importance: *C. sinensis* (L.) Osb. (sweet oranges), *C. reticulata* Blanco (mandarins), *C. paradisi* Macf. (grapefruits), *C. grandis* (L.) Osb. (pummelos), *C. limon* (L.) Burm. f. (lemons), *C. aurantifolia* (Christm.) Swing. (limes), *C. aurantium* L. (sour oranges), and *C. medica* L. (citrons).

Tanaka’s system is better adapted to horticultural traits paying also special consideration to cultivated species. This concerns to *Citrus* genotypes that are widely cultivated and of high economic importance, such as clementine mandarins (*C. clementina* Hort. ex Tan.), satsuma mandarins (*C. unshiu* (Mak.) Marc.), or Rangpur

lime (*C. limonia* (L.) Osb.) among others, for which most citrus researchers use the Tanaka’s classification.

Based on biochemical and molecular marker data, there are only three true *Citrus* species: citron, mandarin, and pummelo. Since the three ancestral species reproduce only sexually and are original from the same geographical area, several generations of hybridization among these species would generate the highest levels of genetic diversity within the genus *Citrus* and sexually compatible relatives. The appearance of facultative apomixis together with the selection of specific genotypes propagated as seedlings by men gave rise to most of the *Citrus* types currently cultivated. Obviously, this has led to a narrow genetic base within *Citrus*, in spite of the wide diversity of horticultural traits that can be found in the cultivated varieties.

Isozymes, organelle genomes, microsatellites, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and sequence-characterized amplified region (SCAR) analyses have provided important clues on the genetic relationships among *Citrus* types. Concerning economically important *Citrus* “species”, sweet oranges are thought to be hybrids between mandarin and pummelo, sour oranges would come from a mandarin genotype introgressed with genes from pummelo, which is the maternal donor, lemon would originate from citron and sour orange (female parent), and lime would also have citron as one of the parents and a *Papeda* (*Citrus micrantha* Webster) species is proposed to be the other parent. Grapefruit is a very recent species, first described in Barbados in 1750, and originated from a natural hybridization between pummelo and sweet orange probably followed by introgression with pummelo (reviewed in Nicolosi, 2007).

1.2.2 Citrus biology: some clues on growth and development

All *Citrus* types are evergreen and do not show winter dormancy but just a bud-resting period. However, the *Poncirus* relative is deciduous, showing winter leaf abscission and bud dormancy. *Citrus* species show a sympodial pattern of growth, based on flushes of growth in which the shoot

apical meristem senesces after the production of several leaves and axillary buds. The closest bud to the senescent shoot apical meristem drives the following growth flush. Whereas in subtropical conditions, three to five flushes occur per year, in tropical areas shoot growth occurs almost uninterruptedly. These growth pattern and number of flushes per year is maintained in plants grown under temperature-controlled greenhouses. However, it can be forced up by increasing day length artificially. Moreover, the elongation of the shoot can be manipulated by controlling light intensity.

Citrus shoots develop several axillary buds in the axil of each leaf, together with an axillary thorn. Shoot length is related with the genotype and the vigor/age of the shoot. Presence of thorns is commonly considered as a juvenile character, but there are genotypes showing thorns being fully mature, as for example some lemon varieties. There are also juvenile genotypes without thorns, such as Cleopatra mandarin. Leaves are unifoliate and in most species the petioles are winged. *Poncirus* shows trifoliate leaves, reminiscent of other *Aurantoideae* genera with composite leaves. Elongated leaf shape and larger petiole wings are considered juvenile characters.

Flower bud differentiation is induced photoperiodically in subtropical areas when the day becomes shorter during winter months. Cold temperatures are also important in floral induction. In the deciduous *Poncirus*, flower bud induction is initiated during late summer. In tropical areas without photoperiod changes, water stress is the major flower-inducing signal. In *Citrus*, blooming usually occurs in spring, following flower development. As evergreen, reproductive and vegetative developments are intimately related, and four main shoot types can be distinguished: vegetative shoots, leafy inflorescences, leafless inflorescences, and solitary flowers. *Poncirus* and *Fortunella* also flower in spring but usually sooner and later than *Citrus*, respectively.

The citrus fruit is a hesperidium, namely a berry arising from growth and development of the ovary, consisting of fleshy parts divided by segments, the whole being surrounded by a separable skin. It is composed of two major regions: the pericarp, commonly known as the peel, and the endocarp, often called the pulp.

The pericarp is composed of external colored peel known as flavedo, and the internal usually white layer known as albedo. Citrus fruits are nonclimacteric, ethylene changes being extremely low during fruit development. During maturation the dark green, photosynthetically active flavedo transforms its chloroplasts into carotenoid-rich chromoplasts. On the other hand, maturation of the pulp is characterized by a decline in acidity and an increase in sugars, the ratio of both components being used to define the “maturity index”. A good summary on citrus biology can be found in Spiegel-Roy and Goldschmidt (1996).

1.3 Economic Importance

Citrus is the most important fruit tree crop in the world, with a production of more than 105 million tons in 2005 (FAO, 2006). It is grown in more than 130 countries all over the world, mainly in tropical and subtropical areas (approximately 40° latitude in each side of the equator) where favorable soil and climatic conditions occur, extending over 7.6 million hectares. Major producing countries include Brazil, the United States, China, Spain, Mexico, India, Iran, Italy, Egypt, Argentina, Turkey, Japan, Pakistan, South Africa, Greece, Thailand, Morocco, Israel, Indonesia, Korea, and Australia, from major to minor. The first five countries account for about 55% of the world production.

Sweet orange represents more than 55% of total citrus production, being marketed as fresh fruit or as processed juice. Traditionally, oranges were consumed as fresh fruits but in the last 30 years consumption of processed oranges (mainly as concentrated fruit juice) has increased extraordinarily all over the world, and especially in Europe and the United States. It represents the primary force supporting expanded world consumption and is the basis of Brazilian and Florida citrus industries. Brazil and the United States produce almost 45% of sweet orange in the world and about 60% is processed.

Mandarins, including clementines, tangerines and satsumas, represent about 20% of total citrus fruit production. The first producer is China, followed by Spain and Japan. Most mandarins are intended for the fresh market and are generally consumed in the country of

production, with the important exception of Spain that is the first producer of clementines and exports more than half of its production. Japanese mandarins are nearly all satsumas. It is difficult to track the production of processed mandarin since most juice is blended with orange juice. There is an increasingly important industry of production of canned fruit segments (mainly from satsuma) developed in Japan and Spain, and more recently in China that has become the first producer.

Lemons and limes differ from other citrus types in that they are typically consumed processed or mixed with other foods. Whereas lemons are widely adapted to different climates, limes are highly sensitive to cold, being grown exclusively in tropical climates. Mexico is the first producer and exporter of limes with a production of almost 2 million tons in 2005, followed by Brazil. India is a major producer of both lemons and limes. Argentina is the first world producer and exporter of lemons. The United States, Spain, and Italy are also major producers of lemon. There is an important industry of processed lemon and lime fruits with the juice and essential oils used as flavorings in beverages and foods.

Production of other citrus types is much smaller compared to the four major groups, no more than few hundred tons in the case of pummelos, citrons, and kumquats, mostly are commercialized in local Asian markets. Processed *Citrus* fruits have many uses apart from those mentioned above. Sour orange fruits are used to produce marmalade and flowers are used to extract neroli oil, which is highly appreciated in perfumery. Peel essential oils, mainly monoterpenes, have a wide industrial use as food additives to provide a citrus flavor, as a fragrance in perfumes, air fresheners and personal care products, and as a natural replacement for petroleum-based solvents in paints and cleaning products. By-products of concentrated citrus fruit juice are used as molasses for animal feed. There is an increasing utilization of citrus trees as ornamental plants in the United States and Europe.

Citrus fruits have been shown to possess many constituents, which have important effects on the human health: vitamin C, carotenoids (as provitamin A), folic acid, flavonoids, monoterpenes/essential oils, limonoids, and others.

1.4 Citrus Scions and Rootstocks: Needs for Genetic Improvement

1.4.1 Origin of currently cultivated citrus scions and rootstocks

Citrus are diploid species having a haploid chromosome number of 9 and an approximate genome size of 0.9 pg (picogram) (~385 Mb). Most *Citrus* rootstocks and varieties grown commercially nowadays have been originated by budsport mutations and chance seedlings and have been selected directly by growers due to their excellent fruit quality, performance, and stress resistance.

In case of sweet oranges, there are two major types: blond oranges, mainly used for juice production, as the most representative Valencia orange, and navel oranges, mainly used for fresh consumption. Valencia orange was probably originated in Azores or Portugal as a mutant from the superior class of oranges brought by the Portuguese from China in the early 16th century. Many nucellar clones and likely budsport mutations of outstanding interest have been generated from the original Valencia, which are currently the basis of the Brazilian and Florida citriculture. The origin of navel orange is uncertain but it has been claimed that it was generated as a limb sport from the blond variety Selecta at Salvador de Bahia (Brazil). Worldwide expansion of this type started after it was brought to the United States and renamed as Washington navel at the end of 19th century. Many early and late-ripening bud mutants have been found covering all seasons with one of the most excellent fresh fruit types achievable in markets. A third group of minor importance is that of blood oranges, characterized by the accumulation of anthocyanins in the flesh and juice, red pigment synthesis being usually dependant on low night temperatures. They probably originated in the Mediterranean area as mutants from blond oranges. Nowadays, blood oranges are only important in Italy, Tarocco being their most popular selection.

Natural and man-made mandarin hybrids have been cultivated in China and Southeast Asia during many centuries. Two of the most commercially successful mandarin types in current times are satsumas in China and Japan, and clementines in Spain and Morocco. The original

satsuma was most probably generated as a chance seedling in Kyushu Island (Japan) around 15–16th century. Many early and late maturing, small and larger fruited types are commercially relevant and all of them have been generated as limb sports, bud sports, or nucellar seedlings from pre-existing scions. Clementine first appeared in the garden of an orphanate near Oran (Algeria) and it was discovered by Father Clement Rodier as a seedling from a cross between a Mediterranean mandarin and an ornamental sour orange known as Granito, according to historical records. However, molecular markers data support that it was originated from a mandarin \times sweet orange cross (reviewed by Nicolosi, 2007). It is not strange that the first describers were confused about the actual pollen donor. Clementine was introduced into Spain from Algeria in 1925, and since then many excellent bud mutants of different fruit size, shape, color and maturing season have been found and propagated, constituting the main basis of the Spanish citrus industry.

Another natural hybrid mandarin is the Ponkan, widely cultivated in Asian citrus countries and in Brazil. Some other mandarin cultivars of relative importance that were originated as chance seedlings are Dancy, from Florida, and Ellendale and Imperial, found in Australia, all in the 19th century. “Murcott” is most probably a tangor (mandarin \times sweet orange) of unknown parentage cultivated in Florida, Brazil, Argentina, and Japan. Another important tangor of natural hybrid origin is “Ortanique”, discovered in Jamaica around 1920. A more recent natural hybrid is “Afourer” or “Nadorcott”, found recently in Morocco (most probably a “Murcott” \times clementine hybrid), which is considered the most significant new mandarin variety currently available.

All important common varieties of lemon and lime are natural hybrids, chance seedlings or budsport mutations selected by men either in ancient time in Southeast Asia or since the 19th century in other major citriculture areas.

Regarding rootstocks, Rangpur lime is the predominant one in Brazil, due to its combined tolerance to several important biotic and abiotic stresses. It is an ancient Asian natural hybrid. *P. trifoliata*, a true species, is widely used in several parts of the world, mainly in Asia, especially due to its cold-hardiness and semi-dwarfing abilities.

Importantly, both are resistant or highly tolerant to the major pathogens *Phytophthora* spp. and *Citrus tristeza virus* (CTV). Other relevant citrus rootstocks, as sour orange, Cleopatra and Sunki mandarins, rough and Volkamer lemons, and alemow or *Citrus macrophylla* are ancient natural hybrids of Southeast Asian origin.

In spite that present-day citriculture is based on cultivars that grower directly selected from the wild or from the orchards, they usually have outstanding quality. This makes very difficult the obtention of new improved cultivars, especially in the case of scion varieties for the fresh fruit market. Citrus rootstocks and varieties of the world are extensively detailed by Saunt (2000).

1.4.2 Needs for genetic improvement

Many different citrus genotypes are commercially grown in a wide diversity of soil and climatic conditions, implicating that trees are subjected to important abiotic and biotic stresses that limit the production and, in some instances, the use of certain rootstocks and varieties. The main abiotic stresses are acid, alkaline, and salty soils, flooding and drought, freezing, and high temperatures.

Citrus trees are also affected by many pests, diseases caused by nematodes, fungi, oomycetes, bacteria, spiroplasmas, phytoplasmas, viruses and viroids, and diseases of unknown etiology. Some diseases are spread throughout the world, as those produced by the oomycete *Phytophthora* spp., or by the CTV, which preclude the use of certain excellent rootstocks, and severely restrict fruit production and quality of important varieties in some countries. Other diseases are restricted to specific geographic areas, as those caused by the bacteria *Xylella fastidiosa* or by citrus sudden death associated virus in Sao Paulo state (Brazil). There are also diseases spread in most citrus areas, as citrus canker, caused by the bacteria *Xanthomonas axonopodis* pv. *citri*. Whereas Brazil has been able to implement a quite successful eradication program, the bacterium is currently expanding without control in Florida. Finally, there are diseases that were locally important but in more recent times have become widely spread and are seriously threatening important citricultures, as it is the case of the Huanglongbing caused by the bacterium *Candidatus Liberobacter asiaticum*,

which affects all citrus varieties. It has impeded the development of citriculture in certain Southeast Asian countries and at present day is devastating millions of trees in Florida and Brazil. In the cases of these three bacteria there are no means for efficient control. At the same time that citrus industry is threatened by important biotic and abiotic stresses, markets demand fresh fruit and juice of increasing quality. In this situation, genetic improvement of citrus has a very high priority.

Major current goals of rootstock breeding are resistance to CTV and *Phytophthora* spp., cold-hardiness in citrus areas as Japan, Florida, or New Zealand, scion size-controlling abilities, higher tolerance to calcareous, and saline soils in areas with poor-quality water, and resistance to the citrus and the burrowing nematodes, particularly in Florida. Scion breeding is mainly focused in resistance against major pests and diseases, and in fruit quality aspects. For the fresh fruit market, major goals include adequate size for each citrus type, easy peeling, seedlessness, attractive color and aroma, compensated acid/sugar content in the fruit, extension of the maturity season for all year round supply, and good storage and shipment. When the fruit is going to be used for juice production, prime goals are juice content of the fruit, good color, and lack of bitterness.

1.5 Limitations of Conventional Breeding, Achievements, and Rationale for Transgenic Breeding

1.5.1 Biological limitations of citrus breeding

Conventional breeding by hybridization has important limitations. *Citrus* species have a complex reproductive biology. Most genotypes are facultative apomictic, which means that adventitious embryos initiate directly from maternal nucellar cells, limiting or precluding the development of less vigorous zygotic embryos. Although this is the basis for propagation of citrus rootstocks, apomixis seriously limits the recovery of sexual progeny populations in breeding programs. Some important genotypes have total or partial pollen and/or ovule sterility and cannot be used as parents in breeding programs; for example, most navel oranges are male sterile

while satsuma mandarins and most navel and Valencia oranges are female sterile. There are many cases of cross- and self-incompatibility. Clementines, grapefruits, and certain important lemons are self-incompatible, and many hybrids between self-incompatible cultivars are also cross-incompatible. They have a long juvenile period and most species need at least 5 years to start flowering in subtropical areas, and usually several years more to achieve fully mature characteristics. Citrus have high heterozygosity, there is a lack of basic knowledge about how the most important horticultural traits are inherited, and they show quantitative inheritance of important characters, many of them related to fruit quality and maturity time. All these features together with their large plant size have greatly impeded genetic improvement of citrus through conventional breeding methods. Moreover, sources of efficient resistance against important pathogens as *Candidatus* L. asiaticum and *X. fastidiosa* have not been found in the citrus germplasm.

1.5.2 Breeding goals and new tools for citrus improvement

In principle, breeding objectives in citrus are different depending on whether improved rootstocks or scions would like to be generated. Citrus trees, mainly sweet orange, were mostly produced from seedlings until mid-19th century, when *Phytophthora* spp. was recognized as a major disease of trees first in Azores (1840) and later in France and Spain. There are records indicating that sweet orange trees were grafted onto different rootstocks as citron, lemon, sour orange and other sweet orange genotypes in certain citrus areas of Spain. After spreading of *Phytophthora* spp. in Spain, only scions grafted onto sour orange remained alive (Wolffenstein, 1880). Since then, all citrus trees were budded onto this rootstock, which is not only resistant to the oomycete pathogen but also provides excellent agronomic attributes, particularly fruit yield, quality, rusticity, and tolerance to calcareous and saline soils. Thus bud grafting of scion varieties onto sour orange became a universal practice. Between 1910 and 1930, trees grafted onto sour orange started to decline with a disease later identified as caused by CTV. Since then,

different citrus genotypes and relatives have been used as rootstocks depending on the requirements of each citriculture area, but very few of them were originated in breeding programs. The first recorded artificial hybridization of citrus was carried out by Swingle and Webber in Florida in 1893 with the aim of incorporating resistance to diseases, but a severe freeze destroyed most of the seedlings. Then, they decided to use the cold-hardy *P. trifoliata* as a parent in crosses to try to incorporate higher cold tolerance to citrus scions. None of the progeny trees combined hardiness with good fruit quality. However, the excellent Carrizo and Troyer citrange hybrids (sweet orange \times *P. trifoliata*) rootstocks resulted undeliberately from these crosses. Both hybrids are highly tolerant to CTV and *Phytophthora* spp., and are widely used as rootstocks in countries as Spain and the United States. Another rootstock hybrid obtained in the same program, though released in 1974, was the Swingle citrumelo, coming from a grapefruit \times *P. trifoliata* cross performed in 1907. It is widely used in Florida and South Africa. All other important rootstocks used now-a-days are true species or ancient natural hybrids.

Very active variety hybridization programs were performed along 20th century in most citrus-growing countries, but none of the important scion varieties cultivated at present day came from such programs though some hybrid scions of relative importance were generated. In Florida, grapefruit \times mandarin hybrids gave rise to the “Orlando” and “Minneola” tangelos. Clementine \times these tangelos yielded a number of hybrids of some importance as “Nova”. In California, Frost performed interesting crosses among different mandarin types between 1914 and 1916, and obtained the “Kinnow” mandarin hybrid (King mandarin \times Mediterranean mandarin), widely cultivated in Pakistan and India. “Fortune”, a hybrid of clementine \times Dancy mandarin made by Furr in California in 1964, has been the most important late mandarin hybrid in Spain in the last two decades of the 20th century. “Kiyomi” is a tangor (satsuma \times sweet orange) obtained in Japan and released in 1979. Ponkan \times “Kiyomi” recently gave rise to “Shiranui” that is probably the best success of hybridization breeding programs in Japan.

Artificial induction of genetic changes was initiated in 1935 in the United States by treating seeds with x-rays. Then the most important results

of irradiation programs came from Henz, who obtained several thousand plants from irradiated seeds and budwood from grapefruit and Valencia orange in Texas, resulting in two of the most widely grown pigmented grapefruit varieties of present times. “Star Ruby” was produced by irradiating seeds from the Hudson variety in 1959, and “Rio Red” was selected as a bud mutation of a tree obtained after irradiation of budwood from a “Ruby Red” seedling (see also **Grapefruit**). More recently, irradiation programs have been carried out for the generation of seedless mandarin hybrids. Examples of recent highly promising seedless releases are “Orri”, generated from the Israeli hybrid “Orah” (“Temple” \times “Dancy”), and “Tango”, originated from irradiated “Afourer”.

Although diploidy is the prevalent state in citrus, tetraploids spontaneously arise due to chromosomal duplication in nucellar cells, which are able to generate embryos and plants, their frequency being highly dependent on genotype and environmental conditions. Tetraploidy has been also induced by treating nucellar callus, and caulinar apices (for monoembryonic cultivars) with colchicine. Some authors have proposed the use of tetraploid citrus as semi-dwarfing rootstocks. Nevertheless, tetraploid genotypes have been mostly used to generate triploids in crosses with diploid parents. Most triploid citrus trees are sterile, producing seedless fruit. A natural triploid variety is “Tahiti” lime (*Citrus latifolia* Tan.), increasingly important for Mexican citrus industry. Seedlessness is very difficult to obtain by conventional hybridization. Production of triploid hybrids is currently the most promising approach to obtain seedless cultivars that do not produce seeds. Recovery of citrus sexual triploid hybrids ($3n = 27$) has been reported since the early 1960s after $2n \times 4n$, $4n \times 2n$, and $2n \times 2n$ crosses (Ollitrault *et al.*, 1998). In the last case, the triploid embryos are originated by the fertilization of an unreduced diploid female gamete with a normal reduced haploid male gamete. Seeds with triploid embryos are generally underdeveloped or aborted due to uneven embryo/endosperm chromosome balance, and it is very difficult to regenerate plants regularly. In addition, analysis of ploidy level of large populations of citrus plants by cytological methods is very difficult. The development of methodologies for *in vitro* culture of embryos and small seeds and for ploidy

analysis by flow cytometry is allowing a much more efficient production of citrus triploid sexual hybrids (Navarro *et al.*, 2003). Several promising triploid mandarin hybrids have been recently released in the United States and Italy.

Another promising technology to generate tetraploid breeding parents is somatic hybridization. It allows producing somatic hybrids that incorporate genomes of the two parents without recombination, thus avoiding the problem of the high heterozygosity in citrus. Somatic hybrids are generally produced from the fusion of protoplasts isolated from embryogenic callus or suspension cultures of one parent with leaf-derived protoplasts of the second parent. Protoplast fusion is induced either by polyethylene glycol (PEG), electrically, or by a combination of both methods. After fusion, the embryogenic parent provides to the hybrid most of the capacity of regeneration through callus formation and somatic embryogenesis. In citrus this technology has been extensively used and has many important applications, including the generation of new tetraploid hybrids, utilizable as parents for triploid breeding or directly as new rootstocks, the production of triploids from haploid + diploid somatic hybridization, and the generation of cybrids, namely new diploid hybrids with the nuclear genome from one parent and either the cytoplasmic genome from the other parent or a combination of both parents (Grosser *et al.*, 2000). There is a large somatic hybridization program in Florida, with many hybrids being tested in the field as potential rootstocks and several flowering ones are being used as pollen donors in a breeding program aimed to produce triploid mandarin hybrids (Grosser and Gmitter, 2005).

Grosser *et al.* (2002) are investigating the phenomenon of somaclonal variation as an alternative method for improving sweet orange. Somaclonal variation is defined as genetic variation that is either induced or uncovered by plant tissue culture *in vitro*. This generally slight variation could modify plant horticultural performance and give rise to new improved germplasm. They are currently field evaluating several Valencia sweet orange selections obtained through somatic embryogenesis from nucellar callus or from protoplasts, which possess the superior fruit color and quality of Valencia orange, but mature earlier or later than the standard variety.

The development of genetic markers is also providing a potential tool for citrus breeding. Linkage maps have been constructed using isozymes, RFLP, RAPD, SCARs, amplified fragment length polymorphism (AFLP), microsatellites (single sequence repeat; SSR), and cleaved amplified polymorphic sequences (CAPs). These studies have served to determine the mode of inheritance of some traits and they could be useful for early selection of the progeny and genotype identification in breeding programs. More recently, other markers as resistance gene candidates (RGCs) and single nucleotide polymorphisms (SNPs) have been developed and are currently being used in citrus breeding.

1.5.3 Rationale for transgenic breeding

In spite of the many efforts put in classical breeding programs in the last 100 years, current citrus industry relies on various groups of varieties of outstanding quality that are grafted onto a narrow diversity of rootstocks adapted to different abiotic and biotic stresses. Most of these excellent genotypes have been generated by chance, mostly as budsports but also as natural hybrids or seedlings selected by men in the wild or in orchards. In addition citrus breeding is complicated due to its complex reproductive biology. In this context, genetic transformation offers an excellent alternative for genetic improvement of citrus because it is based on the introduction of specific traits into known genotypes without altering their elite genetic background. Theoretically, it would be possible to incorporate the CTV-resistance trait into the otherwise primary sour orange rootstock, or seedlessness independent of cross-pollination into clementine mandarins. The transgene of interest could come from another *Citrus* species or relative, from another plant species, or from another organism such as a bacterium, an insect or a virus, widening the possibilities for genetic improvement. Moreover, it allows overcoming the heterozygosity, inbreeding depression, and genetic incompatibility barriers associated with hybridization. Facultative apomixis is in principle an advantage because it could be possible to use vigorous juvenile material genetically identical to the elite mature germplasm as a source of plant tissue for transformation. However, this

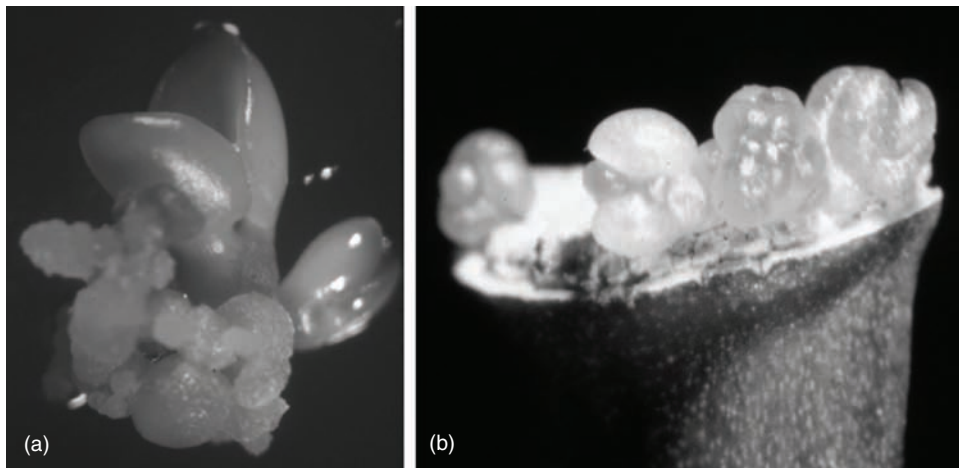


Figure 1 Regeneration of whole citrus plants through somatic embryogenesis and organogenesis. (a) Somatic embryo at the cotyledonary stage developing from nucellar-derived callus tissue. (b) Direct organogenesis from the cut end of an internodal stem segment

cannot be applied to important monoembryonic citrus types as Clementine mandarins. In addition, female sterility is extended in citrus cultivars making it difficult to obtain seeds (e.g., in navel sweet oranges). More important, the juvenile period of citrus is extremely long compared to other fruit trees, taking about 5 years for first flowering and fruit setting, and at least 3 years more to loose completely juvenile growth and developmental characteristics. For the purpose of genetic improvement, transformation of mature tissue then becomes necessary. Sweet orange was the first fruit tree from which adult material was transformed (Cervera *et al.*, 1998a) providing the only biotechnology-based system able to overcome the juvenility constraint of citrus breeding.

2. DEVELOPMENT OF TRANSGENIC CITRUS

2.1 Plant Regeneration: Organogenesis and Somatic Embryogenesis

2.1.1 Source plant material

Compared to other fruit trees, *Citrus*, *Poncirus*, and their hybrids are more amenable to tissue culture. The ability to regenerate whole plants from protoplasts, cell suspensions, callus, tissues

and organs has been fully established (Figure 1), and regeneration studies have been successful for different applications including recovery of pathogen-free plants (Navarro *et al.*, 1975; Navarro, 1992), ploidy manipulation (Ollitrault *et al.*, 1998), generation of new hybrids and cybrids (Grosser and Gmitter, 2005), and genetic transformation (Table 1).

Sweet orange was the first tree crop in which plant regeneration from protoplasts was achieved (Vardi *et al.*, 1982; Kobayashi *et al.*, 1983). Since then, protoplast totipotency has been demonstrated and exploited for most citrus types of interest (reviewed in Grosser and Gmitter, 2005). Embryogenic suspension cultures initiated from ovule-derived nucellar callus have been the most appropriate source of protoplasts able to regenerate plants through somatic embryogenesis.

Protoplasts were the first source of plant material used to attempt genetic transformation in citrus. Kobayashi and Uchimiya (1989) obtained transgenic callus from Trovita sweet orange by PEG treatment of protoplasts with a plasmid containing the neomycin phosphotransferase II (*nptII*) marker gene, but regeneration of transgenic plants from that callus was unsuccessful. Cell suspension cultures were originated from nucellar callus and maintained in MT (Murashige and Tucker, 1969) liquid medium with 10 mg l^{-1} 6-benzylaminopurine (BAP). Two-week-old cells

Table 1 Summary of studies conducted in genetic transformation and regeneration of transgenic citrus plants through somatic embryogenesis (A) and organogenesis (B)

References	Citrus genotypes	Plant material	Vector/method	Results/remarks
A				
Kobayashi and Uchimiya, 1989	<i>Citrus sinensis</i> cv. Trovita	Protoplasts	PEG	<i>nptII</i> No plant regeneration
Vardi <i>et al.</i> , 1990	<i>Citrus jambhiri</i>	Protoplasts	PEG	<i>cat</i> , <i>nptII</i> Scarce plant regeneration
Hidaka <i>et al.</i> , 1990	<i>Citrus sinensis</i> cv. Washington navel and cv. Trovita	Cell suspensions	<i>Agrobacterium tumefaciens</i>	<i>hpt</i> , <i>nptII</i> No plant regeneration
Hidaka and Omura, 1993	<i>Citrus reticulata</i> cv. Ohta ponkan	Protoplasts	Electroporation	<i>uidA</i> No plant regeneration
Niedz <i>et al.</i> , 1995	<i>Citrus sinensis</i> cv. Hamlin	Protoplasts	Electroporation	<i>gfp</i> Efficient plant regeneration
Yao <i>et al.</i> , 1996	Tangelo (<i>Citrus reticulata</i> × <i>Citrus paradisi</i>)	Cell suspensions	Particle bombardment	<i>nptII</i> , <i>uidA</i> No plant regeneration
Fleming <i>et al.</i> , 2000	<i>Citrus sinensis</i> cv. Itaborai	Protoplasts	PEG	<i>gfp</i> No plant regeneration
Li <i>et al.</i> , 2002	<i>Citrus reticulata</i> cv. Ponkan	Embryogenic callus	<i>Agrobacterium tumefaciens</i>	<i>bar</i> , <i>pAT29-barnase</i> Efficient plant regeneration
Olivares-Fuster <i>et al.</i> , 2003	<i>Citrus sinensis</i> cv. Itaborai	Protoplasts	PEG	<i>gfp</i> , CTV-derived sequences No plant regeneration
Li <i>et al.</i> , 2003	<i>Citrus sinensis</i> cv. Valencia	Embryogenic callus	<i>Agrobacterium tumefaciens</i>	<i>bar</i> , <i>pAT29-barnase</i> Efficient plant regeneration
Niedz <i>et al.</i> , 2003	<i>Citrus sinensis</i> cv. Hamlin	Protoplasts	Electroporation	<i>egfp</i> Efficient plant regeneration
Guo <i>et al.</i> , 2005	<i>Citrus sinensis</i> cv. Valencia	Protoplasts	PEG	<i>gfp</i> , <i>TSPME</i> Efficient plant regeneration
B				
Moore <i>et al.</i> , 1992	Carrizo citrange (<i>Citrus sinensis</i> × <i>Poncirus trifoliata</i>)	<i>In vitro</i> internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Scarce transgenic plant regeneration
Kaneyoshi <i>et al.</i> , 1994	<i>Poncirus trifoliata</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Peña <i>et al.</i> , 1995b	Carrizo citrange (<i>Citrus sinensis</i> × <i>Poncirus trifoliata</i>)	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Peña <i>et al.</i> , 1995a	<i>Citrus sinensis</i> cv. Pineapple	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Kobayashi <i>et al.</i> , 1996	<i>Poncirus trifoliata</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>hEGF</i> gene Transgenic plants
Peña <i>et al.</i> , 1997	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Gutiérrez <i>et al.</i> , 1997	Carrizo citrange <i>Citrus aurantium</i> <i>Citrus aurantifolia</i>	<i>In vitro</i> internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-CP gene Transgenic plants
Cervera <i>et al.</i> , 1998a	<i>Citrus sinensis</i> cv. Pineapple	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Mature transgenic plants
Cervera <i>et al.</i> , 1998c	Carrizo citrange (<i>Citrus sinensis</i> × <i>Poncirus trifoliata</i>)	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration

(Continued)

Table 1 (Continued)

References	Citrus genotypes	Plant material	Vector/method	Results/remarks
Bond and Roose, 1998	<i>Citrus sinensis</i> cv. Washington navel	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Gentile <i>et al.</i> , 1998	Troyer citrange <i>Citrus sinensis</i> cv. Tarocco	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>rolA</i> , <i>rolB</i> , <i>rolC</i> genes Aberrant transgenic plants
Pérez-Molphe and Ochoa-Alejo, 1998	<i>Citrus aurantifolia</i> cv. Mexican	<i>In vitro</i> internodal stem segments	<i>Agrobacterium rhizogenes</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Luth and Moore, 1999	<i>Citrus paradisi</i> cv. Duncan	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Ghorbel <i>et al.</i> , 1999	Carrizo citrange <i>Citrus aurantium</i> <i>Citrus aurantifolia</i>	<i>In vitro</i> epicotyl or greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>gfp</i> Efficient transgenic plant regeneration
Kaneyoshi and Kobayashi, 1999	<i>Poncirus trifoliata</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>RolC</i> Better rooting ability of transgenic plants, dwarfism
Cervera <i>et al.</i> , 2000b	Carrizo citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Stability of transgene integration and expression over years
Cervera <i>et al.</i> , 2000a	Carrizo citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>HAL2</i> gene Transgenic plants
Domínguez <i>et al.</i> , 2000	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-CP gene Transgenic plants, transgenic protein accumulation
Ghorbel <i>et al.</i> , 2000	<i>Citrus aurantium</i>	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-CP gene Transgenic plants
LaMalfa <i>et al.</i> , 2000	Troyer citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>gfp</i> Efficient transgenic plant regeneration
Yang <i>et al.</i> , 2000	<i>Citrus paradisi</i> cv. Rio Red	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> , unCTV-CP, <i>gna</i> genes Transgenic protein accumulation
Koltunow <i>et al.</i> , 2000	<i>Citrus aurantifolia</i> cv. Mexican	<i>In vitro</i> hypocotyl and epicotyl segments	<i>Agrobacterium tumefaciens</i>	Genes for decreased seed set Transgenic plants
Wong <i>et al.</i> , 2001	Carrizo citrange <i>Citrus sinensis</i> <i>Poncirus trifoliata</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	CS- <i>ACS1</i> gene Repression of ACC content increase following chilling treatment
Ghorbel <i>et al.</i> , 2001b	Different genotypes	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	Enhancement of <i>A. tumefaciens</i> strain virulence by adding <i>virG</i> genes
Ghorbel <i>et al.</i> , 2001a	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-p23 gene Development of CTV symptoms in transgenic plants
Fagoaga <i>et al.</i> , 2001	<i>Citrus sinensis</i> cv. Pineapple	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>PR-5</i> gene Resistance to <i>Phytophthora citrophthora</i> in one transgenic line

(Continued)

Table 1 (Continued)

References	Citrus genotypes	Plant material	Vector/method	Results/remarks
Peña <i>et al.</i> , 2001	Carrizo citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>AP1</i> , <i>LFY</i> genes Precocious flowering of transgenic plants
Domínguez <i>et al.</i> , 2002c	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-CP gene Protection against CTV in several transgenic lines
Domínguez <i>et al.</i> , 2002a	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	Transgenic plants under <i>nptII</i> -selective and nonselective conditions. Silencing.
Domínguez <i>et al.</i> , 2002b	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	unCTV-CP gene versions Some delay in virus infection
Costa <i>et al.</i> , 2002	<i>Citrus paradisi</i> cv. Duncan	<i>In vitro</i> internodal stem segments	<i>Agrobacterium tumefaciens</i>	Phytoene synthase, phytoene desaturase, lycopene- β -cyclase genes Transgenic plant regeneration
Yu <i>et al.</i> , 2002	Carrizo citrange <i>Citrus sinensis</i>	<i>In vitro</i> internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Mendes <i>et al.</i> , 2002	<i>Citrus sinensis</i> cv. Hamlin	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>gfp</i> Efficient transgenic plant regeneration
Febres <i>et al.</i> , 2003	<i>Citrus paradisi</i> cv. Duncan	<i>In vitro</i> internodal stem segments	<i>Agrobacterium tumefaciens</i>	ntCTV-CP, <i>RdRp</i> , 3' end genes Great variability in virus titer in control and transgenic plants in CTV challenge
Almeida <i>et al.</i> , 2003a	<i>Citrus sinensis</i> cv. Natal, cv. Valencia <i>Citrus limonia</i> cv. Rangpur	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Almeida <i>et al.</i> , 2003b	<i>Citrus sinensis</i>	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Mature transgenic plants
Boscariol <i>et al.</i> , 2003	<i>Citrus sinensis</i> cv. Valencia, cv. Hamlin, cv. Natal, cv. Pera	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>PMI</i> gene (positive selection) Efficient transgenic plant regeneration
Molinari <i>et al.</i> , 2004a	Swingle citrumelo (<i>Citrus paradisi</i> \times <i>Poncirus trifoliata</i>)	<i>In vitro</i> epicotyl thin sections	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> Efficient transgenic plant regeneration
Molinari <i>et al.</i> , 2004b	Carrizo citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>p5cs</i> gene Proline accumulation, superior behavior of transgenic plants under drought stress
Kayim <i>et al.</i> , 1994	Carrizo citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>p12</i> gene (sense and antisense) Transgenic plant regeneration
Domínguez <i>et al.</i> , 2004	Different genotypes	<i>In vitro</i> epicotyl or greenhouse internodal segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> , <i>gfp</i> Study of phenomena as chimeras, escapes or silencing
Peña <i>et al.</i> , 2004a	Different genotypes	<i>In vitro</i> epicotyl or greenhouse internodal segments	<i>Agrobacterium tumefaciens</i>	<i>nptII</i> , <i>uidA</i> , <i>gfp</i> Study of citrus cell transformation process

(Continued)

Table 1 (Continued)

References	Citrus genotypes	Plant material	Vector/method	Results/remarks
Iwanami <i>et al.</i> , 2004	<i>Poncirus trifoliata</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	CiMV-CP gene One immune and several tolerant transgenic lines
Trainin <i>et al.</i> , 2005	<i>Citrus paradisi</i> cv. Duncan	<i>In vitro</i> internodal stem segments	<i>Agrobacterium tumefaciens</i>	Transposable element activator Ac Ac activity maintenance in transgenic plants after 4 years of growing
Endo <i>et al.</i> , 2005	<i>Poncirus trifoliata</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	Ci-FT gene Precocious flowering of transgenic plants
Fagoaga <i>et al.</i> , 2005	<i>Citrus aurantifolia</i> cv. Mexican <i>Citrus aurantium</i> <i>Poncirus trifoliata</i>	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-p23 gene Development of CTV symptoms in transgenic plants
Fagoaga <i>et al.</i> , 2006	<i>Citrus aurantifolia</i> cv. Mexican	Greenhouse internodal stem segments	<i>Agrobacterium tumefaciens</i>	CTV-p23 gene Some CTV-immune transgenic plants
Boscariol <i>et al.</i> , 2006	<i>Citrus sinensis</i> cv. Hamlin	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>attA</i> gene Significant reduction in susceptibility to citrus canker in some transgenic lines
Rai, 2006	<i>Citrus paradisi</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	Ten candidate CTV resistance genes Evaluation of gene expression in transgenic lines and of CTV accumulation in infected plants
Ballester <i>et al.</i> , 2007	Carrizo citrange <i>Citrus sinensis</i> cv. Pineapple	<i>In vitro</i> epicotyl or greenhouse internodal segments	<i>Agrobacterium tumefaciens</i>	<i>ipt</i> gene (positive selection), <i>R/RS</i> recombinase system
Cervera <i>et al.</i> , 2006	Carrizo citrange	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>hpt</i> , <i>bar</i> , <i>gfp</i> Retransformation of early flowering <i>API</i> transgenic plants
Batuman <i>et al.</i> , 2006	<i>Citrus macrophylla</i>	<i>In vitro</i> epicotyl segments	<i>Agrobacterium tumefaciens</i>	<i>ds(p23 + 3'UTR)</i> gene construct Delayed appearance of symptoms but no durable resistance

were transferred to hormone-free MT medium, and 2 weeks later were collected and subjected to protoplast isolation by incubation with an enzyme solution containing macerozyme, cellulase Onozuka, and driselase in $\frac{1}{2}$ MT salts plus mannitol as osmoprotectant. Incubation was carried out at 25 °C in a rotary shaker (25 rpm) for 16 h in dark. Protoplasts were filtered through a nylon mesh and washed with mannitol and MT plus mannitol through low centrifugation cycles to remove the enzymatic solution. Protoplasts were purified from a sucrose-mannitol gradient (Kobayashi *et al.*, 1985), suspended in a mannitol

solution and mixed with a plasmid vector solution. After 5 min, a PEG solution was added to the mixture. Dilutions were made with a glucose-containing solution to adjust the osmotic level of the mixture. Protoplasts were then cultured in MT plus mannitol medium in small Petri dishes. The plates were sealed and maintained under 16-h/day illumination (500 lux) at 26 °C. After 2 weeks, the medium was diluted with an equal volume of MT plus mannitol. Two weeks later, the medium was solidified by an equal MT plus mannitol medium supplemented with 50 mg l⁻¹ kanamycin and 1.2% agarose. After 2

months, cell colonies were transferred to MT plus 0.8% agar and 25 mg l^{-1} kanamycin for a second round of selection. Only eight colonies larger than 1 mm were able to survive the two rounds of selection. They were transferred to kanamycin-free MT medium supplemented with 5 mg l^{-1} BAP, 0.8% agar, but apparently they did not progress further. Integration of the *nptII* transgene was demonstrated in 4–5 of the callus lines by Southern blot analysis.

Vardi *et al.* (1990) produced transgenic callus from rough lemon (*Citrus jambhiri* Lush.) by PEG treatment of protoplasts with a plasmid containing the marker genes chloramphenicol acetyltransferase (*cat*) and *nptII*, and obtained several stably transgenic embryogenic lines, and at least two of them regenerated whole plants. Nucellar callus subcultured at least twice on MT was macerated in an enzyme solution containing macerozyme, cellulase Onozuka, and driselase in $1/2$ MT salts plus mannitol and sucrose. Protoplasts were isolated by sequential filtering through nylon screens, and washed several times by centrifugation at $100 \times g$ in MT plus sucrose/mannitol solutions. Washed protoplasts were resuspended and centrifuged in a Ficoll/mannitol gradient. Intact protoplasts formed a prominent band at the upper interface while the debris remained at the bottom. Then, protoplasts were collected and rewashed. For transformation, protoplasts were cocultivated with the linearized plasmid vector in a buffer medium containing PEG. Protoplasts were layered on top of a feeder layer consisting of γ -irradiated *Citrus* \times *P. trifoliata* protoplasts plated in small Petri dishes. According to Vardi and Galun (1989), the feeder layer would promote protoplast division. However, the PEG treatment delayed the initiation of cell division from protoplast-derived cell colonies, and only about 8 weeks after transformation protoplast-derived microcallus attained about 0.5 mm. Then, it was exposed to paromomycin selection ($20\text{--}40 \text{ mg l}^{-1}$) in MT plus 4% sucrose, since the more common aminoglycoside antibiotic kanamycin did not provide a reproducible inhibition curve. Green embryoids were formed from 21 callus colonies, which were picked up and plated on a medium devoid of paromomycin to promote further growth and development. Individual embryos were isolated and regenerated

to plants. Integration of the *nptII* gene in several plants and embryos was demonstrated by Southern blot analysis, as well as *cat* and *nptII* expression in putative transgenic embryos.

Hidaka *et al.* (1990) produced transformed callus of Washington navel and Trovita sweet oranges by co-cultivation of embryogenic cell suspension lines with *Agrobacterium tumefaciens*, but only one transgenic plantlet of Washington navel was regenerated. Six- to eight-year-old nucellar callus from Washington navel orange, Ohta ponkan, and Kara mandarin, and pollen-derived somatic callus from Trovita sweet orange, all of them maintaining their embryogenic potential, were transferred to liquid MS (Murashige and Skoog, 1962) medium supplemented with 0.2 M sucrose and $50 \mu\text{M}$ kinetin. Cultures were incubated at 25°C , 16-h light period, in an orbital shaker at 130 rpm, and refreshed at least three times at 2-week intervals. Seven days after subculture, cell colonies were suspended in MS-sucrose liquid medium and inoculated with *A. tumefaciens* at a ratio of 100–200 bacteria per cell colony. Two bacterial strains were used, each carrying the *nptII* or the hygromycin phosphotransferase (*hpt*) marker genes within the transfer-DNA (T-DNA) of their disarmed Ti plasmids. After 3, 5, or 7 days of co-cultivation, cell colonies were washed several times with MS plus 0.2 M sucrose supplemented with 100 mg l^{-1} kanamycin or 20 mg l^{-1} hygromycin, and then they were spread on the same medium but gelified with 0.8% agar. A second round of selection was performed with double antibiotic concentration. Aliquots from both the first and the second round of selection were spread on MS plus 0.2 M sucrose for callus proliferation, or on MS plus 0.1 M galactose and 0.1 M sorbitol for embryoid differentiation. Co-cultivation of 3 days provided the best results. Antibiotic pressure, even at low concentration, inhibited callus proliferation. However, green embryoids were formed from Washington navel and Trovita orange callus after 6–10 weeks of culture in the first selection medium. Transfer of embryoids and callus to the second selection medium precluded further progress in most cases but 15 embryoids in total were able to survive. At least one Washington navel orange embryoid was able to regenerate a whole transgenic plant, which was confirmed as *hpt*-positive by Southern blot.

Hidaka and Omura (1993) obtained transformed ponkan mandarin callus by electroporation of protoplasts, but no plants were regenerated. Ohta ponkan nucellar callus was transferred from liquid MS and subcultured at least three times at 2-week intervals. Then cell suspensions were transferred to liquid MS plus 0.1 M sorbitol and 0.1 M galactose for other three subcultures. One week after the last subculture, protoplasts were isolated according to Vardi *et al.* (1990). The maceration medium consisted of $\frac{1}{2}$ MS with 0.3% macerozyme Onozuka, 0.3% cellulase Onozuka, 0.35 M mannitol, and 0.35 M sorbitol. Protoplasts were resuspended in electroporation buffer containing 0.6 M mannitol, and plasmid DNA was added at a concentration of 20 mg l^{-1} . The plasmid used carried the β -glucuronidase (*uidA*) gene as reporter marker. Samples were subjected to electroporation with an exponential decay pulse provided by a pulse generator. After electroporation, the suspension was transferred to a buffer containing 0.25% gelrite, 0.15 M sucrose, and 0.45 M glucose. Callus colonies formed after 2 months of culture were stained with 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc) for testing histochemical GUS (β -glucuronidase; *uidA*) expression. Apparently, regeneration of transgenic plants was not attempted in this study.

Yao *et al.* (1996) reported transformation of Page tangelo embryogenic cells using particle bombardment, and produced 15 transgenic embryo lines, but they did not progress further. Highly embryogenic nucellar callus was used also here as source of tissue for transformation. Cell suspensions were prepared in liquid MS without growth regulators basically as described before. They were collected on filter paper containing sorbitol + mannitol, and were bombarded with tungsten particles coated with plasmid DNA using a Biolistic PDS-1000/HE Particle Delivery System. The plasmid vector used carried the *uidA* reporter and the *nptII* selectable marker transgenes. Stably transformed cells were detected by GUS staining at 8 weeks post bombardment. Transgenic cells and callus passed through rounds of 100 and 200 mg l^{-1} kanamycin selection. Between 10 and 100 embryos were produced and transferred to germination medium, but conversion of embryos to plantlets was generally unsuccessful. Integration of the *nptII* transgene in the callus lines was demonstrated by Southern blot analysis.

J. Grosser's laboratory has used extensively protoplasts for citrus genetic transformation (Fleming *et al.*, 2000; Olivares-Fuster *et al.*, 2003; Guo *et al.*, 2005). The protoplast transformation protocol was adapted from the PEG protoplast fusion method developed by Grosser and Gmitter (1990) for citrus somatic hybridization. Cell suspensions are prepared from ovule-derived nucellar callus with high embryogenic potential in a basal medium free of growth regulators. Protoplast isolation medium must be fine-tuned for every genotype but basically consists of the three enzymes mixture, mannitol, and buffer solutions. The suspension culture is digested overnight in the isolation medium, protoplasts are purified by centrifugation in a sucrose-mannitol gradient, and resuspended in 0.6 M modified MT medium (BH3; Grosser and Gmitter, 1990). Protoplast suspension is aliquoted and plasmid DNA is added followed by a 40% PEG solution. After several cycles of incubation, centrifugation, and washings with different culture media, protoplasts are plated in small petridishes on BH3 medium, and incubated at 25°C under low light for 4 weeks. Although transgenic callus and embryoid production is generally very efficient, whole transgenic plant regeneration is difficult, and transgenic plants usually show atypical morphology. This has been attributed to the use of long-term cultures as starting materials.

Niedz *et al.* (2003) used a 2-year-old embryogenic callus line from Hamlin sweet orange for protoplast transformation by an improved electroporation method. In this case, many normal transgenic plants were regenerated as it was demonstrated by Southern blot analysis of 18 lines. In a previous report, Niedz *et al.* (1995) electroporated embryogenic protoplasts of sweet orange with a plasmid vector, but no transgenic plants were regenerated, probably due at least in part to the toxicity of the reporter marker gene used.

Li *et al.* (2002, 2003) exploited the embryogenic potential of different citrus (Ponkan, Valencia sweet orange) callus lines to attempt the *Agrobacterium*-mediated transformation system. The use of proper vector, co-cultivation, and selection conditions allowed producing whole transgenic plants at high frequencies. All relevant reports on genetic transformation of citrus through somatic embryogenesis are summarized in Table 1(A).

2.1.2 Source plant material: citrus seedling explants

Plant regeneration through organogenesis has been reported for many *Citrus* types and relatives, and from different tissues and explants, including leaf pieces, epicotyl segments, stem internodes, root segments, thin layers, and other tissues, being epicotyl and stem segments the most preferred ones. First works on citrus organogenesis reported the regeneration of whole plants from callus tissue formed from the primary explants (Grinblat, 1972; Chatuverdi and Mitra, 1974; Barlass and Skene, 1982; Edriss and Burger, 1984), likely reflecting how responsive they are to the addition of growth regulators *in vitro*. The cytokinin BAP has been essential for secondary organogenesis from disorganized callus, with auxins having only a marginal effect (García-Luis *et al.*, 1999). A promotive effect in shoot regeneration is shown at low BAP concentration range, but raising the concentration over 5 mg l^{-1} usually inhibits bud formation and shoot regeneration while enhances callusing (Moreira-Dias *et al.*, 2000). In the absence of BAP, direct shoot regeneration occurs from epicotyl and stem segments (García-Luis *et al.*, 1999; Bordón *et al.*, 2000).

In early transformation works, Moore *et al.* (1992) compared the organogenic ability of 0.7 cm leaf disks and stem segments of different lengths from *in vitro*-grown 2–4-month-old seedlings of several citrus genotypes. Stem segments of about 1 cm in length were the most effective in shoot production. Consequently, they decided to use this material as source explant for *A. tumefaciens*-mediated genetic transformation. A disarmed *A. tumefaciens* strain that contained two different transformation vectors carrying *nptII* and *uidA* marker transgenes was used in the experiments. Internodal stem segments were inserted vertically with either the basal or the apical end protruding from a medium consisting of MT with 5% sucrose, 5 mg l^{-1} BAP, and 0.8% agar, pH 5.7. The protruding ends were inoculated with an overnight culture of *A. tumefaciens* by placing a small drop of the culture on the end of the segment with a syringe. After 2–3 days of co-cultivation, explants were transferred to the same medium supplemented with the antibiotic kanamycin (100 mg l^{-1}), as selective agent, and mefoxin (200 mg l^{-1}), to control bacterial growth.

At 4 weeks, shoots started to arise from the protruding cut ends of the explants with little or no callus production. They were excised and rooted in cups containing sterile potting soil moistened with $\frac{1}{2}$ MT medium. More than 95% of the regenerated shoots were GUS-negative, suggesting that kanamycin was not a reliable indicator of transformation in this system. In addition, rooting of GUS-positive shoots was highly inefficient, so only two whole transgenic Carrizo citrange plants could be produced.

Kaneyoshi *et al.* (1994) established the first efficient protocol for transformation of seedling plant material and applied it to the generation of transgenic *P. trifoliata* plants. Importantly, they used 1 cm long etiolated epicotyl segments from 20-day-old *in vitro*-grown seedlings as starting material for transformation, because they had previously shown that epicotyl segments were highly responsive to shoot regeneration. Seeds were sterilized with 1% sodium hypochlorite solution containing 0.1% Tween-20 for 20 min, and rinsed three times with sterile distilled water. Seeds, with their seed coats peeled off, were placed on MS plus 5% sucrose and 0.8% agar, and then incubated at 27°C in darkness. A disarmed *A. tumefaciens* strain was used as transformation vector, and *nptII* and *uidA* were used as marker transgenes. Explants were immersed in a bacterial suspension at 5×10^8 cells/ml for 15 min, blotted on sterile filter paper, and transferred to co-cultivation medium consisting of hormone-free MS plus acetosyringone at $100 \mu\text{M}$ for 3 days. Then, explants were subcultured to MS plus 5 mg l^{-1} BAP, 0.1 mg l^{-1} α -naphthalene acetic acid (NAA), supplemented with kanamycin at 100 mg l^{-1} for transgenic selection, and cefotaxime at 500 mg l^{-1} to prevent bacterial growth. Explants regenerating shoots were transferred to a new medium with much reduced BAP concentration (0.5 mg l^{-1}) and increased kanamycin level (200 mg l^{-1}) to favor transgenic shoot development. GUS assays revealed that more than 50% of the regenerants were transgenic, suggesting that kanamycin was a reliable selectable marker in this system. Attempts to avoid escape regeneration by growing the explants in 200 mg l^{-1} kanamycin were unsuccessful. Elongated shoots were rooted in MS plus 0.5 mg l^{-1} NAA without problems. Efficient stable integration was demonstrated by Southern blot analysis of *uidA* gene in several transformants.

Authors claimed that this procedure permitted them to generate more than 100 transgenic plants within 2–3 months with an average transformation efficiency (transgenic shoots \times 100 per total number of explants) higher than 60%. Moreover, it has been successfully used by this and other groups to incorporate transgenes of potential interest into *P. trifoliata* (Kobayashi *et al.*, 1996; Kaneyoshi and Kobayashi, 1999; Wong *et al.*, 2001; Iwanami *et al.*, 2004; Endo *et al.*, 2005).

Peña *et al.* (1995b) used a similar protocol to transform Carrizo citrange, but it had to be modified because this genotype responded much worse than *Poncirus* to *Agrobacterium*-mediated transformation and shoot rooting. Stored seeds coming from the same tree stock were peeled, removing both seed coats, disinfected for 10 min in a 0.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20, and rinsed three times with sterile distilled water. Five-week-old germinating seedlings were used as the starting material for genetic transformation. These seedlings were grown in MS salt solution plus 10 g l⁻¹ agar, pH 5.7, at 26 °C in darkness for the first 2 weeks, and under a 16-h photoperiod and illumination of 45 $\mu\text{E m}^{-2} \text{s}^{-1}$ for three additional weeks. An *A. tumefaciens* strain carrying a transformation plasmid with *nptII* and *uidA* marker transgenes was used as vector system for transformation. Bacteria were cultured overnight in an orbital shaker at 28 °C and 200 rpm in Luria Broth (LB) medium containing the proper antibiotics to grow the binary system. Bacterial cells were pelleted at 3500 rpm for 10 min, resuspended and diluted to 4×10^7 or 4×10^8 cells/ml in liquid inoculation medium, which consisted of MS salt solution, 0.2 mg l⁻¹ thiamine hydrochloride, 1 mg l⁻¹ pyridoxine hydrochloride, 1 mg l⁻¹ nicotinic acid, and 3% (w/v) sucrose, pH 5.7.

Either epicotyl or internodal stem segments (about 1 cm long) were cut transversely and incubated for 15 min in 10-cm-diameter plates containing 15 ml of the bacterial suspension in inoculation medium by gentle shaking. The infected explants were blotted dry on sterile filter paper and placed horizontally on plates with the same medium but gelified with 0.8% agar for a 2-day co-cultivation period. In parallel, co-cultivation was tested as in Moore *et al.* (1992), by placing a drop of the bacterial culture on the

cut end of the segments inserted vertically in the co-cultivation medium.

After co-cultivation, the explants were blotted dry with sterile filter paper and transferred to shoot regeneration medium (SRM), which consisted of MS salts, 0.2 mg l⁻¹ thiamine hydrochloride, 1 mg l⁻¹ pyridoxine hydrochloride, 1 mg l⁻¹ nicotinic acid, 3% (w/v) sucrose, 1% (w/v) agar, pH 5.7, plus 100 mg l⁻¹ kanamycin for the selection of transgenic shoots, and 250 mg l⁻¹ vancomycin and 500 mg l⁻¹ cefotaxime to control bacterial growth. This medium was supplemented with 3 mg l⁻¹ BAP. Cultures were maintained in the dark for 4 weeks at 26 °C and then were transferred to 16-h photoperiod, 45 $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination, and 26 °C. A high frequency of GUS-positive shoots (55.1%) was obtained when explants were disposed horizontally in co-cultivation and regeneration medium, and when the bacterial culture was used at 4×10^7 . However, escapes (21%) and GUS-chimeric shoots (23.9%) were also produced. Attempts to root the transgenic regenerants were unsuccessful. Alternatively, shoots were excised from the explants and cut in two pieces. The basal portion was GUS-assayed and, if the reaction was positive, the apical part was grafted *in vitro* onto a nontransgenic decapitated *in vitro*-grown seedling (Navarro, 1992). This resulted in 100% recovery of transgenic shoots. About 3–4 weeks after shoot-tip grafting, plantlets were again grafted on a vigorous seedling rootstock in a greenhouse at 18–27 °C. The system resulted in transformation efficiency higher than 20% and allowed to generate Carrizo citrange transgenic plants routinely.

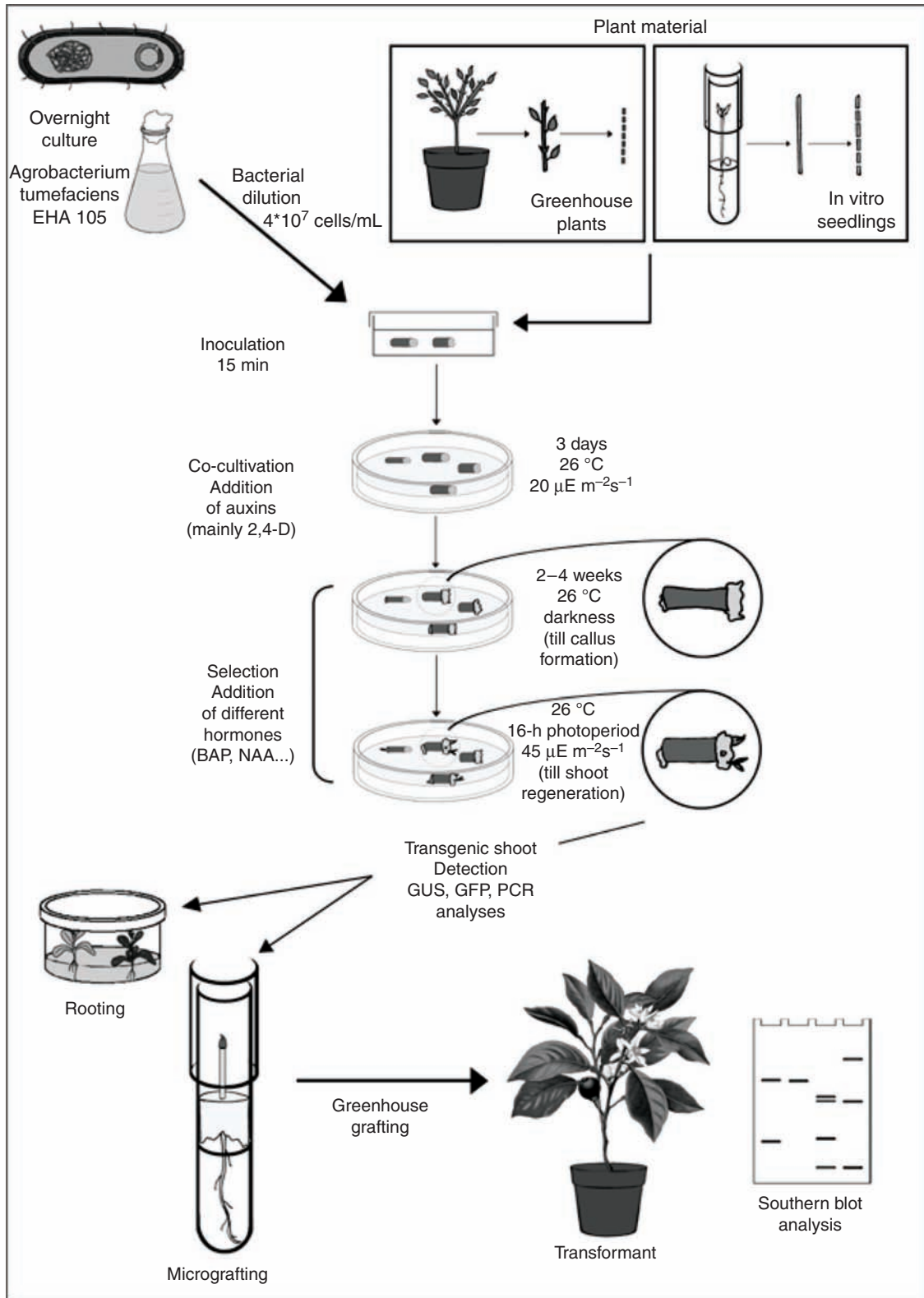
Gloria Moore's group also compared co-cultivation and regeneration/selection of the explants in vertical and horizontal orientations and concluded that horizontal disposition permitted to perform a better kanamycin selection. However, the use of older (3–4-month-old) seedlings, inefficient selection, and poor rooting frequency allowed them to only produce two sour orange, nine lime, and nine Carrizo citrange transgenic plants (Gutiérrez *et al.*, 1997). The same group later found that the transformation system previously established for *P. trifoliata* by Kaneyoshi *et al.* (1994) also worked for transformation of Duncan grapefruit, though at much lower efficiency (Luth and Moore, 1999; Costa *et al.*, 2002; Febres *et al.*, 2003).

Leandro Peña's group improved the transformation system of Carrizo citrange epicotyl segments by studying critically several factors affecting transformation and regeneration (Cervera *et al.*, 1998c). It was determined that co-cultivation of 3 days with *A. tumefaciens* in a medium rich in auxins and postcultivation in regeneration/selection medium for 4 weeks in darkness increased transformation efficiency to 41.3%, making possible to produce so many transgenic plants as needed in 3–6 months. The same basic procedure with more or less similar modifications has been used by other laboratories to transform Carrizo citrange (LaMalfa *et al.*, 2000; Wong *et al.*, 2001; Yu *et al.*, 2002; Kayim *et al.*, 2004), Washington navel orange (Bond and Roose, 1998), Tarocco sweet orange (Gentile *et al.*, 1998), Rio Red grapefruit (Yang *et al.*, 2000; Rai, 2006), Mexican lime (Koltunow *et al.*, 2000), Xuegan sweet orange (Yu *et al.*, 2002), Rangpur lime, Valencia, Natal, Pera, and Hamlin sweet oranges (Mendes *et al.*, 2002; Almeida *et al.*, 2003a; Boscariol *et al.*, 2003, 2006), Duncan grapefruit (Trainin *et al.*, 2005; Rai, 2006), and Ruby Red grapefruit (Rai, 2006) (Figure 2).

Pérez-Molphe and Ochoa-Alejo (1998) used *Agrobacterium rhizogenes* as transformation vector and *in vitro*-grown internodal stem segments of Mexican lime as explants. In this case, the bacteria carried the root inducing plasmid (pRi) plasmid, which is necessary for transformation and confers the characteristic hairy root phenotype, and the disarmed Ti plasmid with *nptII* and *uidA* transgenes within the T-DNA. Explants were inoculated by immersion in the bacterial culture diluted at 1×10^8 cells/ml in MS liquid medium plus B5 vitamins and 3% sucrose during 45 min, and then were placed horizontally on the same medium but with 5% sucrose and gelified with 0.8% agar for a 3-day co-cultivation period at 28 °C in darkness. Infected explants were washed for 45 min with liquid MS medium containing 750 mg l⁻¹ cefotaxime to eliminate *A. rhizogenes*. Finally, the explants were transferred to the medium used for co-cultivation but supplemented with 80 mg l⁻¹ kanamycin and 300 mg l⁻¹ cefotaxime, and with two different growth regulators treatments: without regulators or with 7.5 mg l⁻¹ BAP and 1 mg l⁻¹ NAA. In the first case, transformed roots appeared directly from the cut ends of the explants 30 days after

infection. Some explants produced shoots and roots simultaneously, but in general the frequency of regenerated shoots was low. When growth regulators were added to the regeneration medium, a pale green callus was formed at the cut ends and about 60 days later shoots were formed without hairy root phase. Transformed roots transferred to a medium with growth regulators also produced shoots at high frequency. Shoots were excised from explant and root segments and easily rooted in a medium without growth regulators. This profuse organogenic response was due to the integration and expression of the root loci (*rol*) genes from the Ri plasmid of *A. rhizogenes* in the transformed cells. The survival rate upon transfer to soil was 79%. Stable transformation was confirmed by Southern blot analysis of the *nptII* transgene. This is a rather efficient transformation system for citrus but it is not being widely used due to the integration of the T-DNA genes from the Ri plasmid in the transgenic cells. Their expression caused not only active regeneration but also important phenotypical alterations, including short internodes, reduced apical dominance, and wrinkled leaves in the transgenic plants, seriously limiting the biotechnological possibilities of the procedure.

Several groups have modified the original epicotyl transformation system with the aim of increasing the wounded area of the explants and the number of explants generated per seedling. Yu *et al.* (2002) proposed cutting longitudinally the epicotyl segments in two halves to enhance both regeneration and transformation frequency. This was used for Carrizo citrange transformation (Yu *et al.*, 2002; Kayim *et al.*, 2004), but attempts to adapt it to other citrus genotypes have been generally unsuccessful mainly due to problems associated to *Agrobacterium* overgrowth (Cervera and Peña, unpublished results; W. Guo, personal communication). Another alternative has been using thin layers of about 1–2 mm cut transversally from etiolated epicotyls. This explant type was highly organogenic, as demonstrated by Le *et al.* (1999) in *P. trifoliata*, and reduced the occurrence of escapes in Swingle citrumelo and Carrizo citrange transformation (Molinari *et al.*, 2004a, b). However, transformation efficiency is much lower than using 1 cm long explants, probably due to the toxicity of *A. tumefaciens* inoculation and co-cultivation of so small explants (Molinari *et al.*, 2004b; Cervera and Peña, unpublished results).



Although using either embryogenic nucellar callus or epicotyl segments from *in vitro*-grown seedlings as source material for transformation has led to workable transformation efficiencies for some citrus genotypes and relatives, transgenic plants were juvenile. In addition, regeneration from these materials could result in some cases in production of plants that are not true-to-type (Cervera *et al.*, 2000b; Grosser *et al.*, 2002; Cervera and Peña, unpublished results). Given the long juvenile period of most citrus genotypes, several years of cultivation would be needed before horticultural and commercial traits of the transgenic plants could be evaluated, if juvenile tissues were used for transformation. Therefore, development of transformation systems from mature plants was very important in citrus to be able to overcome juvenility.

The first approach in such direction was the use of source material coming from the greenhouse. Peña *et al.* (1995a) used 6–12-month-old greenhouse-grown (18–27°C) seedlings of Pineapple sweet orange as the source of tissue for transformation. Internodal stem segments (0.5–1 cm in length) from stem pieces (20 cm in length) were inserted vertically in SRM (Peña *et al.*, 1995b) without antibiotics and inoculated with *A. tumefaciens* carrying *nptII* and *uidA* marker transgenes by placing a drop of the bacterial culture on the cut end of the segment protruding from the medium. The explants were co-cultivated for 2 days with the bacteria, blotted dry with sterile filter paper and transferred to SRM supplemented with kanamycin, as selectable agent, and cefotaxime and vancomycin to prevent further bacterial growth. The pots were maintained in

dark at 27°C during 8 weeks and then at 25°C, 16-h photoperiod, illumination of $10 \mu\text{E m}^{-2} \text{s}^{-1}$ and 60% relative humidity during 4 weeks. Regenerated shoots of 0.2–0.3 cm height were harvested from the stem segments. Portions of 0.1–0.2 cm were excised from the shoot basal ends and assayed for GUS activity, and the remaining portions were shoot-tip grafted on Troyer citrange seedlings, as in Peña *et al.* (1995b). As transformation efficiency was low using this system (7.9%), test inoculation was done by immersion in the bacterial culture diluted at 10^7 cell/ml in SRM with BAP at 1 mg l^{-1} , and co-cultivation by disposing the explants horizontally under two different conditions: 2 days in SRM with BAP 1 mg l^{-1} , agar 0.8%, or 3 days on tomato feeder layers. Feeder plates were prepared by pipetting 2 ml of 6–7-day-old tomato cell suspensions on the surface of 25 ml of tomato cell suspension (TCS) solid medium with sterile Whatman 5 filter paper on the top in $10 \times 1.5 \text{ cm}$ (diameter \times height) plates. TCS medium consisted of MS salts, 1 mg l^{-1} thiamine hydrochloride, 1 mg l^{-1} pyridoxine hydrochloride, 1 mg l^{-1} nicotinic acid, 3% sucrose, 2 mg l^{-1} indole-3-acetic acid (IAA), 1 mg l^{-1} 2-isopentenyl-adenine (2-iP), 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8% agar, pH 5.7. TCSs were maintained in TCS liquid medium in a shaker at 100 rpm and 25°C and were subcultured in fresh medium every 15 days. After co-cultivation, the explants were blotted dry with sterile filter paper and transferred to SRM (BAP 1 mg l^{-1}) plates containing 100 mg l^{-1} kanamycin, for selection and 500 mg l^{-1} cefotaxime, 250 mg l^{-1} vancomycin to control further bacterial growth. The cultures were maintained in the dark for

Figure 2 Procedure for the genetic transformation of citrus plants. Aseptically germinated 4–5-week-old seedlings are used as the starting material for genetic transformation of *P. trifoliata* and Carrizo citrange. Older tissues, such as shoots from 4- to 12-month-old glasshouse-grown vigorous seedlings, are also used as the source of tissue for transformation of sweet orange, sour orange, lime, alemow, lemon, and mandarins. For transformation of mature tissues, new shoots elongated from buds, collected from trees maintained in a screenhouse (pathogen-free Germplasm Bank Collection of the IVIA), grafted onto seedlings of a vigorous rootstock grown under glasshouse conditions, are used as starting material. *Agrobacterium tumefaciens* strain EHA 105 carrying a binary plasmid is used for transformation. Bacteria are cultured overnight in an orbital shaker at 28°C and 200 rpm in LB medium containing the appropriate antibiotics. After bacterial cells are pelleted at 3500 rpm for 10 min, they are resuspended and diluted to 4×10^7 cells/ml in liquid inoculation medium. Epicotyl or internodal stem segments (1 cm long) are cut transversely and incubated for 15 min in 15 ml of bacterial suspension by gentle shaking. The infected explants are blotted dry on sterile filter paper and co-cultivated for 3 days on co-cultivation medium. After co-cultivation, explants are blotted dry with sterile filter paper and transferred to selection/regeneration medium. Cultures are grown in the dark for 2–4 weeks at 26°C and then maintained under a 16-h photoperiod with $45 \mu\text{E m}^{-2} \text{s}^{-1}$ of light intensity. Shoots usually develop from the cut ends 3–8 weeks after co-cultivation. Their transgenic nature is evaluated by histochemical assays for GUS activity or by visualizing GFP expression. The apical portions of transgenic shoots are then grafted *in vitro* onto decapitated seedlings of a citrange rootstock. Rooting is generally not easy in many citrus genotypes. New grafting of the *in vitro*-grown plants on vigorous rootstocks in the greenhouse allows the rapid acclimatization and development of putative transgenic plants. Genetic transformation is then confirmed by Southern blot analysis

15 days at 26 °C and then transferred to 16-h photoperiod, 45 $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination, 26 °C, and 60% relative humidity. For Mexican lime, co-cultivation in feeder plates allowed to increase transformation efficiency twice (Peña *et al.*, 1997).

2.1.3 Source plant material: citrus mature material

Once Leandro Peña's were able to achieve 15–20% transformation efficiency working with greenhouse-grown juvenile material, they attempted transformation of mature tissues (Cervera *et al.*, 1998a). However, in the first preliminary experiments, internodal stem segments from aged mature citrus trees showed very limited regenerative potential. Then, buds from adult trees were grafted on vigorous seedlings. Buds were collected from Pineapple sweet orange trees maintained in a screenhouse (pathogen-free Germplasm Bank Collection of the IVIA) and were grafted onto seedlings of a vigorous rootstock under greenhouse conditions (18–27 °C), and newly elongated shoots were used as starting material. Stem pieces (20 cm in length) were stripped of their leaves and thorns disinfected for 10 min in a 2% (v/v) sodium hypochlorite solution and rinsed three times with sterile distilled water. Regeneration from stem segments from the first, second, and third flushes of newly grafted invigorated mature sweet orange plants was evaluated in comparison to regeneration from stem segments from juvenile plants. Results indicated that explants from the first and second flushes produced similar regeneration frequencies, significantly higher than that of the explants from the third flush. As expected, stem segments from juvenile plants produced the highest regeneration frequency. The first flush of the adult plants was selected as the source of tissue for genetic transformation experiments. Different *A. tumefaciens* concentrations and co-cultivation time and media for transformation were tested. A bacterial concentration of 10^7 cells/ml and 3-day co-cultivation in tomato feeder plates provided the best results and were used in further experiments. Regeneration/selection medium and conditions were identical to those used for transformation of juvenile material. Shoot regeneration was observed after 2–5 months on

selective medium, and transformation efficiency achieved was of about 6%, approximately threefold lower than that obtained for juvenile material. It is probable that aging decreased the susceptibility of plant cells to *Agrobacterium*-infection as well as their organogenic potential. Southern blot analysis confirmed the stable integration of *uidA* and *nptII* gene cassettes into the plants' genome. The putative mature transgenic sweet orange plants showed morphology and growth habits of an adult plant, as compared to control mature plants. In fact, whereas juvenile plants showed a pronounced thorniness, transgenic mature plants were almost thornless, similar to the mature plants from which the explants were taken for transformation. After 14 months in the greenhouse, the transgenic and control plants started to flower, confirming their mature nature (Figure 3a). Both flowers and immature fruits from the transgenic plants showed a dark blue color after overnight incubation in X-Gluc, a substrate of the β -glucuronidase enzyme (Figure 3b). These results confirmed the maintenance of the ontogenic mature stage of the invigorated mature plants as well as the transgenic plants. Interestingly, transgenic events kept their epigenetic mature state even after a process of de-differentiation, induction and redifferentiation, necessary to shift the cells to a competent state for transformation. Therefore, Cervera *et al.* (1998a) directly transformed and regenerated mature tissues of citrus, bypassing the juvenile stage. This process greatly shortens the period of time until flowering and bearing fruits and decreases the time to achieve horticulturally acceptable characteristics by years. Pineapple sweet orange transgenic juvenile plants generated in the same series of experiments needed at least 5 years to start flowering, and 4 years later still keep juvenile characteristics. This transformation procedure for mature citrus tissues has been patented in Spain (no 9700491/X), Europe (no EP 0870838 B1), and United States (no 6.103.955). Moreover, it could be worth trying to apply this transformation and regeneration strategy to transgenic plant production of other woody fruit species with long juvenile periods.

In subsequent experiments, Leandro Peña's group investigated the influence of the components of the feeder plates, namely the TCSs, the filter paper layer, and the TCS culture medium rich in auxins, separately and in combination,

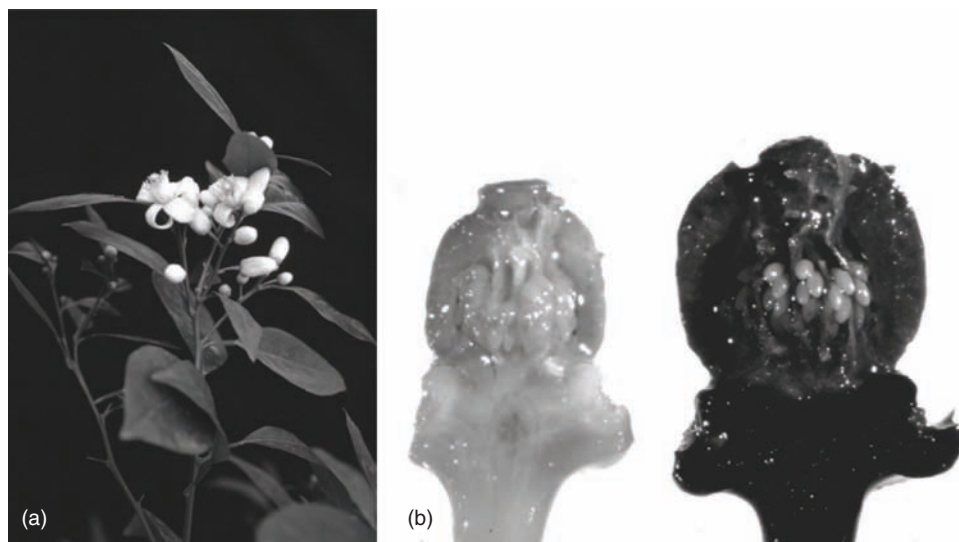


Figure 3 Genetic transformation of mature citrus plants. (a) About 1 year after transformation, regenerated plants started to flower and set fruit, confirming their mature nature. (b) GUS expression in a longitudinal cut from a developing fruit of a transgenic mature sweet orange plant (right) compared to the corresponding nontransgenic control (left)

in the genetic transformation of Mexican lime explants. TCS basal medium was responsible for the increased lime transformation frequency previously attributed to the feeder plates as a whole. The role of the TCSs and the filter paper was even detrimental, as they drastically decreased transformation frequency in comparison with co-cultivation of explants in TCS. Not only was the transformation frequency much higher in explants co-cultivated in TCS, but also the percentage of explants with GUS-positive spots and the maximum number of GUS-positive spots per explant. The liquid layer provided by the cell suspension affected negatively the explants, favoring excessive formation of abnormal callus. On the contrary, the filter paper layer between the basal medium and the explants precluded callus formation at their cut ends (Domínguez *et al.*, 2000). Therefore, TCS was used for co-cultivation in further experiments with Mexican lime and other citrus genotypes. As it was previously shown for Carrizo citrange epicotyl segments (Cervera *et al.*, 1998c), phytohormones (mainly auxins) in TCS seemed to play a crucial role in inducing de-differentiated cells competent for transformation.

The use of vigorous shoots from plants growing in the greenhouse as source of material for

transformation in the case of citrus varieties provides with the possibility of using the same procedure first established to transform juvenile tissues of a given species and also to transform adult materials from the same species (Figure 2). Thus, Leandro Peña's group extended the genetic transformation system for juvenile material to mature citrus tissues of many citrus types of interest, including several sweet orange varieties, sour orange, Mexican lime, Fino lemon, rough lemon, Cleopatra mandarin, and *C. macrophylla* (reviewed in Peña *et al.*, 2004a), and more recently also clementine (Cervera *et al.*, 2008). Alternative to invigoration by grafting, Leandro Peña's group directly used new shoots from the Pineapple sweet orange tree maintained in a screenhouse (at the Germplasm Bank Collection) as starting material for mature transformation (Peña *et al.*, 2004a). Although transgenic plants were generated, the efficiency of the system was much lower (Cervera and Peña, unpublished results). Almeida *et al.* (2003b) used this system with minor modifications to transform mature Hamlin sweet orange with low efficiency. They also used leaf disks from mature plants as explants but poor bud induction and *Agrobacterium* overgrowth even at 1-day co-cultivation impeded transgenic plant recovery. A summary of reports on regeneration of transgenic

citrus plants by organogenesis is shown in Table 1(B).

2.2 Methods Employed for Transformation

2.2.1 Electroporation and PEG-mediated transformation of citrus protoplasts

First reports on genetic transformation of citrus used PEG, electroporation or *A. tumefaciens* as systems for protoplast transformation. Electroporation or addition of PEG to a protoplast suspension induces pore formation in the membranes, so plasmid DNA is able to pass through them and in some cases the DNA becomes integrated in the nuclear genome. Kobayashi and Uchimiya (1989) mixed 10 µg of the circular form of the bacterial plasmid vector carrying the *nptII* selectable marker gene with a protoplast suspension (2.2×10^6) in 0.6 M mannitol. Vardi *et al.* (1990) also used PEG 6000 for protoplast transformation but in this case the plasmid was linearized and calf thymus DNA was used as a carrier. Preliminarily, these authors compared transient transformation mediated either by PEG or by electroporation and found that both the systems worked well, but since the former procedure was simpler and resulted in more consistent transgene transient activity, further stable transformation experiments were PEG-mediated. Electroporation was also attempted by Hidaka and Omura (1993) and conditions were fixed to get transgenic callus colonies. Whereas maximal transgene transient expression was shown in protoplasts electroporated at 1200 V/cm, callus colonies were only formed using voltages between 200 and 600 V/cm. A heat shock treatment at 49 °C before electroporation greatly enhanced transient transformation, as well as using 0.1 mM CaCl_2 in the electroporation buffer. Plasmid DNA concentration of 100 µg/ml and a capacitance of 5.5 µF were also determined as optimal for transient transgene expression.

More recently, PEG-mediated citrus protoplast transformation is being routinely used in J. Grosser's laboratory (Fleming *et al.*, 2000; Olivares-Fuster *et al.*, 2003; Guo *et al.*, 2005), as an extension of their PEG protoplast somatic hybridization method (Grosser and Gmitter, 1990). The protoplast suspension at 2×10^6 cells/ml is aliquoted into 15 ml round-bottomed

tubes at 0.5 ml per tube and 20 µg of plasmid DNA in buffer or water is added to each tube, followed by 0.5 ml of a 40% PEG solution. Niedz *et al.* (2003) optimized electroporation conditions for protoplast transformation. They found that an electric field strength of 375–450 V/cm, a vector DNA concentration of 100 µg/ml, a carrier DNA (salmon sperm DNA) concentration of 100 µg/ml, the use of electroporation buffer at pH 8.0, and preelectroporation heat shock of protoplasts for 5 min at 45 °C, were optimum for DNA uptake into protoplasts as determined by assaying GUS activity 24 h after electroporation. These improvements allowed producing transgenic plants quite efficiently.

2.2.2 *Agrobacterium*-mediated transformation of citrus epicotyl and internodal stem segments

Moore *et al.* (1992) tested Carrizo citrange, Swingle citrumelo, Mexican lime, and Hamlin sweet orange for susceptibility to various *A. tumefaciens* wild-type strains by injecting seedling hypocotyls with bacterial cultures. Although the detailed results of these experiments were not shown, it was clear that all tested citrus types were susceptible to *Agrobacterium* as evidenced by the production of tumors at the wound site following inoculation, and that the succinamopine strain A281 gave rise to very large, rapidly growing tumors. Because of the apparent hypervirulence of its Ti plasmid, pTiBo542, a nononcogenic derivative of this strain, EHA 101, was used in subsequent transformation experiments. Due to the low transformation efficiency achieved, in a second work the same group attempted using the oncogenic strain A518 harboring a binary plasmid with the aim of enhancing transgenic shoot regeneration. GUS-positive shoots were obtained, sometimes at higher frequencies than those obtained with EHA 101, but all of them also contained the oncogenic T-DNA, appearing morphologically abnormal. Therefore, EHA 101 was used in further experiments.

Peña *et al.* (1995a, b, 1997) used *A. tumefaciens* strain EHA 105 for transformation of epicotyl segments from *in vitro*-grown seedlings and of internodal stem segments of greenhouse-grown juvenile plants. EHA 105 is a derivative of EHA

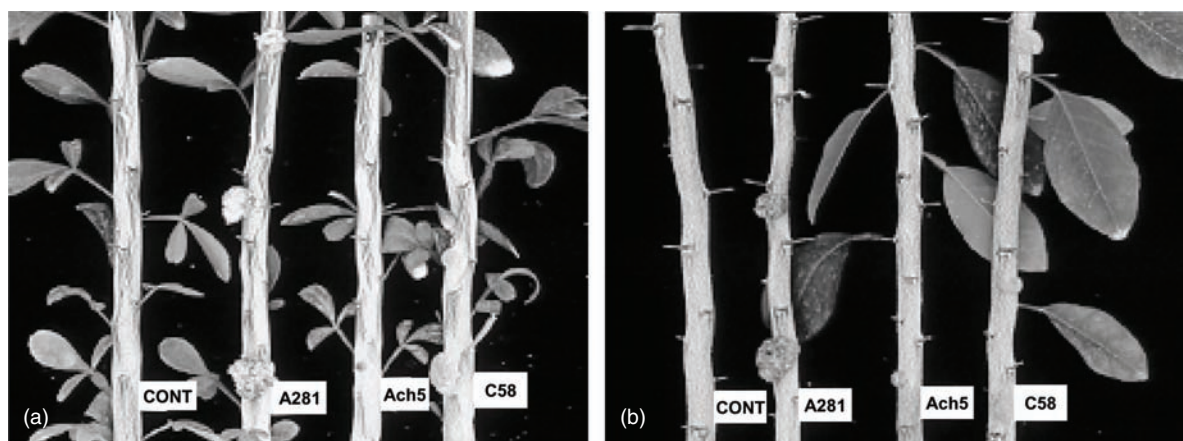


Figure 4 Tumors incited by *Agrobacterium tumefaciens* strains A281, Ach5, and C58 on: (a) Carrizo citrange and (b) Mexican lime. On each photograph, stem marked with CONT corresponds to a mock-inoculated plant. Photographs were taken 28 weeks after inoculation. Three inoculations were performed per stem segment

101 in which the bacterial kanamycin resistance gene from the disarmed Ti plasmid has been removed. Thus, this *Agrobacterium* strain can be used with most binary plasmid systems, as pBin or pCambia vectors, which harbor bacterial resistance to kanamycin. In order to choose a proper *A. tumefaciens* strain for transformation of mature material, we investigated the virulence of three different wild-type strains by inoculating stems of greenhouse-grown sweet orange seedlings. These three strains were A281 (succinamopine type), Ach5 (octopine type), and C58 (nopaline type). *A. tumefaciens* A281 produced the earliest and the highest frequency of tumor formation. Therefore, Leandro Peña's group followed using *A. tumefaciens* EHA 105, a nononcogenic derivative of this strain, as favorite vector for citrus transformation (Cervera *et al.*, 1998b).

To gain more insight on the relationships between *A. tumefaciens* strains and citrus, and to aid in the choice of proper *Agrobacterium* vectors for genetic transformation, Cervera *et al.* (1998b) investigated the virulence of *A. tumefaciens* strain A281, compared to that of Ach5 and C58 on a wide range of agronomically important woody fruit crops. This study included Pineapple sweet orange, Mexican lime, Clemenules clementine, and Carrizo citrange as citrus types. Plants (4–6-month-old) were grown in individual 2.5 l pots and were fertilized weekly. They were kept inside temperature-controlled greenhouses at 24–26/15–16 °C day/night temperatures, with rel-

ative humidity of 60–80%. For each *Agrobacterium* strain and host pair, three inoculations per plant were performed at the same time in all the tested species, at intervals of 5–7 cm and practicing the first wound at 5 cm from the soil. Five microliters of *A. tumefaciens* suspensions in water ($OD_{595} \text{ nm} \approx 0.3$), from 48-h-old cultures, were added to the wounds with a micropipette. The wounds were then covered with plastic wraps for 2 weeks to prevent bacterial desiccation. All the inoculations were performed in April, when plants were actively growing up, and tumor development was allowed to progress for 8–28 weeks. Strain A281 was the most virulent in all citrus types, since it produced earlier appearance and higher tumor formation frequency. On sweet orange and lime, tumor weight was comparable in plants inoculated with strains A281 and C58. However, strain C58 was incapable of inciting tumors in clementine. On citrange, A281 produced earlier-appearing tumors but C58 was as efficient as A281 in tumor formation and incited larger tumors than A281 (Figure 4). Interestingly, tumors induced by strain A281 on the four citrus hosts showed a progressive blackening after 3 months from inoculation. When tumors incited by A281 were excised, 7 months postinoculation, necrosis was severely affecting their morphology and final weight, thus explaining the larger size of tumors produced by strain C58 on citrange, and equivalent sizes of tumors produced by A281 and C58 on sweet orange and Mexican lime. Since tumor necrosis seems to be strongly correlated with

the supervirulent phenotype induced by A281, and this strain incited earlier-appearing tumors and at higher frequencies than the other *Agrobacterium* strains, A281 can be considered supervirulent on the four citrus hosts tested. Strain Ach5 was not effective on sweet orange and clementine, and only produced small tumors at low frequencies on Mexican lime and citrange.

Later, Leandro Peña's group extended this study to other five agronomically important citrus types, Fino lemon, Cleopatra mandarin, *C. macrophylla*, sour orange, and Mediterranean mandarin. Tumors were induced by the three strains in all the genotypes tested, but time of appearance of tumors, tumor frequency, and final tumor weight were clearly different depending on the bacterial strain. A281 was again the most virulent strain, because it induced earlier appearance of tumor and higher tumor formation frequency in most of the hosts, at least until 2 months postinoculation. However, in the cases of Mediterranean and Cleopatra mandarins, C58 was as virulent as A281. Tumors induced by A281 in all the citrus hosts started to become necrotic 2–3 months postinoculation. Such necrosis possibly had a slight effect on final tumor formation frequency, but clearly affected tumor development. Whereas tumors induced by strains Ach5 and, over all, C58 continuously grew until 4 months postinoculation, tumors produced by A281 grew more slowly when necrosis started to affect them. As a consequence, final tumor weight was higher for C58 than for A281 in Cleopatra mandarin and *C. macrophylla*, and similar for C58 and A281 in Mediterranean mandarin. Ach5 incited in general less tumor frequency and weight than the other two strains (Ghorbel and Peña, unpublished results).

Interestingly, the octopine strain LBA 4404, which is a disarmed derivative of Ach5, has been widely used to transform *P. trifoliata* (Kaneyoshi *et al.*, 1994; Kobayashi *et al.* 1996; Kaneyoshi and Kobayashi, 1999; Iwanami *et al.*, 2004; Endo *et al.*, 2005). However, this citrus relative has also been successfully transformed with strain EHA 105 (Wong *et al.*, 2001; Fagoaga *et al.*, 2005), suggesting that it is highly receptive to *Agrobacterium*-mediated gene transfer and that several strains could be used to obtain efficient transformation.

Experiments from Leandro Peña's group clearly indicated that C58 and A281 were appropriate

strains for citrus transformation, A281 being generally much more virulent. A281 is a transconjugant of C58 with pTiBo542 instead of pTiC58, meaning that both strains should interact identically with citrus cells. Therefore, differences in virulence would probably reflect differences in transformation and/or expression of T-DNA and/or *vir* genes. To investigate this, Ghorbel *et al.* (2001b) tested stable transformation in internodal stems segments from Fino lemon, Cleopatra mandarin, *C. macrophylla*, sour orange, and Mediterranean mandarin co-cultivated with the disarmed *Agrobacterium* strains C58 (pMP90) and EHA 105, carrying a binary plasmid (p35SGUSINT) containing *uidA* and *nptII* gene cassettes in the T-DNA. After co-cultivation in the medium rich in auxins that favors transformation (Cervera *et al.*, 1998c), the explants were transferred to a regeneration medium containing kanamycin to allow growth of transformed tissues only. GUS assays were performed 6 weeks after bacterial infections, when T-DNA gene expression was fully stable. For all the citrus genotypes tested, transformation frequency produced by both strains was very different. C58 (pMP90)/ p35SGUSINT produced very low transformation in lime, sour orange, lemon, and *C. macrophylla*, and no transformation in Cleopatra mandarin, while EHA 105/p35SGUSINT produced much higher transformation in all cases, from sixfold in sour orange to 22-fold in lime. Although recovery of transgenic citrus plants was not the main objective of these experiments, transformation frequency provided by EHA 105/p35SGUSINT was so high that we were able to regenerate transgenic shoots from lime, lemon, *C. macrophylla*, and Cleopatra mandarin, just 6 weeks postinoculation. Since the chromosomal background from C58 as well as the T-DNA from p35SGUSINT are identical for the bacterial strains tested, the supertransformation ability of EHA 105/p35SGUSINT in citrus may be attributed to the *vir* region of its Ti plasmid pTiBo542 (Ghorbel *et al.*, 2001b). Consequently, EHA 105 has been the most commonly used *Agrobacterium* strain in citrus transformation.

Bond and Roose (1998) published that C58 was more efficient than EHA 101/105 for transformation of Washington navel sweet orange epicotyl segments. However, EHA 101 and EHA 105 were indistinguishable in their analysis,

being probably very different strains in terms of genetic transformation efficiency in this case, since kanamycin was used as selection marker of transgenic cells being EHA 101 also resistant to kanamycin. Yang *et al.* (2000) also reported that C58 was more efficient than EHA 105 for Rio Red grapefruit transformation, but their experiments were based in transient expression analyses (3 days after co-cultivation) with few explants. More recently, the same group has been using only EHA 105 for transformation of three different grapefruit genotypes including Rio Red (Rai, 2006).

As the supertransformation ability of EHA 105 was attributed to the *vir* region of pTiBo542, Ghorbel *et al.* (2001b) investigated whether supplementary copies of *vir* genes could increase transformation frequency in citrus. The helper plasmid pCH30 (Hamilton *et al.*, 1996) was introduced into C58 (pMP90)/p35SGUSINT and EHA 105/p35SGUSINT. C58 (pMP90) is a disarmed derivative of C58. p35SGUSINT is a binary plasmid carrying *nptII* and *uidA* with an intron, as selectable and reporter marker transgenes, respectively. pCH30 provides additional copies of *virG* from pTiBo542, so this allowed also to investigate the role of this *virG* in the supertransformation ability provided by this Ti plasmid in citrus. Transformation of citrus explants *in vitro* was carried out as described before, and stable transformation was tested 6 weeks after inoculation by performing GUS assays. Two different citrus species were chosen for carrying out these experiments: lime, which was efficiently transformed by EHA 105/p35SGUSINT, and lemon, in which transformation mediated by EHA 105/p35SGUSINT was rather low. C58 (pMP90)/p35SGUSINT produced extremely low transformation frequency in both citrus species. In lime, additional copies of *virG* from pTiBo542 increased transformation frequency induced by C58 (pMP90)/p35SGUSINT about 80-fold, reaching similar transformation frequency than that provided by EHA 105/p35SGUSINT. However, additional copies of *virG* from pTiBo542 in EHA 105/p35SGUSINT did not allow increasing transformation frequency. No further increase in transformation frequency was shown by introducing pCH32 (carrying *virG* and *virE*) instead of pCH30 in any of the two bacterial strains. In lemon, when pCH30 was introduced

into C58 (pMP90)/p35SGUSINT, an increase in transformation frequency of sixfold was obtained, resulting in a transformation frequency similar to that provided by EHA 105/p35SGUSINT, and affording the possibility of regenerating transgenic plants with the use of this strain. Strikingly, pCH30 also enhanced the transformation frequency provided by EHA 105/p35SGUSINT in 1.7-fold. pCH32 provided a similar increase in transformation frequency as pCH30 for both the bacterial strains (Ghorbel *et al.*, 2001b).

VirG is a transcriptional activator that specifically recognizes a 14bp DNA sequence, called the *vir* box, which is found in all *vir* gene promoter regions. The supervirulent phenotype of A281 in its infection to certain hosts has been correlated with higher expression of *vir* genes after induction. Ghorbel *et al.* (2001b) have shown that extra supply of only *virG* from pTiBo542 greatly increased *Agrobacterium*-mediated stable transformation of several citrus genotypes, demonstrating the importance of *vir* induction, and mainly *virG* activation, in the supertransformation ability of this Ti plasmid in citrus. In addition, Ghorbel *et al.* (2001b) have shown that extra copies of this *virG* could complement *vir* induction of pTiC58, providing also to C58 (pMP90)/p35SGUSINT the capacity to supertransform citrus. Looking back to our results on comparison of virulence induced by oncogenic strains in different citrus genotypes, there are genotypes for which C58 is as efficient as A281 for tumor induction, and cases in which A281 incites a too severe reaction at the wounds. In current experiments, Leandro Peña's group is finding citrus types that are severely affected by inoculation and co-cultivation with EHA 105, making very difficult further progress to induce shoot regeneration. For these citrus types, alternative *A. tumefaciens* strains become an almost indispensable need. Using a disarmed derivative of C58 carrying additional copies of *virG* is representing an excellent option in our hands (Peña, unpublished results).

Vir gene inducers, such as acetosyringone, wounded cell extracts, feeder cells, sugars, hormones, wounding, etc. have been extensively used to enhance genetic transformation frequency in many plants. The aim of these treatments was to stimulate *virG* activation and *virA* sensing. Acetosyringone is a phenolic compound produced

during the wounding of plant cells that induces the transcription of the virulence genes of *A. tumefaciens*. Its beneficial role has been demonstrated in the genetic transformation of many plants, including some woody fruit species, as apple (James *et al.*, 1993) and kiwifruit (Janssen and Gardner, 1993). It has being also widely used in citrus transformation, during *Agrobacterium* culture and/or during co-cultivation (Kaneyoshi *et al.*, 1994; Bond and Roose, 1998; Luth and Moore, 1999; Yang *et al.*, 2000; Molinari *et al.*, 2004a), normally at 100–200 μM concentration. However, its role as transformation enhancer was not investigated. Cervera *et al.* (1998c) showed that its addition during co-cultivation increased transformation frequency twofold in Carrizo citrange explants, but when the medium rich in auxins was used for co-cultivation, its effect was not so clear (Cervera *et al.*, unpublished results). Because of this, acetosyringone was not further used in Leandro Peña's lab. More recently, Almeida *et al.* (2003a) used a co-cultivation medium supplemented with acetosyringone at 0, 100, or 200 μM for transformation of epicotyl segments from Rangpur lime and Valencia and Natal sweet oranges. Transgenic shoots were only generated in absence of acetosyringone. It is possible that the effect of acetosyringone in citrus transformation is dependent on the genotype, explant type, and co-cultivation conditions.

2.2.3 *Agrobacterium*-mediated transformation of citrus embryogenic cells and callus

There are a few reports on the use of *A. tumefaciens* as vector for embryogenic callus transformation. Hidaka *et al.* (1990) tested two disarmed *A. tumefaciens* strains and co-cultivation of 3, 5, or 7 days for transformation of cell suspensions coming from embryogenic callus cultures. The bacterial strains were octopine LBA 4404 carrying a binary vector with the *nptII* marker gene, and the nopaline GV3031 harboring a cointegrate vector system with the *hpt* transgene as selectable marker. In general, transformation was very low but 3-day co-cultivation provided a higher number of cell colonies formed with both bacterial vectors. A major problem in the system was that citrus cells were very sensitive to both kanamycin

and hygromycin selective antibiotics, since even concentrations of 10 and 20 mg l^{-1} , inhibited callus proliferation.

Li *et al.* (2002) successfully used the strain EHA 105 for transformation of embryogenic callus from Ponkan by immersion for 25 min in the bacterial culture and 3-day co-cultivation in MT plus 100 μM acetosyringone, in darkness. Transformation conditions were optimized for adapting this procedure to Valencia sweet orange, by preculturing the callus 4 days in MT liquid medium with 0.5 g l^{-1} malt extract, 1.5 g l^{-1} glutamine, and 50 g l^{-1} sucrose. Almost no resistant callus was obtained if the co-cultivation medium lacked acetosyringone.

2.2.4 Less-used transformation systems: *A. rhizogenes* and particle bombardment systems

There is only one report on the use of each of *A. rhizogenes* and particle bombardment systems for citrus transformation. Yao *et al.* (1996) bombarded embryogenic callus cultures of Page tangelo with M-10 tungsten particles coated with plasmid DNA using the Biolistic PDS-1000/HE particle delivery system. Transgenic embryos were produced but most of them were abnormal and conversion to plantlets was generally unsuccessful. Although these results were not satisfactory, Leandro Peña and his co-workers still think that gene bombardment could be a promising transformation system, especially for those explant types that are highly sensitive to *Agrobacterium* inoculation/co-cultivation but have high organogenic/embryogenic potential.

The *A. rhizogenes* A4 agropine-type strain was used for transformation of internodal stem segments of *in vitro*-grown Mexican lime seedlings (Pérez-Molphe and Ochoa-Alejo, 1998). Transformation efficiency achieved was very high, but the aberrant phenotype, the integration of the T-DNA from the Ri plasmid provided to the transformants, has probably precluded its subsequent use for incorporation of transgenes of agricultural interest into citrus types. In spite of this, several authors have proposed the use of *rol* genes from the Ri plasmid as transgenes to produce dwarf *P. trifoliata* and citrange rootstocks (Gentile *et al.*, 1998; Kaneyoshi and Kobayashi, 1999).

2.2.5 Optimizing *A. tumefaciens* and cell/tissue co-cultivation conditions: cell competence for transformation in citrus

As *A. tumefaciens* has been the most commonly used transformation vector for citrus, different research groups have attempted to establish proper infection and co-cultivation conditions with the aim of enhancing transformation frequency. Thus, there are as many protocols on citrus epicotyl segment transformation as laboratories working on this issue. For instance, bacterial inoculation time fluctuates between 5 min (Molinari *et al.*, 2004a) and 20 min (Yang *et al.*, 2000; Almeida *et al.*, 2003a), bacterial concentration for inoculation varies between 4×10^7 (Peña *et al.*, 1995b; Yu *et al.*, 2002) and 5×10^8 (Kaneyoshi *et al.*, 1994; Bond and Roose, 1998; Luth and Moore, 1999), co-cultivation time is usually of 2 or 3 days, and co-cultivation temperature varies between 19 °C (Li *et al.*, 2002, 2003) and 28 °C (Luth and Moore, 1999). Very few papers compare different treatments and then recommend the best conditions for transformation of each citrus genotype, and even less reports investigate why a given treatment is better than others to enhance transformation.

In spite of the high number of studies showing organogenesis and attempting genetic transformation in citrus, little is known about how these two events together contribute to the success of the entire transgenic plant production process. The use of the *uidA* reporter gene allowed

to localize competent cells for transformation in callus presumably formed from the cambium tissue of citrus explants in early steps after co-cultivation with *A. tumefaciens* (Peña *et al.*, 1997; Cervera *et al.*, 1998c). Furthermore, treatments favoring the development of such callus tissue, as co-cultivation in a culture medium rich in auxins and exposure of the explants to darkness during the first 2–4 weeks after bacterial inoculation, greatly increased transformation frequencies and consequently regeneration of transgenic shoots (Cervera *et al.*, 1998c) (Figure 5a). Then, we decided to investigate cell competence and the role of phytohormones for transformation and regeneration of shoots from citrus explants. For this purpose, we used a highly responsive citrus genotype as Carrizo citrange and well-defined tissue culture conditions to perform a histological examination of morphogenesis from citrus explants after co-cultivation with *Agrobacterium* in different culture media: without growth regulators, with BAP at 1 mg l⁻¹, and with 2 mg l⁻¹ 2,4-D, 2 mg l⁻¹ IAA, and 1 mg l⁻¹ 2-iP. Moreover, we used flow cytometry to investigate the role of auxins in the co-cultivation medium as possible enhancers of transformation. Although in all culture media tested regeneration proceeded through indirect organogenesis, co-cultivation in media without phytohormones or with BAP at 1 mg l⁻¹ and subsequent culture in medium with BAP at 3 mg l⁻¹ promoted a faster differentiation response and bud formation. A conspicuous callus was formed from the cambium cells in explants cocultivated in the medium rich in auxins

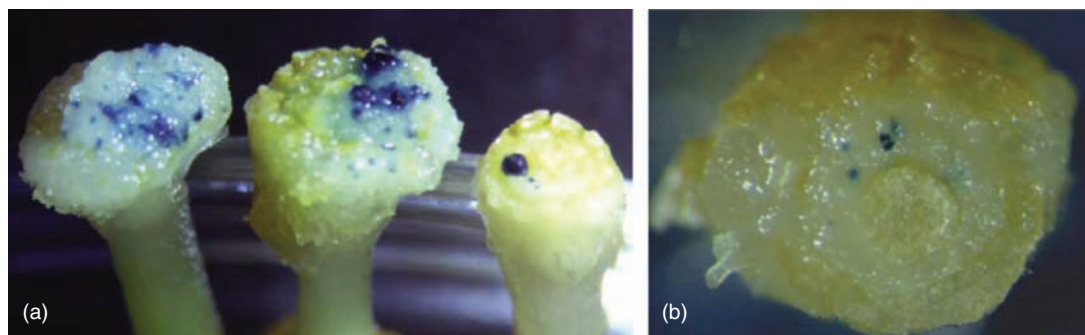


Figure 5 Histochemical GUS-positive spots in cut ends from transformed epicotyl segments. (a) Computing the average number of GUS-positive events at the cut end of inoculated explants is very useful to determine optimal transformation culture media and conditions. (b) Upper view of the cut end of an explant revealing localization of sites of transgenic GUS expression in callus derived from cambial cells

and bud differentiation did not occur during the first weeks after *Agrobacterium*-inoculation. This is consistent with the totipotent state of cambial cells that were able to rapidly respond to external stimuli during *in vitro* culture. Thus competence for regeneration and cell division were strongly related in citrus epicotyl segments. Interestingly, GUS-assayed explants showed that transgenic sectors were only localized in callus cells coming from the cambium, clearly indicating that the development of the cambial callus was also essential to obtain transformation from citrus explants. Therefore, cells competent for transformation and for regeneration were localized in the same callus tissue.

Flow cytometry analysis revealed that co-cultivation in the medium rich in auxins rapidly favored cell entry into the cell cycle because a higher rate of cells at the cut ends were actively dividing and duplicating their DNA after 2 days of culture in the medium rich in auxins. Thus, addition of auxins to the culture medium promoted active cell division and de-differentiation. This coincided with a much higher transformation frequency in the cut ends of such explants. On the other hand, a lower ratio of S-phase cells was found in the explants during co-cultivation in other culture media, and much lower stable transformation was obtained. Taken together, our data suggest that de-differentiation is crucial for transformation of citrus cells.

Another remarkable observation was that callus from the cambium and therefore stable transformation was preferentially produced at the basal end of the explants, probably reflecting an increase in auxin concentration at the basal ends due to the basipetal auxin transport. Then, it could be hypothesized that citrus cells at the explants would be highly responsive not only to an exogenous auxin supplement but also to endogenous auxin accumulation. Leandro Peña's group results demonstrated that auxins play an essential role in cell competence for transformation in citrus. The promotive effect of phytohormones in shifting cells to a competent state for transformation, especially in the case of auxins is well known. Leandro Peña's group observed that addition of BAP promoted cell division and rapid differentiation of buds, as shown by García-Luis *et al.* (1999), but most of these buds were escapes, indicating that cell

division and callus formation were not enough to ensure efficient transformation. Only buds regenerating later, after more prominent callus formation, were transgenic. Leandro Peña's group results demonstrated that de-differentiation was required to shift citrus cells to a competent state for stable transformation, and that de-differentiation was especially triggered by the addition of auxins (mainly 2,4-D) to the co-cultivation medium and possibly by their endogenous accumulation in specific explant sectors. At the same time, co-cultivation in the medium, rich in auxins, could prevent early differentiation of citrus cells into meristemoids that would result in regeneration of escapes. Once transformation at high frequency was achieved in the proper co-cultivation medium, addition of BAP to the regeneration medium could promote differentiation of the transgenic events into buds and shoots. All these results together indicate that the proper combination of phytohormones in the co-cultivation medium shift the cells at the cut ends of the explants to a competent state for integrative transformation (Peña *et al.*, 2004b). These conclusions have been later extended and applied to the genetic transformation of juvenile and mature internodal stem segments from many different citrus types (Ghorbel *et al.*, 1999; Peña *et al.*, 2004a; Rai, 2006; Peña *et al.*, unpublished results).

2.3 Selection of Transformed Tissue

The *nptII* transgene, conferring resistance to aminoglycoside antibiotics as kanamycin by inhibiting protein synthesis, has been the most widely used selectable marker in citrus transformation and regeneration. However, the first reports on citrus transformation already indicated that kanamycin selection was not working efficiently to regenerate transformants. Vardi *et al.* (1990) showed that kanamycin did not provide a reproducible inhibition curve in Rough lemon protoplast transformation experiments. Therefore, they preferred to use paromomycin, another aminoglycoside antibiotic, over kanamycin, as the selective agent, because it inhibited growth of microcalli at 20 mg l⁻¹. In any case, a few whole plants were generated. Kobayashi and Uchimiya (1989) got transgenic sweet orange microcalli from protoplasts, but these did not develop further by

using kanamycin at 25 mg l^{-1} as selective agent. Hidaka *et al.* (1990) found that embryogenic callus lines from different sweet orange genotypes were highly sensitive to kanamycin and hygromycin even used at low doses.

When internodal stem and epicotyl segments coming from either *in vitro* or greenhouse-grown seedlings were used, kanamycin at 100 mg l^{-1} did not control the regeneration of escapes (Moore *et al.*, 1992; Peña *et al.*, 1995a, b; Gutiérrez *et al.*, 1997). The number of escapes was higher than 90% of the regenerated shoots when the explants were inserted vertically in the culture media (Moore *et al.*, 1992; Peña *et al.*, 1995a; Gutiérrez *et al.*, 1997), but this frequency was strongly reduced (to 45% in Carrizo citrange) when epicotyl segments were disposed horizontally in the media (Peña *et al.*, 1995b). Transformation frequency in etiolated epicotyl segments from *P. trifoliata* was rather high, but even with this species, escapes were produced (16–45%) with kanamycin at 100 mg l^{-1} . When kanamycin concentration was raised to 200 mg l^{-1} , transformation efficiency became lower because high kanamycin dose inhibited the proliferation of transformed as well as untransformed cells (Kaneyoshi *et al.*, 1994). The same effect was shown by Peña *et al.* (1997) in internodal stem segments of Mexican lime and by Cervera *et al.* (1998c) in Carrizo citrange epicotyl segments. The aminoglycoside antibiotic geneticin was also tested at 10 mg l^{-1} as an alternative to kanamycin, but it was toxic for transgenic shoot regeneration in Mexican lime (Peña *et al.*, 1997). Another phenomenon apparently associated to the regeneration of escapes was the production of chimeric shoots at high frequencies, which were likely regenerating from transformed and nontransformed cells (Peña *et al.*, 1995a, b, 1997).

As attempts to raise concentrations of kanamycin and use of alternative aminoglycoside antibiotics failed, utilization of the reporter marker gene *uidA* (or *gus* gene from *Escherichia coli*), present in most transformation vectors used in the 1990s, for shoot screening and selection became the best option to enhance citrus transformation efficiency, because screening revealed transformation more efficiently than lethal selection. In this way, part of all shoots regenerating under a 100 mg l^{-1} kanamycin regime was necessarily screened for histochemical GUS expression, and only the few positive

ones were selected for whole plant production. Therefore, the *uidA* reporter transgene was used as a second selectable marker in most citrus transformation protocols. In addition, *uidA* was instrumental for developing appropriate inoculation, co-cultivation, and regeneration media and conditions. Computing the average number of GUS-positive events at the end of inoculated explants was useful to determine in different genotypes the optimal values for factors affecting transformation (Figure 5a). Moreover, in Leandro Peña's laboratory, *uidA* was very important to localize the sites of transgene expression in citrus explants in order to favor the regeneration of whole plants from such competent cells (Figure 5b) (Peña *et al.*, 1997; Cervera *et al.*, 1998c).

Another major step for the improvement of citrus transformation systems was the employment of the green fluorescent protein gene (*gfp*, from the jellyfish *Aequorea victoria*) as a screenable marker. In contrast to *uidA* and other reporters, the fluorescence emission of GFP only requires excitation of living cells by ultraviolet (UV) or blue light, which results from an internal p-hydroxybenzylideneimidazolinone chromophore generated by an autocatalytic cyclization and oxidation of a Ser-Tyr-Gly sequence at amino acid residues 65–67 (Cody *et al.*, 1993; Heim *et al.*, 1994). It does not require either exogenous substrates or cofactors, thus their applications are not limited by problems in substrates' penetration. Moreover, GFP assays are not destructive, allowing performing *in vivo* monitoring of genetic transformation. Earlier attempts to stably incorporate the wt-*gfp* into citrus protoplasts and plants failed, probably due to the toxicity of the GFP protein to citrus protoplasts (Niedz *et al.*, 1995). In Leandro Peña's laboratory, several different *gfp* gene versions were tested, and the so-called *sgfp* gene provided the most easily identifiable GFP expression in citrus explants. The *sgfp* gene is a re-engineered *gfp* gene sequence with the favored codons of highly expressed human proteins, designed to encode a peptide sequence identical to wild-type protein, combined with the replacement of the serine at position 65 with a threonine. It had given a high GFP expression in maize, tobacco, onion, and *Arabidopsis* cells and in stably transformed tobacco plants (Chiu *et al.*, 1996). Leandro Peña's group first used it for *Agrobacterium*-mediated transformation of epicotyl segments from Carrizo

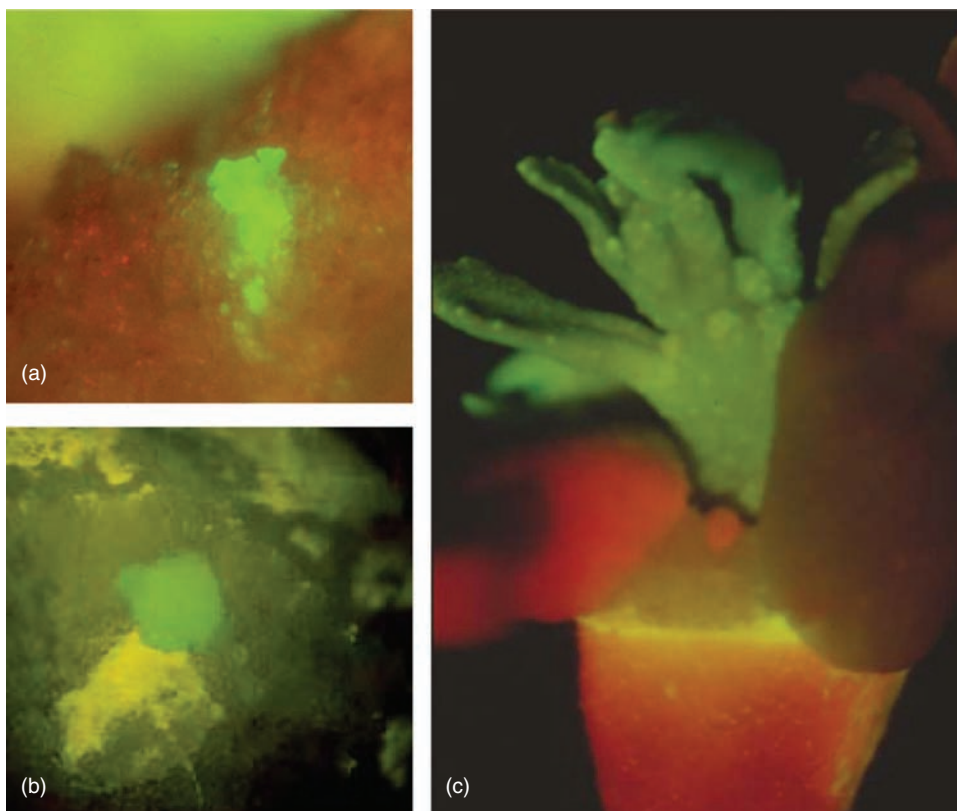


Figure 6 Use of *gfp* as reporter gene for the localization of competent cells and the recovery of transgenic citrus plants. (a) Green fluorescent cell cluster developed in callus tissue from the cut end of an explant. (b) Upper view of an explant showing a green fluorescent transgenic event at the callus formed from the cambium. (c) GFP expression permits a rapid and easy discrimination of transgenic (green) and escape (red) shoots

citrange and internodal stem segments from Mexican lime and sour orange (Ghorbel *et al.*, 1999). After 1 month in darkness, explants developed callus at the cut ends, which was formed from the cambium tissue. When this callus was examined under a stereomicroscope with 480 nm-excited blue light, it exhibited yellow autofluorescence probably emitted by the walls of de-differentiated cells. In some callus tissue, red autofluorescence cell clusters were also visualized. Since chlorophyll fluoresces red at the wavelength used for GFP excitation, these clusters could be considered nontransformed differentiated cells initiating shoot regeneration. Green fluorescent sectors were also observed that were clearly distinguishable from the red and yellow ones. Green fluorescent cell clusters corresponded to transgenic events (Figure 6a). As each GFP molecule represents one fluorophore, high-level

expression was detected in these cells. Furthermore, continuous monitoring of transformation events led us to observe that they always occurred in callus tissue formed from the cambium in all the three citrus genotypes analyzed. Thus, *in vivo* monitoring of *Agrobacterium*-inoculated plant tissues allowed us to localize competent cells for transformation (Figure 6b).

Mexican lime and citrange shoots started to regenerate from the explants after 1–4 weeks in the light. When they were excited at 480 nm blue light, transgenic shoots showed a bright green fluorescence, whereas escapes and nontransformed control shoots appeared red, again because of the autofluorescence of the chlorophyll. GFP expression permitted a rapid and easy discrimination of transgenic and escape shoots of citrus (Figure 6c). Competition for growth between transformed and nontransformed shoots

could be avoided eliminating the escapes soon after arising. In some cases, Mexican lime and citrange regenerated shoots showed sectorial expression of GFP. These plantlets were derived from transgenic and nontransgenic cells originating from one shoot. Transgenic chimeras could be recognized at a very early stage of development and tissue pattern expression could be followed during the whole life of the plant (Ghorbel *et al.*, 1999).

GFP expression was usually detected in transgenic cells, buds, and shoots, without interfering with the transformation and regeneration of plants. All cells or tissues from leaves of the transgenic plants showed green fluorescence. However, fluorescence intensity varied among different parts of the plant, being higher in new growing leaves than in old ones. In old tissues, lower metabolic activity and chlorophyll accumulation partially masked the green fluorescence provided by GFP. Furthermore, differences in green fluorescence intensity could be observed associated to different GFP expression levels in apical leaves from independent transgenic lines. Therefore, GFP could be used as reporter of gene expression for early nondestructive identification and selection of transgenic buds or shoots expressing the highest levels of protein (Ghorbel *et al.*, 1999).

Efficient monitoring by using GFP for early identification and rescue of transgenic buds could circumvent the use of antibiotic or herbicide marker genes to produce transgenic plants. Interestingly, green fluorescent shoots were regenerated from explants inoculated with *Agrobacterium* but cultured in a medium without kanamycin. These results opened up the possibility to produce transgenic plants without using selective agents. Nontransformed buds could be periodically eliminated to avoid competition with the transgenic ones to favor proper development of shoots only from the transgenic events and thus to increase the frequency of GFP-positive shoots regenerating in a medium without kanamycin selection (Ghorbel *et al.*, 1999). This GFP system has also been used to monitor the process of somatic hybridization through protoplast fusion between a GFP-positive leaf parent and a nontransgenic callus donor, and to successfully localize and select hybrid colonies, callus, embryos, and plants (Olivares-Fuster *et al.*, 2002). GFP-based screening/selection has been widely used for citrus protoplast transformation, since they

seem to be highly sensitive to antibiotics even used at low concentrations. Following protoplast culture in liquid medium and transfer to solid medium, transformed calli could be identified through *in vivo* monitoring of GFP expression, physically separated from nontransformed tissue, and cultured on somatic embryogenesis induction medium (Fleming *et al.*, 2000; Olivares-Fuster *et al.*, 2003; Guo *et al.*, 2005; Niedz *et al.*, 2003).

Even with the use of reporter genes as *uidA* and *gfp* as efficient screenable markers, the number of nontransgenic regenerants and chimeras was high enough to become a problem in most citrus transformation systems. Because of this, we decided to investigate in detail the origin and frequency of escape regenerants in transformation of citrus explants, specifically in Carrizo citrange epicotyl and Mexican lime internodal stem segments. Several possibilities have been proposed to explain the regeneration of escapes: transient expression of the selectable marker transgene in many plant cells during the first transformation steps, selection of mutant plant cells resistant to the selective agent, endogenous nonspecific tolerance of plant cells to the selective agent, protection of the nontransformed cells from the selective agent by the surrounding transformed cells, and persistence of *A. tumefaciens* in infected tissues (Peña *et al.*, 1995b). NPTII accumulation in leaves from 20 randomly selected GUS or GFP-positive plantlets of each genotype ranged from 1100 to 6000 and from 2900 to 9100 ng NPTII/mg total protein in citrange and lime, respectively. No NPTII activity was detected in leaves from control plantlets regenerated from noninoculated explants cultured in regeneration medium without kanamycin. Taken together the lack of escapes from noninoculated explants cultured in selection medium, and the absence of endogenous NPTII activity in nontransformed regenerated control shoots, the hypothesis of endogenous nonspecific tolerance of citrus cells to kanamycin to explain the regeneration of escapes and chimeric shoots can be discarded (Dominguez *et al.*, 2004).

To investigate the origin of GUS and GFP-negative/chimeric regenerants, citrange and lime explants were analyzed for GUS expression with the histochemical GUS assay, and for GFP expression under blue light, respectively, from a few weeks to 5 months after infection, when most shoots had already arose. In citrange, most

GUS-negative/chimeric shoots regenerated close to or even from GUS-positive spots. In lime also, a very high rate of GFP-negative/chimeric regenerants was observed coming from GFP-positive transgenic events. This strongly suggested that protection of nontransformed cells from kanamycin by the surrounding transformed cells accounted for most of the escapes and chimeric shoots generated in both citrus genotypes. In fact, detailed observation of certain prominent transgenic events allowed us to observe that in many cases they were composed of mixtures of GUS or GFP-positive and GUS or GFP-negative cells. However, a considerable number of GUS and GFP-negative/chimeric regenerants arose from cut ends without any transgenic event indicating that factors other than protection of escapes by close transgenic cells should also be considered (Domínguez *et al.*, 2004). It has been proposed that transient expression of the selectable marker transgene in many plant cells could play an important role in generation of this type of nontransgenic shoots during genetic transformation of tobacco leaf disks (Park *et al.*, 1998). However, this is unlikely to occur in citrus because shoot regeneration is slow, starting from 1 month after co-cultivation.

Then Leandro Peña's group tested persistence of *Agrobacterium* in citrus explants. Nine months after co-cultivation, lime explants were subcultured to selection medium without cefotaxime and vancomycin for 3 months. During this time, 80–90% of the explants became contaminated by *Agrobacterium* overgrowth, as confirmed by polymerase chain reaction (PCR) analysis of the agrobacterial-like colonies. The remaining explants were analyzed for the presence of the bacteria at the cut ends by culture of basal sections in selective media and subsequent PCR for identification. In two experiments, still 49% and 12.5% of the explants cultured in selective medium contained persisting *Agrobacterium* cells. Interestingly, these frequencies reached 65% and 45% when the explants were cultured in enriched selective medium, indicating that a more sensitive analysis revealed a higher frequency of bacterial detection in the explants. Moreover, these frequencies could be even higher because nonculturable *Agrobacterium* cannot be detected using this method. After co-cultivation, citrus explants are transferred to a regeneration/selection

medium containing the antibiotics cefotaxime and vancomycin to control *Agrobacterium* overgrowth. However, complete elimination of the bacteria seemed to be difficult mainly because the antibiotics used are bacteriostatic rather than bactericidal. It is also possible that resistance of the engineered *Agrobacterium* strains used to kanamycin could provide a selective advantage to bacterial cells over nontransformed plant cells and tissues in the *in vitro* culture medium. In any case, it is clear that the consistent presence of bacterial colonies resistant to kanamycin in certain tissues at the cut ends of the explants could detoxify the surrounding nontransgenic tissues and favor the regeneration of escapes. This could especially explain the regeneration of escapes in cut ends without any transformation event (Domínguez *et al.*, 2004).

In addition, Southern blot characterization of individual propagations from GUS-positive shoots revealed that 12% were actually chimeras from either transgenic and nontransgenic events or from different transformation events, indicating that chimeric shoots resulted not only from the convergence of transgenic and nontransgenic cells as shown by reporter gene expression, but also from the union of different transgenic events (Domínguez *et al.*, 2004).

These facts have important implications in the production of transgenic citrus plants. With the aim of preventing the generation of escapes and chimeras, strategies directed to avoid *Agrobacterium* persistence in plant tissues, and the use of alternative, preferably antibiotic-free positive selection marker genes that could not be expressed in the bacteria would be advisable. The use of alternative selective agents has been attempted. Li *et al.* (2002) used the phosphinothricin acetyl transferase (PAT) *bar* gene, providing resistance to glufosinate ammonium (active ingredient of the BASTA[®] herbicide), to transform Ponkan calli with *A. tumefaciens* and efficiently regenerate whole plants through somatic embryogenesis in culture media supplemented with Basta at 50 mg l⁻¹. Few escapes and no chimeras were generated with this transformation system. One year later, the same group (Li *et al.*, 2003) also used the *hpt* gene, providing resistance to the antibiotic hygromycin, as selection marker gene for developing successful *Agrobacterium*-mediated embryogenic callus transformation of

Valencia sweet orange. We have tested both *bar* and *hpt* as selectable markers for epicotyl and internodal stem segment transformation of several citrus genotypes. Initial regeneration experiments were performed in order to choose the appropriate hygromycin and basta concentrations to control regeneration from nontransformed explants. In the case of hygromycin, regeneration was impeded from a concentration of 10 mg l^{-1} in Carrizo citrange epicotyl segments. A slight toxicity was, however, noticed at this and at lower concentrations, causing developmental anomalies in regenerated shoots. Results from tests using bialaphos as regeneration-controlling agent indicated that a concentration of 5 mg l^{-1} was appropriate for citrus transformation. In transformation experiments, the use of any of both selective agents allowed to produce transgenic plants (Cervera *et al.*, 2006; Cervera *et al.*, unpublished results).

Most of the selectable markers genes used to date, including *nptII*, *bar*, and *hpt*, are based on the addition to the culture media of a substance that is toxic to untransformed cells. There is a concern that the transformation efficiencies are suboptimal with toxic substrates because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells (Haldrup *et al.*, 1998). Alternatively, the *manA* gene, which codes for phosphomannose isomerase (PMI), is an example of a conditional-positive selection system where the selection substrate is not toxic. In this system, the substrate mannose is unable to act as a carbon source for untransformed cells but it will promote the growth of cells transformed with *manA*. This system has been used for sweet orange transformation from epicotyl segments (Boscardi *et al.*, 2003). Mannose used as the sole carbon source, at 73–112.3 mM concentrations depending on the genotype, promoted transgenic shoot regeneration. Transformation efficiency achieved varied between 3% and almost 24%, but escape regeneration was not avoided and about 57% of the total regenerants were not transgenic.

Nonconditional positive selection systems do not require external substrates, yet promote the selective growth and differentiation of transformed material. An example is the isopentenyl transferase gene (*ipt*) from *A. tumefaciens*, which catalyzes the

production of a precursor of several cytokinins that enhance shoot development by modifying the plant hormone levels endogenously. Leandro Peña's group have used this system in citrus transformation and compared it with *nptII* selection. An important characteristic of *ipt* used as positive selectable marker in genetic transformation of citrus was that *ipt* overexpression was sufficient to promote shoot organogenesis in citrus (Figure 7). Regeneration was comparable to that obtained by supplementing a cytokinin (BAP) to the regeneration medium. Moreover, *ipt* was a reliable positive selectable marker that permitted to select transgenic sweet orange shoots from internodal stem segments at the first stages of development, since they showed a clearly distinctive phenotype. In citrange epicotyl segments, *ipt* overexpression induced adverse pleiotropic effects in the explants and their regenerants, and transgenic selection was only possible after PCR analysis of the *ipt* transgene in the regenerated shoots or plantlets. These results suggest that citrange cells are highly sensitive to IPT accumulation, likely causing hormonal imbalances detrimental for development. Compared to kanamycin selection, the *ipt* system permitted to increase transformation efficiency in sweet orange about three times, but was much less efficient in Carrizo citrange (Ballester *et al.*, 2007). Due to the induction of significant alterations in the morphology, development, and physiology of the transgenic plants, this marker had to be combined with a site-specific recombination and DNA removal system to generate *ipt*-free plants (Ebinuma *et al.*, 1997; Sugita *et al.*, 1999).

2.4 Regeneration of Whole Plants

For regeneration of whole transgenic citrus plants through organogenesis and somatic embryogenesis, tissue culture media and conditions are similar to those fully established for several other biotechnological applications. However, these media are usually supplemented with antibiotics, which are toxic to most cells/tissues in culture, since only a few cells of those put in contact with the vector become transformed. In addition, gene transfer itself provokes important alterations in transformed cells and regenerated shoots or embryos. Therefore, the ideal regeneration medium should facilitate the development of

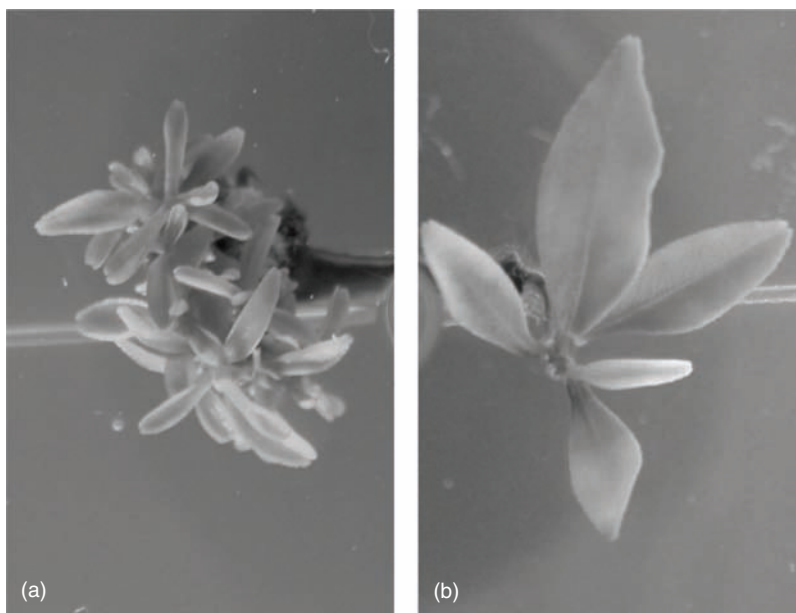


Figure 7 Isopentenyl transferase gene (*ipt*) as positive selectable marker in genetic transformation of citrus. *ipt* overexpression is sufficient to promote shoot organogenesis in citrus. Different phenotypes are exhibited by *ipt*-positive (a) and *ipt*-negative (b) Pineapple sweet orange shoots

buds or microcalli from transgenic cells, and at the same time avoid the generation of escapes. As escape regeneration is a major obstacle in all citrus transformation systems and with all marker genes tested to date, regeneration media and conditions have been adapted to favor the growth and development of transgenic events. Peña *et al.* (1995a, b, 1997) proposed the cultivation of explant in darkness for 2–4 weeks in regeneration/selection medium after co-cultivation with *A. tumefaciens*. This enhanced not only transformation frequency, but also promoted the generation of large transgenic events able to regenerate whole transformed buds (Domínguez *et al.*, 2004; Peña *et al.*, 2004b). Its beneficial role for citrus explant transformation has been demonstrated in several laboratories (Cervera *et al.*, 1998c; Yu *et al.*, 2002; Almeida *et al.*, 2003b; Boscardioli *et al.*, 2006). For some genotypes and in general for mature tissue transformation, the addition of NAA at different concentrations to the regeneration medium during cultivation in darkness strongly promoted the development of the transgenic events (Ghorbel *et al.*, 2000; Almeida *et al.*, 2003b; Rodríguez *et al.*, 2008). Molinari *et al.* (2004a) suggested that transfer

of the explants after *Agrobacterium* co-cultivation to a medium without selective agent for a week, followed by 25 mg l⁻¹ kanamycin for 3 weeks, and then to 50 mg l⁻¹ kanamycin was optimal for regeneration of transgenic Swingle citrumelo plants from thin layer explants.

The most important problem of whole transgenic plant production in citrus has been the extraordinary difficulty found in rooting transgenic shoots and embryos, which seriously reduces transformation efficiencies. Probably, the only exceptions are *P. trifoliata*, grapefruit, and Swingle citrumelo, which could be rooted in MS supplemented with NAA (Kaneyoshi *et al.*, 1994; Luth and Moore, 1999; Molinari *et al.*, 2004a).

As an alternative method for the generation of whole transgenic plants, shoot tip grafting (Navarro *et al.*, 1975) was introduced by the procedure of Peña *et al.* (1995a, b). Small pieces of the shoots emerging from the explants were assayed for reporter marker activity and, when the result was positive, apical portions were grafted *in vitro* onto Troyer citrange seedlings. Rootstock preparation was as follows: Troyer citrange (or any other rootstock) seeds were peeled removing both

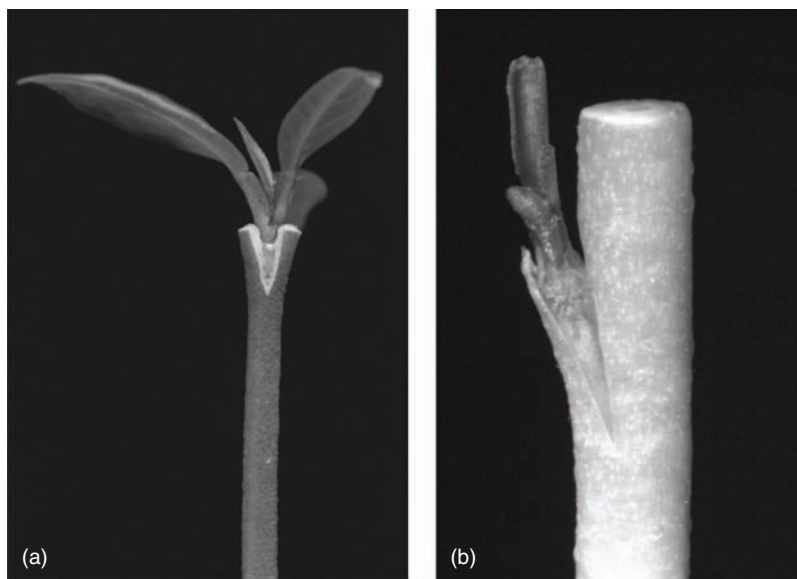


Figure 8 Apical portions from transgenic regenerated shoots are grafted *in vitro* on citrange seedlings. Regenerated shoot apical ends are placed on the top cut surfaces of the decapitated citrange epicotyls, in contact with the vascular ring, or, when larger than 0.4 cm, they are inserted into a vertical incision along the length of the epicotyl, starting at the point of decapitation (a) or in a lateral incision (b)

seed coats, disinfected for 10 min in a 0.5% sodium hypochlorite solution containing 0.1% Tween-20 and rinsed three times with sterile water. The germination medium was MS salts with 10 g l⁻¹ agar, pH 5.7. Seeds were sown individually in tubes and grown in dark at 27 °C for 2 weeks. Troyer citrange seedlings were decapitated leaving 1–1.5 cm of the epicotyls. The roots were shortened to 4–6 cm and the cotyledons and their axillary buds were removed. Then, the regenerated shoot apical ends were placed on the top cut surfaces of the decapitated citrange epicotyls, in contact with the vascular ring, or when larger than 0.4 cm, they were inserted into a lateral incision or in a vertical incision along the length of the epicotyl, starting at the point of decapitation (Figure 8). Grafted plants were cultured in a liquid medium composed of MS salts, 100 mg l⁻¹ m-inositol, 0.2 mg l⁻¹ thiamine-HCl, 1 mg l⁻¹ pyridoxine-HCl, 1 mg l⁻¹ nicotinic acid, 75 g l⁻¹ sucrose, pH 5.7. The cultures were kept at 25 °C, 16 h of photoperiod, 45 µE m⁻² s⁻¹ of illumination. Shoots with only 0.1 cm in length could be used to regenerate transgenic plants following this protocol. A frequency of 100% successful grafts is usually obtained. Scions develop two to four expanded leaves, 3–4 weeks

after grafting. A new grafting of the *in vitro*-grown plants on vigorous rootstocks in the greenhouse allows the rapid acclimatization and development of the plants. Grafted plants are kept in a shadow area and are covered with a closed plastic bag for about 1 month. Then the bag is progressively opened, and when the shoot starts to actively grow, plant is transferred to a greenhouse area with normal illumination. This system is routinely used in our laboratory and has permitted to recover whole transgenic plants from all citrus types tested, even in cases in which the transgene of interest induced malformations in the regenerated shoots (Fagoaga *et al.*, 2005).

The grafting system has been demonstrated to be reliable and widely applicable for whole plant production also in other laboratories and for many different citrus genotypes, including *P. trifoliata* and grapefruit (Kobayashi *et al.*, 1996; Bond and Roose, 1998; Gentile *et al.*, 1998; LaMalfa *et al.*, 2000; Wong *et al.*, 2001; Mendes *et al.*, 2002; Yu *et al.*, 2002; Almeida *et al.*, 2003a, b; Boscariol *et al.*, 2003; Iwanami *et al.*, 2004; Endo *et al.*, 2005). It has been mostly used to recover whole plants from shoots regenerated through organogenesis, but also to regenerate plants from germinated

somatic embryos (Fleming *et al.*, 2000; Olivares-Fuster *et al.*, 2003; Guo *et al.*, 2005; Niedz *et al.*, 2003). Moreover, this method could be applied to the recovery of transgenic plants from other woody plants, in which shoots are difficult to elongate and/or root, and escapes regenerate at high frequencies competing with the transgenic shoots.

Alternatively, for certain purposes, rooting of the transgenic shoots allow performing rapid analyses of the effect of the transgene of potential interest even *in vitro* when the new inserted trait could affect not only to the aerial part of the plant but also the roots (i.e., higher tolerance to salinity, modification of plant size or architecture, etc.). Although development of whole plants is slower and less efficient than performing *in vitro* grafting, rooting can be obtained by cutting 0.5–1 cm regenerated shoots from the explants and transferring them to SRM supplemented with 0.3 mg l⁻¹ BAP for 7–10 days, and then to SRM medium supplement with 5 mg l⁻¹ indolebutyric acid. At least for Mexican lime and Fino lemon, roots develop within a 3–6 weeks period with 90–100% efficiency (Peña, unpublished results).

2.5 Incorporation of Transgenes of Interest Into Citrus

Although there are many reports on introduction of transgenes of potential interest into citrus, few of them describe how the expression of the transgene affects the phenotype of the modified rootstock or variety genotype. This is due in part to the low number of transgenic plants generated in some cases, to the fact that juvenile tissues were transformed in most cases and consequently fruit characteristics could not be evaluated yet, and also due to the difficulties of performing challenge assays against pathogens with woody plants.

In the first attempts to transform citrus, only marker genes as the selectable marker *nptII* and the reporter marker *uidA* were used. Moreover, constitutively expressed promoter and terminator sequences, as those from the 35S gene of *Cauliflower mosaic virus* (CaMV) or from the nopaline synthase (*NOS*) gene of *A. tumefaciens*, were predominantly used. Availability of a genetic transformation system for *P. trifoliata* (Kaneyoshi *et al.*, 1994) allowed to efficiently incorporate

the human epidermal growth factor (hEGF) (Kobayashi *et al.*, 1996) and the *rolC* gene from *A. rhizogenes* (Kaneyoshi and Kobayashi, 1999) into this species. In their first paper, Kaneyoshi *et al.* (1994) used the *uidA* gene under the control of the 35S promoter, and under the control of the promoter from the *rolC* gene. As expected, the 35S promoter directed GUS expression in all leaf, stem, and root tissues of the transgenic regenerants. Interestingly, histochemical GUS expression was only detected in phloem cells when the *rolC* promoter was used. As the precise role of hEGF *in vivo* was unknown, the work of Kobayashi *et al.* (1996) only served to demonstrate that it was possible to express human bioactive peptides in transgenic trees. Eleven plantlets were obtained from 282 treated segments (4% transformation efficiency), and seven were regenerated into whole plants by grafting. The highest hEGF achieved was 113 pg mg⁻¹, which was considered low.

2.5.1 Tree performance

The following report by Kaneyoshi and Kobayashi (1999) was more interesting from an agricultural point of view since its purpose was to produce dwarf rootstocks able to impart dwarfism to the scion. The *rolC* gene was used for this purpose under the control of either the 35S promoter or its own promoter. Transgenic plants were produced with both constructs at high efficiency. Most transgenic lines were dwarf, and the degree of dwarfism differed among lines; 35S::*rolC* transformants were 40% shorter than controls, while *rolC*::*rolC* lines ranged from 10% to 120% shortenings. Six transformants (three from each group) were assayed for rooting of cuttings. Five of them rooted better and one *rolC*::*rolC* line (with the longest internodes) rooted similarly to controls. As next step, Kaneyoshi and Kobayashi (1999) planned to graft some citrus cultivars onto the transgenic plants and check their potential as dwarfing rootstocks, but no report has been published to date on this aspect. Gentile *et al.* (1998) regenerated transgenic plants of Tarocco sweet orange and Carrizo citrange with *rolABC* genes from *A. rhizogenes*. Although plant phenotypes were not characterized in detail, preliminary observations indicated that they grew with difficulties and

showed morphological anomalies, as small and narrow leaves.

Fagoaga *et al.* (2007) have produced transgenic Carrizo citrange plants overexpressing a citrus gibberellin (GA) 20-oxidase cDNA (*CcGA20ox1*) in sense or antisense under the control of the 35S promoter to modify plant architecture. *CcGA20ox1* is a key enzyme of gibberellin biosynthesis. Expression of the transgenes was assayed by Northern blot and Western blot analyses in both antisense and sense transgenic lines. In sense transgenic lines, the level of *CcGA20ox1* protein was high and correlated with plant height and internode length. The leaf area was considerably smaller than that of the control plants. In antisense transgenic lines, down-regulation of *CcGA20ox1* resulted in both shorter internodes and dwarfing with larger number of branches. No adverse pleiotropic phenotypic effects were observed in these plants. As expected, taller (sense) and shorter (antisense) phenotypes correlated with higher and lower levels, respectively, of active GA1 in growing shoots of the transgenic plants (Fagoaga *et al.*, 2007). We are currently investigating whether antisense transgenic Carrizo citrange used as rootstock is able to reduce the size of nontransgenic scions. Potential reduction of scion plant stature by down-regulating GA20ox of a well known and widely used rootstock would provide a considerable benefit to citriculture by allowing higher planting density, easier management and fruit harvesting, thus reducing labor costs.

2.5.2 Higher tolerance to abiotic stresses

Soil salinity significantly limits citrus production in several areas worldwide. Carrizo citrange, considered an excellent citrus rootstock, is very sensitive to salt stress, which restrains its use in salty soils. We have successfully transformed plants of Carrizo citrange with the halotolerance gene *HAL2* under control of 35S promoter (Cervera *et al.*, 2000a). This gene was originally isolated from yeast and is implicated in salt tolerance mechanisms (Murguía *et al.*, 1995). Plants showing higher transcription levels in Northern blot analyses were chosen to accomplish *in vivo* salt stress tolerance assays, by using transgenic plants as rootstocks for a sensitive citrus

variety. However, transgenic lines did not show higher tolerance to salinity than controls.

The accumulation of proline represents a general response to stress in many organisms, including higher plants, exposed to environmental stresses such as water deficit, high salinity, high temperature, freezing, UV radiation, and heavy metals. Molinari *et al.* (2004b) have incorporated a Δ^1 -pyrroline-5-carboxylate synthetase mutant gene (*P5CS*) from *Vigna aconitifolia* driven by the 35S promoter into Carrizo citrange plants, with the objective of increasing proline accumulation in all tissues and consequently enhancing tolerance to drought stress. In well-watered conditions, transgenic plants accumulated proline in leaves up to eightfold higher than in control plants. As compared to controls of the same age, no apparent differences in growth and morphology were observed in the transgenic Carrizo citrange plants after 5 months in the greenhouse. Ten days after water had been withheld, when water present in substrate was not available to the plant, there was an accentuated fall in photosynthetic rate and increase in the values of stomatal resistance in nontransgenic plants, while the transgenic plants showed comparatively minor changes in these two parameters. The values of stomatal resistance and transpiration during the experiment period showed that transgenic plants were able to support the applied water deficit stress. The adoption of well-established citrus rootstocks with enhanced drought tolerance could represent a promising strategy in maintaining productivity in citrus-growing regions with water deficit.

2.5.3 Rapid cycling citrus trees

Results of genetic improvement programs for citrus are very limited, due, among other reasons, to the extremely long juvenile phases of the trees, which prolong the time required to analyse late traits like fruit features. With the aim to accelerate their flowering time, we have transformed juvenile Carrizo citrange seedlings to constitutively express the *Arabidopsis* *LEAFY* (*LFY*) or *APETALA1* (*API*) genes that are sufficient to promote flower initiation in *Arabidopsis* (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Chimeric genes with their coding regions fused to the 35S promoter were used. Expression of the 35S::*LFY* and



Figure 9 Overexpression of *APETALAI* from *Arabidopsis* in Carrizo citrange transgenic plants induces an early flowering phenotype. Transgenic *APETALAI* plants flowering 6 months after sowing (four plants on the right) compared to a nontransformed control plant (plant on the left)

35S::*API* transgenes was confirmed by Northern blot hybridization. In contrast to the development of regenerated nontransformed control plants, most of the 35S::*LFY* transgenic trees developed thin stems with a weeping growth habit and with a rapid reduction in the number and size of thorns. Leaf size was also reduced, and leaves displayed a variable degree of curling in different transgenic lines. The most extreme phenotypes were observed in transgenic plants showing a higher level of accumulation of *LFY* transcript in leaves. Flowering time was strongly accelerated in many of those plants, which initiated flowering in spring, 12–20 months after their transfer to the greenhouse. Transgenic trees carrying the 35S::*API* construction displayed more normal growth than the 35S::*LFY* trees. Many plants showed rapid signs of phase change, evidenced by the reduction in the size and number of thorns. Furthermore, leaves displayed heart-shaped leaflets, typical of adult citrange trees (Peña *et al.*, 2001).

Both types of transgenic citrus plants produced fertile flowers and fruits as earlier as the first year, notably through a mechanism involving a dramatic shortening of their juvenile phase. Furthermore,

expression of *API*, being as efficient as *LFY* in the initiation of flowers, did not produce any severe developmental abnormality. Both types of transgenic trees flowered again in consecutive years and their flowering response was under environmental control (Figure 9). In addition, sexual and nucellar-derived transgenic seedlings had a very short juvenile phase and flowered in their first spring, demonstrating the stability and inheritance of this trait (Peña *et al.*, 2001). This opens the possibility to use independent *API* transgenic plants as parents in crosses with nontransformed genotypes that would yield 50% of the progeny flowering and setting fruits in 1–2 years, thus providing the opportunity to evaluate fruit features very early and to rapidly advance generations. On the other hand, by retransformation of *API* transgenic citrus plants it could be possible to rapidly test the effect of the expression of certain transgenes under flower organ or fruit specific promoters as a system to look for biotechnological strategies to develop seedless varieties, modify fruit color and aroma, or favor easy-peeling characteristics. Retransformation of *API* Carrizo citrange plants has been successfully achieved in Leandro Peña's

laboratory (Cervera *et al.*, 2006). In addition, the 35S::*API* construction has been incorporated into other *Citrus* genotypes and it also promoted early flowering and fruiting features (Cervera *et al.*, unpublished results).

FLOWERING LOCUS T (FT) is another flowering time gene of *Arabidopsis* and is characterized as a floral pathway integrator. An *FT* homolog (*CiFT*) was found in the expressed sequence tag catalog of a cDNA library from the fruit of satsuma mandarin and its overexpression induced an early flowering phenotype in *Arabidopsis*. Endo *et al.* (2005) introduced *CiFT* under the control of the 35S promoter into *P. trifoliata* and generated transgenic plants that flowered in general in less than 8 months. Four out of six transgenic lines developed normal fruits with intact seeds. All lines of the 35S::*CiFT* plants had a leafy inflorescence architecture, in which flowers and leaves concurrently developed. In contrast, wild-type *P. trifoliata* plants usually develop solitary flowers in the axils of leaves. Differentiation of floral buds starts in early summer and is completed prior to the onset of winter dormancy. In the transgenic plants, the development of flowers was not interrupted by a dormant period. The tree shape of transgenic plants was dwarfed and branched in comparison with that of the controls, and the leaf shape was morphologically altered. Moreover, the leafy inflorescence was often accompanied with gradual changes of thorns into flowers. Nucellar and sexual progeny seedlings from these lines showed extremely early flowering, developing floral buds almost immediately after germination. Although *CiFT* induced many pleiotropic effects on plant growth and development, early flowering *CiFT-P. trifoliata* plants could be a helpful tool for functional genomics studies in citrus. It could be also very interesting to verify the effect of 35S::*CiFT* in *Citrus* genotypes.

2.5.4 Improvement of fruit quality

Li *et al.* (2002, 2003) reported the generation of Ponkan and Valencia sweet orange transgenic plants, respectively, through *Agrobacterium*-mediated transformation of embryogenic calli with a chimeric ribonuclease gene (*barnase*) derived from *Bacillus amyloliquefaciens* under the control

of an anther tapetum-specific promoter (pTA29). The aim of the work was to produce pollen sterile transformants, and subsequently seedless fruit. More than 20 lines from each genotype were generated. Since transformants were juvenile, several years of cultivation are needed to evaluate possible male sterility. The same can be applied in part for Koltunow *et al.* (2000) who produced juvenile transgenic Mexican limes containing genes for decreased seed set. However, the juvenile period of Mexican lime is one of the shortest among citrus types. In spite of it, to our knowledge there are no published data on the phenotype of the mature plants and their fruits.

Costa *et al.* (2002) introduced several carotenoid biosynthetic genes under the control of constitutively expressed promoters into juvenile Duncan grapefruit (*see* Grapefruit for description of this work). Wong *et al.* (2001) introduced a 1-aminocyclopropane-1-carboxylate synthase gene (*CS-ACS1*) isolated from *C. sinensis* under the control of a double 35S promoter into *P. trifoliata* and Carrizo citrange seedlings in antisense orientation. Eight and 13 Southern blot-positive plants were generated, respectively. Two and seven of these plants, respectively, expressed the antisense messenger-RNA (mRNA) at detectable levels. Those transgenic lines producing higher levels of antisense mRNA were found to partially repress the increase of ACC accumulation after a chilling treatment. The authors speculated that the reduced level of ACC in transgenic citrus tissues following the chilling treatment could be useful to reduce the damages caused by chilling injury in citrus fruits. However, several years are needed to get mature plants producing enough number of transgenic fruits.

Guo *et al.* (2005) introduced a pectin methylesterase gene (*Cs-PME4*) isolated from sweet orange into Valencia sweet orange protoplasts. *Cs-PME4* cDNA was cloned in sense between a double 35S promoter and a *gfp* (*e-gfp*) marker gene, so the promoter was controlling the expression of both cistrons. Several transgenic plants were recovered but all of them came from the same transformation event. In addition, the plants exhibited a slightly abnormal morphology, likely attributable to the *gfp* version used. Transgenic plants were juvenile and require years to flower and fruit. Then, it will be possible to investigate whether *Cs-PME4* overaccumulation

occurs in fruit cells. From a biotechnological perspective, it would be interesting to down-regulate *Cs-PME4* with the aim of preventing juice cloud separation.

2.5.5 Resistance to pathogens

Phytophthora citrophthora is the most widely spread oomycete all over the citrus-growing areas and represents one of the major causes of crop losses. Constitutive overexpression of proteins involved in plant defense mechanisms is one of the strategies proposed to increase plant tolerance to fungal pathogens. P23 is a 23-kDa pathogenesis-related protein induced in tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) plants when these are infected with citrus exocortis viroid and its antifungal activity has been shown in *in vitro* assays (Rodrigo *et al.*, 1993). Fagoaga *et al.* (2001) have successfully produced transgenic Pineapple sweet orange plants with a chimeric gene construct comprising the coding region of the *P23* gene under the control of the 35S promoter. Nine transgenic lines accumulated the P23 protein, as determined in Western blot analyses, and in general P23 accumulation levels were high in most lines. Transgenic lines constitutively expressing the *P23* transgene were challenged with *P. citrophthora* by using detached bark and whole plant assays. One of them achieved consistently reduced susceptibility in both assays. This line showed plant survival rates higher than the control when whole transgenic plants were inoculated with fungal cultures, since only one of 10 inoculated plants died at the end of the 2-year assay, while 50% of the controls have died 6 months after inoculation. The exact nature of tolerance to *P. citrophthora* provided by constitutive tomato *P23* (PR-5) expression in transgenic orange plants is unknown. These results provide evidence for the antifungal activity *in vivo* of the P23 pathogenesis-related protein against *P. citrophthora* and suggest that this may be employed as a strategy aimed at the engineering of *Phytophthora* disease resistance in citrus (Fagoaga *et al.*, 2001).

CTV is the causal agent of the most important virus disease of citrus in the world. It produces two main field syndromes: common strains cause decline and death of most scion varieties grafted on sour orange rootstock, whereas highly virulent

strains additionally cause stem pitting, stunting, low yield, and poor fruit quality of some varieties of sweet orange, limes, and grapefruits, regardless of the rootstock used. Several strategies have been used to engineer plant resistance to viral pathogens. Most are based on the concept of pathogen-derived resistance (PDR), which proposes that the introduction and expression in plants of viral sequences could interfere with the life cycle of the same or a closely related challenging virus, thus providing resistance to infection. Gutiérrez *et al.* (1997) produced a few transgenic plants of sour orange, Mexican lime, and Carrizo citrange with the *p25* major coat protein gene from CTV. However, challenge analysis of these plants has not been reported to date.

Domínguez *et al.* (2002c) generated more than 40 transgenic Mexican lime lines, 25 containing the *p25* gene from the severe CTV strain T305, and 17 from the mild strain T317. Transgene integration patterns were usually complex, with almost half of the plants showing T-DNA truncations and a copy number of the *p25* transgene ranging from one to six. Accumulation of *p25* was detected in most of the transgenic lines (Domínguez *et al.*, 2000). When 8–10 propagations of each transgenic line were graft- and aphid-inoculated with CTV isolates T305 and T300, respectively, two types of responses to viral challenge were observed: most lines developed CTV symptoms similar to those of the nontransgenic controls, but six of them exhibited resistance against the virus. This consisted of a fraction of plants, ranging from 10% to 33%, that were immune to CTV, with the rest showing a significant delay in virus accumulation and symptom onset in at least three consecutive flushes (about 1 year) after inoculation (Domínguez *et al.*, 2002c). These results were reproduced with four of the six transgenic lines in an additional challenge experiments in which propagations were again graft-inoculated with CTV T305: three *p25* resistant lines accumulated *p25* at high levels, suggesting that a protein-mediated resistance mechanism was operative in these lines, whereas the fourth transgenic line, called SCP.15, showed almost undetectable *p25* accumulation and consistent resistance. Moreover, this line exhibited silencing of the *uidA* transgene, used as reporter in the T-DNA of the transformation vector, as determined by the lack of histochemical GUS expression in leaf pieces. Post-transcriptional

gene silencing (PTGS) of the *uidA* transgene was confirmed in Northern blot hybridization and nuclear run-on analyses (Domínguez *et al.*, 2002a; Domínguez and Peña, unpublished results). When eight plants propagated from the SCP.15 line were graft-inoculated with CTV T305, two were immune to virus challenge and the *uidA* transgene remained silenced in their leaves along the 1-year-duration of the experiment, but the other six plants became direct antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) positive for CTV and started showing symptoms at the second or third flush postinoculation. Interestingly, infection of these plants was directly related with activation of GUS expression, indicating that CTV might encode a suppressor of gene silencing which could restore GUS expression in infected transgenic plants carrying the *uidA* silenced. This result also suggested that PTGS of the *p25* transgene was involved in the propagations that remained immune to CTV. This was the first demonstration of PDR against a member of the genus *Closterovirus* in its natural host. In the frame of a collaboration project of the IVIA with the University of Hawaii (M. Melzer, S. Ferreira, and J. Hu) and the USDA (D. Gonsalves), seeds from the most promising *p25*-transgenic lines were sent to Hawaii in 2004 for a field trial to test the potential resistance of these plants to natural challenge in an area where severe CTV strains and the efficient vector *Toxoptera citricida* are predominant.

The *p25* gene of CTV has also been inserted into sour orange, and transgenic plants accumulating or not the *p25* protein have been generated (Ghorbel *et al.*, 2000). However, resistance of these plants to tristeza decline was not evaluated because this syndrome is not usually reproduced under greenhouse conditions and a permit to transfer these plants to the open field could not be obtained yet. The inverted-graft test (Pina *et al.*, 2005) was used as alternative. It consists on grafting the scion genotype onto CTV-infected sweet orange rootstocks in the greenhouse. Those genotypes that are resistant to tristeza decline are able to sprout and develop normally, while susceptible genotypes show serious difficulties in sprouting and usually abscise a few weeks after grafting. Unfortunately, none of the *p25*-sour orange transgenic lines resulted resistant to tristeza decline in this assay.

In an attempt to develop RNA-mediated resistance against CTV, Mexican lime plants were transformed with two untranslatable versions of the *p25* gene from CTV isolate T305: (1) the full-length *p25* gene modified by introducing two consecutive stop codons three nucleotides downstream the start codon, and (2) the 3' half of *p25* gene fused to the *sgfp* marker gene, both under the control of the 35S promoter. Of the 48 transgenic lines obtained, 35 harbored the untranslatable full-length *p25* gene and 13 carried the truncated version, as revealed by Southern blot hybridization. Furthermore, run-on assays and cytosine methylation analysis showed that the *p25* transgene was post-transcriptionally silenced in some of the lines (Domínguez *et al.*, 2002a). After graft-inoculation with CTV T305, all transgenic plants developed symptoms, though some delay in virus infection and symptom attenuation was observed in the first two flushes postinoculation, especially in lines transformed with the full-length nontranslatable construct. However, in the third and following flushes, all transgenic plants accumulated CTV as the nontransgenic controls (Domínguez *et al.*, 2002b).

Yang *et al.* (2000) reported production of Rio Red grapefruit transgenic plants containing an untranslatable version of the *p25* coat protein gene from CTV and the *Galanthus nivalis* agglutinin gene, a plant-derived insecticidal gene.

Febres *et al.* (2003) transformed Duncan grapefruit with several CTV-derived sequences, including *p25* from three different strains and a nontranslatable version of the same gene, *p27*, the replicase gene, and a 400 bp segment of the 3' end of the genomic RNA in either sense or antisense orientation (*see Grapefruit* for description of these works).

Olivares-Fuster *et al.* (2003) used two constructs derived from the 3'-UTR of CTV T36, under the control of the 35S promoter, to transform Itaborai sweet orange protoplasts. They were used in sense orientation and were able to transcribe the 393 and 742 3'-terminal nucleotides of the viral genome. Protoplast lines were challenged with CTV, and 4–5 days later viral replication was tested by Northern blot. Two of the clones showed reduced replication and one clone carrying the larger construct showed null replication. Unfortunately, attempts to regenerate whole plants from these clones were unsuccessful. Leandro

Peña's group has used a construct derived from the 3'-terminal 550 nucleotides from CTV T36 to transform Mexican lime. It was engineered to express this fragment either in sense or in antisense orientation, under the control of the 35S promoter. More than 20 independent transgenic lines were generated with each construct. After propagation and challenge by graft-inoculation, none of them was immune to infection, though several plants showed delayed infection (Fagoaga *et al.*, unpublished results).

The 3'-terminal gene of CTV codes for a 23-kDa protein (p23), which is an RNA-binding protein that contains a motif rich in cysteine and histidine residues in the core of a putative zinc-finger domain (López *et al.*, 2000). This protein is involved in regulating the balance of plus and minus RNA strands during replication, with the zinc finger domain and the adjacent basic region being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana *et al.*, 2002). Considering its regulatory role in CTV replication, we decided to explore whether overexpression in transgenic plants of this protein could affect the normal CTV infectious process, we have produced more than 50 transgenic lime plants carrying the *p23* gene from CTV T36, or a truncated version thereof, both types under the control of the 35S promoter. Constitutive expression of *p23* induced phenotypic aberrations resembling symptoms incited by CTV in nontransgenic lime plants, whereas transgenic plants expressing the *p23*-truncated version were normal. The onset of CTV-like symptoms in *p23*-transgenic plants was associated with the expression of p23, and its accumulation level paralleled symptom intensity (Ghorbel *et al.*, 2001a).

To determine whether expressing ectopically the *p23* gene from a severe and a mild CTV strain induced similar phenotypic effects on Mexican lime, a second set of transgenic limes was produced using the *p23* gene from either the severe strain T36 or the mild strain T317. Ectopic expression of the *p23* gene induced in Mexican lime aberrations resembling viral leaf symptoms of similar intensity, irrespective of the pathogenicity of the CTV strain from which *p23* was obtained, and this was correlated again with accumulation of p23 protein. Transformation with *p23*-T36 of other CTV susceptible citrus species, including sweet and sour orange, and of the CTV-resistant *P. trifoliata*,

also led to CTV-like symptoms that were not visible when these plants were transformed with a truncated *p23* version. The intensity of CTV-like symptoms in citrus species and relatives other than Mexican lime correlated with levels of *p23* transcripts, but the p23 protein was barely detectable in these hosts. The higher accumulation of p23 in Mexican lime with respect to sweet and sour orange was also observed in nontransgenic plants inoculated with CTV, suggesting that even minimal levels of p23 cause deleterious effects in the latter two species. In contrast, transgenic expression of *p23* in CTV nonhost *Nicotiana tabacum* and *Nicotiana benthamiana* species led to consistent accumulation of p23 without phenotypic aberrations. Altogether, these results indicate that p23 is an important CTV pathogenicity determinant that interferes with plant development specifically in citrus species and relatives (Fagoaga *et al.*, 2005). In addition, p23 has been found to act as an RNA silencing suppressor in *N. tabacum* and *N. benthamiana* plants (Lu *et al.*, 2004).

In course of the experiments to incorporate *p23* into Mexican lime, 3 out of 60 lines carrying the *p23* gene of CTV T36 and 2 out of 20 lines carrying that of CTV T317, were visually normal and developed as controls transformed with the empty vector or nontransformed (Figure 10). These five lines displayed characteristics typical of PTGS: multiple copies of the transgene and methylation of the silenced transgene as revealed in Southern blot analyses, low levels of the corresponding mRNA in Northern blots, and accumulation of *p23*-specific siRNAs as shown in ribonuclease protection assays. When propagations of these silenced lines were graft- or aphid-inoculated with CTV T308 and T300, respectively, some were immune: they neither expressed symptoms nor accumulated virions and viral RNA as estimated by DAS-ELISA and Northern blot hybridization, respectively. Other propagations were moderately resistant because they showed delayed expression of leaf symptom and attenuated stem pitting compared to the controls. The susceptible propagations, in addition to normal symptom expression and elevated virus titer, accumulated *p23*-specific siRNAs at levels significantly higher than immune or noninoculated propagations, and showed transgene demethylation (Fagoaga *et al.*, 2006).



Figure 10 Engineering resistance to citrus tristeza virus (CTV). Nontransgenic control (left) and *p23*-silenced transgenic (right) Mexican lime plants graft inoculated with CTV. Transgenic plant shows resistance to viral infection while control plant shows CTV symptoms such as vein clearing and leaf cupping

A characteristic of the *p23* transgene-mediated PTGS was that vegetative propagations from the same transgenic line showed different responses against CTV, with some propagations being immune and others susceptible to viral challenge. This variable response among clonal transformants carrying viral-derived transgenes indicates that factors other than the genetic background of the transgenic plant, such as environmental conditions or the developmental stage, play a key role in PTGS-mediated resistance. As mentioned above, Fagoaga *et al.* (2006) also observed a high accumulation of viral-specific siRNAs in CTV-inoculated nontransgenic (or transformed with the empty vector) Mexican lime plants, indicating a strong natural PTGS-mediated antiviral response in this host. These results indicate that most of the siRNAs detected in susceptible *p23*-transgenic plants come from degradation of viral RNAs, and that the resistance obtained in immune plants was likely triggered by the small amounts of the transgene-specific siRNAs existing before CTV inoculation. A connection can also be inferred between the high accumulation level of siRNAs in CTV-infected plants and cross-protection induced by mild CTV strains: in line with the accepted PGTS model (Hammond *et al.*, 2000), the siRNAs derived from mild cross-protecting CTV strains could prevent or attenuate the subsequent invasion by a severe CTV strain through their incorporation into an RNA-induced silencing complex targeting for degradation the genomic and subgenomic viral RNAs.

More recently, Batuman *et al.* (2006) have engineered a construct consisting of the *p23* gene

plus 3'-UTR from CTV in sense and antisense, separated by the Castor bean catalase intron, under the control of the 35S promoter, which once expressed in transgenic cells should be folded into a double stranded (ds) RNA structure theoretically able to trigger PTGS. It was used to transform *N. benthamiana* and *C. macrophylla*. Integration and expression of the transgene was demonstrated by Southern and Northern blot, respectively. Seventy *C. macrophylla* transgenic plants coming from 35 independent lines were challenged with CTV by graft-inoculation, but only 9 lines showed a delay in viral infection. None showed durable protection against CTV. Nevertheless, transgenic lines accumulated transgene-derived mRNA usually at high level, and neither siRNA accumulation nor transgene methylation was characterized. We have used a similar construct to transform Mexican lime plants, selected those lines carrying a single copy transgene and accumulating siRNAs at high levels, and challenged 6–11 propagations from them with CTV by graft-inoculation. A variable number of propagations from some of these lines were fully resistant to CTV challenge (Cervera *et al.*, unpublished results).

In summary, these studies demonstrate that PDR can be extended to a member of the family Closteroviridae and to its natural hosts. Whether transgenic citrus plants expressing CTV-derived sequences could be an efficient alternative to cross protection for controlling stem pitting CTV strains in the field, and whether resistance to tristeza decline could be incorporated to the sour orange rootstock, remains to be tested.

An alternative strategy to look for resistance against CTV could be using plant-derived resistance genes. General resistance to CTV has been found in *P. trifoliata*, and a resistance gene (*Ctv*) has been characterized and mapped (Gmitter *et al.*, 1996; Mestre *et al.*, 1997; Fang *et al.*, 1998). Because of the complex genetics of citrus, it is extremely difficult to introgress this resistance gene into citrus varieties by conventional breeding. However, cloning of this gene is underway in several laboratories (Deng *et al.*, 2001; Yang *et al.*, 2003). A bacterial artificial chromosome (BAC) library developed from *P. trifoliata*, homozygous to *Ctv*, was used for a 1.2 Mb genome walk spanning the region between *Ctv*-flanking markers. Sequencing of a set of four overlapping BAC clones in this region, using shotgun sequencing and resolution of their ends by sequencing of additional BAC clones and their use as anchors, further localized *Ctv* to a 282 kb region, comprising 22 predicted genes (Yang *et al.*, 2003). Sequence analysis of the *Ctv* locus in this region identified 61 SSRs, which were used to further narrow down the locus in the *Poncirus* genome to 121 kb, comprising 10 genes. Each of the 10 genes in this region has been individually cloned in *Agrobacterium*-based binary vector and used to transform susceptible Ruby Red, Rio Red, and Duncan grapefruit varieties in Eric Mirkov's laboratory (Rai, 2006). Although results from the CTV challenge experiments are still preliminary, transgenic lines carrying and expressing either of the 10 candidate genes were susceptible to CTV infection, suggesting that more than one gene in the locus is involved in resistance to CTV or that the role of other genomic loci has been overlooked (*see also Grapefruit*).

Citrus mosaic virus (CiMV) is a serious constraint for citrus production in Japan. Infected trees grow poorly and often develop ringspot symptoms on the fruit, which drastically reduce their commercial value. Iwanami *et al.* (2004) reported the incorporation of the coat protein gene from CiMV into *P. trifoliata*, the main citrus rootstock in Japan. More than 30 transgenic lines were produced and characterized by Southern blot. Attempts to detect coat protein immunoreaction in the transgenic plants by Western blot failed. More than 20 transgenic lines were propagated and mechanically inoculated with the virus. One line showed resistance to virus challenge (7.1%

infection, compared to 65.1% infection in controls at 60 days postinoculation) while the other lines showed responses ranging from susceptibility to more moderate resistance at the same time period. There are plans to transfer the most promising lines to the field and challenge them against the virus under natural conditions.

Citrus canker, caused by the bacterium *X. axonopodis* pv. *citri*, is one of the most important diseases in several citrus areas, and there are no ways to control it other than accomplishing very expensive eradication programs in regions where limited number of foci are periodically identified, or applying massive doses of chemicals that only make a partial control and cause adverse effects in the environment. Attacins belong to a class of antimicrobial peptides, which are secreted by several insect species into the haemolymph in response to bacterial infections. Transgenes encoding attacin precursors have been used to reduce susceptibility to *Erwinia* species in transgenic pear, apple, and potato plants, but the mechanism of resistance is still unknown. Boscardi *et al.* (2006) introduced the attacin A gene (*attA*) from *Tricloplusia ni* under the control of a double 35S promoter into Hamlin sweet orange seedlings. Interestingly, the transgene-derived protein carried a native signal peptide that secreted it to the apoplast, as it was demonstrated by transiently expressing *attA-gfp* and *attA-uidA* fusion constructs in onion cells. Propagated plants from eight Southern and Northern blot-positive lines were spray-inoculated with a 10⁶ CFU/ml canker bacterial suspension and incubated in a growth room set at 27°C during 1 month, when disease severity was assessed by calculating diseased leaf area in 15 young leaves per transgenic line. Seven lines showed a significant reduction in susceptibility to citrus canker, and in two of them the reduction was of 55% and 60% compared to the control.

Citrus blight is a devastating disease of unknown etiology, which causes general decline of trees in hot and humid areas. A pathogenesis-related (PR) protein of about 12 kDa can be detected in trees affected by blight, and it is not found in citrus trees with other disorders and diseases. The *p12* gene was isolated from a cDNA library from roots of a tree with citrus blight and used in sense or antisense, under the control of the 35S promoter, to transform Carrizo citrange plants.

Stable integration was confirmed by Southern blot, and expression was verified by Northern blot and by Western blot (in sense plants). Accumulation of the p12 protein in sense plants did not provoke any symptom or developmental abnormality, suggesting that this protein is not involved in symptom expression. The precise role of p12 in citrus blight requires further investigation (Kayim *et al.*, 2004).

2.6 Stability of Transgene Expression and Phenotype; and Effects on Growth, Yield, and Quality

A major requisite for evaluating the validity of genetic transformation technology in improvement programs is the stability of the modified genome and the stability of transgene integration and expression over long periods of time, especially in vegetatively propagated and long-lived perennial fruit crops. However, reports on transgenic stability in citrus are almost nonexistent, and most works are focused in introducing a transgene of potential agricultural interest into a given genotype and evaluating the phenotype of the regenerating plants only after few months of growth in the greenhouse and normally just pertaining the trait, which is tentatively modified.

However, somaclonal variation induced during the *in vitro* culture phase has been extensively reported in greenhouse experiments and field trials of transgenic plants. It has been used in citrus as a strategy to generate genetic variation that could be potentially useful in improvement programs (Grosser *et al.*, 2002), but somaclonal variation could also affect plant phenotypes producing abnormal morphologies, which has been correlated with genomic changes as chromosomal deletions and alterations in ploidy. In addition, expression of transgenes can be affected by numerous causes, as transgene loci number, genomic context of the integrated loci or position effects, abnormal configurations of the integrated T-DNA, or even environmental conditions that may contribute to differences in expression between plants with unlikely integration patterns.

Cervera *et al.* (2000b) maintained a collection of 70 transgenic citrange plants grown in 50l-pots in a screenhouse in order to investigate, for a period of 4–5 years, (1) the origin of

morphological variants in the transgenic population, (2) how factors related to T-DNA integration, regeneration process and expression of transgenes may be influenced by transformation conditions, (3) whether transgenes are stably integrated and expressed over long time periods in citrus plants grown under natural environmental conditions, and (4) whether correlation between integration patterns and transgene expression could be established in these plants (Cervera *et al.*, 2000b).

Transgenic plants used for this analysis were generated earlier (Peña *et al.*, 1995b; Cervera *et al.*, 1998c). Briefly, *A. tumefaciens* EHA 105 containing the binary plasmid p35SGUSINT was used as vector for transformation of epicotyl segments from *in vitro*-grown seedlings. Two co-cultivation media were used: in the first case it contained BAP at 1 mg l^{-1} as growth regulator (type 1), and in the second, co-cultivation was performed in the medium rich in auxins (2 mg l^{-1} 2,4-D, 2 mg l^{-1} IAA, and 1 mg l^{-1} 2-iP as phytohormones) (type 2). All the transgenic plants were confirmed as *nptII*- and *uidA*-positive by Southern blot analysis. First of all, isozyme analysis allowed to confirm that the same banding pattern was found for all the samples, which corresponded exactly to the pattern shown by the female parent from which the starting seeds were taken. These results indicated that no zygotic plants had been recovered from our transformation experiments and that all the plants were nucellar, maintaining the Carrizo citrange maternal genotype, with the exception of differences in transgenes integration loci.

Four phenotypic variants were detected among the transgenic population. Several morphological features made these plants distinguishable from the maternal phenotype: they developed thicker and broader leaves, with darker green color, and usually with broader petiole wings, and showed a slower growth. Ploidy tests of the whole plant collection demonstrated that all the plants in the population were diploid except these four plants, which were tetraploid. Transgenic tetraploids could have been originated either by a process of polyploidization during *in vitro* culture or more likely by the regeneration of transgenic shoots from tetraploid plant tissue, since more than 10% of the Carrizo citrange seedlings germinated *in vitro* are tetraploid.

Somaclonal variation may be common after exposition of explants to auxins, which is used as stimulus for cell de-differentiation, division, and consequently callus induction. This de-differentiated state makes cells more likely to integrate foreign DNA (Peña *et al.*, 2004b) but at the same time more predisposed to genomic alterations. Nevertheless, we did not find phenotypical differences between plants of type 1, obtained after transformation on a culture medium without auxins, and plants of type 2 that were obtained after transformation on a culture medium rich in auxins and thus after regeneration through a prominent callus phase.

The follow up of the population of transgenic plants by means of histological and fluorimetric GUS analyses confirmed the stable expression of the *uidA* transgene in all the plants over a period of 4–5 years. Patterns of expression were comparable for each line in successive histochemical analyses, although differences of 40% on measured values could be detected in fluorimetric assays, making the quantification of transgene activity over years very difficult. These fluctuations in time could be attributed to the developmental and physiological state of the plants. Almost one third of the population exhibited a typical pale blue staining pattern, recurrent in histochemical analyses performed during several years that correlated with very low values of expression measured by fluorescence analyses. These plants seemed to be undergoing a process of transgene silencing. A correlation between the pale blue color in leaf pieces and PTGS phenomena in citrus was corroborated in subsequent experiments (Dominguez *et al.*, 2002a, 2004). Any transgenic plant with complete silencing was not detected among the population, which may be reasonable since the recovery of transgenic plants was performed after a histochemical GUS assay of regenerating shoots, and only those showing a detectable GUS blue staining were shoot-tip grafted and allowed to progress into whole plants. This selection scheme was possibly delimiting the recovery of plants in which total silencing phenomena could be occurring.

The most frequent number of T-DNA inserts was one and two, but plants having possibly more than six *uidA* copies were also found. According to the data from Southern blot analyses, it is suggested that at least 35% of the analyzed plants

had integrated multiple T-DNA rearranged inserts at one locus. A significant tendency to low *uidA* expression levels was confirmed for transgenic citrus plants with more than two T-DNA copies, but levels of expression were highly variable for plants having only single copy. Our data agree in general with the results described by other authors, since although it has been frequently reported that single copies of a transgene are generally more stably expressed than multiple or rearranged insertions, a clear-cut correlation has not been found. Copy number, position effects, and organization of a given insert could account as a whole for the highly variable levels of expression displayed by the transgenic plants.

To investigate more deeply the actual incidence of transgene silencing in citrus, we decided to use a population of transgenic Mexican lime plants regenerated under *uidA/nptII* selection (type A), and a second one regenerated without marker selection but confirmed as transgenic by PCR (type B) (Dominguez *et al.*, 2002a). Irrespective of the recovery scheme used, most of the transformants (at least 59%) showed T-DNAs arranged as tandem repeats. The high frequency at which these complex structures were found, and their configuration as direct repeats (DRs) or inverted repeats (IRs), either on the right border (RB) or on the left border (LB), supports the hypothesis that the T-DNA is made double stranded prior to integration and that repeats are formed by extrachromosomal homologous recombination.

Regeneration of transgenic limes under nonselective conditions resulted in the production of plants with silenced transgenes at high frequency. Furthermore, silencing affected all the transgenes of the T-DNA in all silenced lines. When organization of T-DNA was analyzed in silenced and nonsilenced lines, it was observed that IRs were exclusive of silenced transformants, establishing a clear-cut correlation between both the phenomena. About 30% of the lines regenerated under nonselective conditions were silenced, whereas none of the lines recovered after *nptII* and *uidA* selection showed transgene silencing. These results indicated that under selective conditions only transgenic events expressing the marker genes could be detected, thus precluding the possibility of recovering transgenic plants with silenced transgenes along the whole T-DNAs. Moreover,

more than 5% of the regenerants obtained without marker selection were actually transgenic plants, opening the way for the production of citrus transformants without marker genes by PCR screening.

Gene silencing in citrus was further investigated by Domínguez *et al.* (2004). In this report, all Mexican lime regenerants obtained after kanamycin selection that resulted either GUS-positive or GUS-negative were analyzed by Southern blot. Interestingly, one-fourth of the GUS-negative plants were not escapes but transgenic plants showing *uidA* silencing, indicating that the transformation efficiency was underestimated when it was based in reporter marker gene expression.

Although genetic instability is usually a non-desirable trait in transgenic plants, Trainin *et al.* (2005) proposed the use of genetic transformation as a tool to induce mutagenesis in citrus by transposon tagging that could be used to identify mutants in genes of interest. Unless silenced, an intact transposon introduced into a plant will keep transposing and generating mutations for years converting transformed trees into mutation machines. With this purpose, Trainin *et al.* (2005) transformed Duncan grapefruit plants with a construct consisting of the *uidA* gene controlled by the 35S promoter but with the *Ac* transposable element cloned between the promoter and the marker gene, so GUS activity would result only if *Ac* excision occurred. However, this system had major drawbacks because transposition could not be controlled that caused in most cases tiny sectors if the transgenic plants, making detection of any mutant phenotype very difficult.

To investigate the effects of transgene incorporation and expression on tree growth and development, and fruit yield and quality, it is necessary to perform field trials. Unfortunately, there are only two field trials with citrus trees so far in which genetically modified plants are growing in the field over years under diverse environmental conditions. A release of transgenic citrus plants under controlled field conditions is being performed at the IVIA (Valencia, Spain) since 1997. The release site is located in an experimental field with an area of 1638 m². There are 130 trees, including 16 transgenic plants of Pineapple sweet orange, 16 transgenic plants of Mexican lime, and 16 transgenic plants of Carrizo citrange.

There are two plants from eight independent transgenic lines for each case. In all cases, the transgenes integrated were 35Spro::*uidA*::NOSter and NOSpro::*np1II*::NOSter, providing GUS expression and resistance to kanamycin, respectively. In addition, there are 8 nontransgenic control plants from each of the species and an external border of 58 nontransgenic trees of *Clemenules clementine* (Figure 11).

The purpose of the release is to investigate (1) morphological and phenological characteristics of the transgenic trees, (2) expression of the transgenes in different tissues and organs, (3) stability of the transgenes, (4) transmission of the transgenes to the progeny, and (5) possibility of transgene dispersal through the pollen to nontransgenic monoembryonic citrus trees (*Clemenules clementine*). The trial was approved by the Spanish Ministry of Environment (permit Nr. B/ES/96/15) and was in accordance with Article 9 of Directive 90/220/EEC of the European Union. This was the first release in the world of transgenic citrus plants to the field. Until now, transgenic plants are morphologically and phenologically normal, as nontransgenic controls, and transgenes are stably expressed over different seasons and in different plant tissues and organs. Tree phenology and fruit quality have been evaluated during three consecutive years for each tree. Phenological calendar was made by observation and description of phenological stages of development every 2 weeks. Fruit analysis is being made once a year when fruit is ripe and full colored. The parameters analyzed to determine fruit quality are weight, volume, caliber, color, acidity, Brix, juice weight, juice volume, % solid weight (juice weight/fruit weight) and maturity index (Pons and Peña, unpublished results).

A second field trial is being performed in Weslaco (Texas, USA) with Rio Red grapefruit transgenic plants (*see Grapefruit*). In addition, seeds from Mexican lime transgenic plants carrying a CTV-derived transgene encoding the major coat protein gene *p25* were sent to Hawaii in 2004 to carry out a field trial to test the potential resistance of these plants to CTV under natural challenge conditions, as part of a collaboration project of the IVIA with the University of Hawaii (M. Melzer, S. Ferreira, and J. Hu) and the USDA (D. Gonsalves).



Figure 11 Release of genetically modified citrus plants under controlled field conditions at the IVIA. (a) Schematic diagram of the trial. There are 130 trees, including 16 transgenic plants of Pineapple sweet orange (Pi), 16 transgenic plants of Mexican lime (MI), and 16 transgenic plants of Carrizo citrange (Cc) (2 plants from 8 independent transgenic lines numbered from 1 to 8; gray circles). In addition, there are 8 nontransgenic control plants from each genotype (black circles). There is also an external border of 58 nontransgenic trees of *Clemenules clementine* (CN; white circles). (b) Photograph taken 10 years after the field trial was established. It shows the Pineapple sweet orange row (left), the Carrizo citrange row (centre), and the Mexican lime row (right). Most proximal and surrounding trees correspond to the *Clemenules clementine* border

2.7 Specific Regulatory Measures Adopted

The European Union currently regulates the activities performed with genetically modified organisms (GMOs) through two basic Directives: Directive 98/81/CE, on the confined utilization of GM microorganisms, including transgenic plants, and Directive 2001/18/CE (repealing the above-mentioned Directive 90/220/EEC) on the deliberate release into the environment of GMOs. Several other regulations have been adopted later,

as those related to GMOs in human food and animal feed, other on the labeling and traceability of GMOs, or other on cross-border movement of GMOs.

Both these European Directives were incorporated to the Spanish legislation through the Law 9/2003 on the confined use, deliberate release, and commercialization of GMOs. Regulation 178/2004 approved and established the general framework for the development and execution of the Law 9/2003. In Spain, competencies on

confined utilization and deliberate release rely on Regional Governments, and commercialization entirely depends on the National Government. At the practical level, confined utilization of transgenic citrus plants, either in laboratories, phytotrons, or special greenhouses (those in which transgenic pollen release is prevented), is usually considered at the type 1 level (lowest risk in the scale of 1–4) by the competent authorities. If this is the case, it only requires a notification of the activities that are being carried out but does not need any specific authorization by the competent regulators. Regarding deliberate release of transgenic plants, much more detailed information is requested in any submitted notification, including data on the genetically modified plant (identity of the recipient, description of modified traits, type of genetic modification, etc.), information relating to the experimental release (purpose of the release, geographical location, size, etc.), potential environmental impact of the release, description of any measures taken by the notifier for the control of risks including isolation designed to limit dispersal, and planned field trials designed to gain new data on the environment and human health impact of the release (where appropriate). Notifications are publicly available. In Spain, there is a Biosafety National Committee, which is a technical board mostly composed of well-recognized independent experts that recommends to the competent National and Regional Authorities on approval or rejection.

It is important to point out that the Article 4 of the Directive 2001/18/CE says that “Member States and the Commission shall ensure that GMOs, which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment, with a view to identifying and phasing out antibiotic resistance markers in GMOs, which may have adverse effects on human health and the environment. This phasing out shall take place by the December 31, 2004 in the case of GMOs placed on the market and by December 31, 2008 in the case of GMOs authorized for deliberate release.”

In Valencia region, the legislation on confined utilization and deliberate release of GMOs was approved in 2006, meaning that during the precedent 8 years Leandro Peña’s group could

not submit any notification on these issues because there was no competent authority in our region. From now on, we are preparing five new notifications for deliberate release of transgenic citrus trees, which have been modified in disease resistance, tree performance, and fruit quality aspects.

3. FUTURE ROAD MAP

3.1 Expected Products

There are citrus areas seriously threatened by diseases caused by bacteria. It is the case of Huanglongbing, induced by the bacterium *Candidatus* L. asiaticum, which has impeded the development of citrus industries in Southeast Asian countries. It is also a limiting factor for increasing citrus productivity in China. The bacterium affects all citrus types making trees unproductive, and there are no means of efficient control other than almost permanent insecticidal treatments to fight against the psyllid vector. *Candidatus* L. asiaticum and the apparent variant *Candidatus* Liberibacter americanus were first detected in Sao Paulo state (Brazil) in 2004 and an eradication program was started 1 year later. In about 1 year, more than half million affected trees have been removed. *Candidatus* L. asiaticum was also found in Florida (USA) in 2005 and the disease is spreading from south to north without control. In this area, the situation is even worse due to the impossibility to eradicate citrus canker, caused by the bacterium *X. axonopodis* pv. *citri*, and affecting all economically important varieties cultivated in the region. Therefore, looking for resistance mainly against *Liberobacter* spp. but also against *X. axonopodis* pv. *citri* is a major priority for the most important citrus industries in the world. Probably, the only opportunity for getting durable resistance against these diseases could come from the incorporation of transgenes able to efficiently protect the most relevant variety and rootstock genotypes grown in these areas from the bacteria and/or their vectors.

There are other pests and diseases caused by other bacteria, viruses, fungi, nematodes, etc. that are not so serious but limit production and fruit quality in certain citrus areas depending on the pathogen. Among them, much effort has been

invested in attempting to incorporate transgenic resistance to CTV into citrus genotypes used as rootstocks and varieties. It can be predicted that this will continue as an important objective, and that strategies based on either PDR or plant-derived resistance will be further investigated.

Since markets of developed countries demand fresh fruit of increasing quality and with less agrochemical treatments, and also better and richer juice, more research will focus in understanding genetic control of metabolic pathways regulating carotenoid, flavonoid, limonoid, and monoterpene/essential oil biosynthesis with the aim of modifying fruit color and aroma, increasing vitamin content and reducing bitterness. In the same way, a better knowledge of citrus maturation and of determinants of sugar and acid content is important to attempt modulation of these traits in transgenic fruits. Seedlessness is another trait that has been successfully achieved in annual crops, and similar strategies could be used to afford this in citrus. Most strategies related to transgenic improvement in citrus fruits would require the use of tissue-specific promoters.

Considering that citrus trees are composed of scion and rootstock genotypes and the lack of current acceptance of transgenic foods by the consumer, it is logical to think that genetic modification of rootstock traits will be emphasized in the near future. In this sense, increasing tolerance to abiotic stresses (salinity, drought, etc.), to rootstock diseases (CTV, *Phytophthora* spp., nematodes, etc.), and adapting tree architecture to managing schemes requiring less labor and land, could become research areas of major interest.

In spite of the increasing number of laboratories working on development of genetic transformation systems for citrus and on the search for potentially useful transgenes derived from widely diverse source organisms, the lack of basic knowledge on citrus and citrus pathogens biology and the difficulties of working with genetically complex tree species, make very difficult to produce new improved transgenic genotypes of real agricultural importance.

3.2 Addressing Risks and Concerns

To our knowledge, the only transgenic citrus field trial in which environmental risk is being assessed

is that performed at the IVIA, and researchers are exclusively investigating the frequency of transgenic pollen dispersal. Since 2001, the number of transgenic seeds expressing the *uidA* gene has been yearly assessed in fruits produced by the nontransformed monoembryonic Clemenules clementine border trees surrounding the transgenic trees. Clementines are self-incompatible and parthenocarpic, meaning that they can produce fruits without seeds. Seeded fruit results only in case of cross-pollination. As border trees are very close to the transgenic trees (3–6 m), these are the most probable pollinators of clementine flowers. Even under this situation, the frequency of GUS-positive seeds is being less than 1% year by year (Pons and Peña, unpublished results).

In addition, there are characteristics of citrus biology and cultivation that should be strongly considered when environmental risks are assessed in transgenic field trials, and that depends on the citrus area of the world and the transgenic genotypes that are going to be evaluated. Commercial citrus varieties are propagated vegetatively by grafting of well-known genotypes onto well-known rootstocks. In our Mediterranean conditions and considering the citrus genotypes used, it is not possible that transgenic plants could become weeds. There are no wild citrus species and relatives in Europe, so there are no possibilities of compatible interactions between transgenic and wild plants. The situation would be completely different in Southeast Asia regarding this issue.

Citrus cultivars grown in some citrus areas are sexually compatible with many transgenic citrus genotypes that would be desirably produced. Under natural conditions, cross-pollination between transgenic lines and cultivated genotypes would be theoretically possible in many cases. Pollination in citrus is exclusively performed by insects, bees being the most successful pollinators. In areas where citrus is commercialized as fresh fruit, most varieties are sterile or self-incompatible, but the former are usually cross-compatible, leading to seed development. As presence of seeds in the fruit drastically reduces its price, affecting possible commercialization, cross-pollination in citrus is usually prevented by farmers by using different cultural practices and treatments. Most citrus species are parthenocarpic. In contrast, in areas in which fruit industry is based on juice production,

cross-pollination and presence of seeds is not a matter of concern. In any case, if cross-pollination occurs, transgenes will only be expressed in the seed, which is never consumed and is not used for propagation of varieties. In the incidental case that transgenic seedlings could germinate in an orchard, they could be removed by farmers as it is usually done with any citrus seedling germinating in any orchard in most citrus areas. Moreover, these seedlings would never flower before being removed because citrus seedlings are juvenile and thus, they need several years to start flowering.

With the aim to assess the potential risk of horizontal gene transfer between GM-citrus plant material and food-associated bacteria, Weiss *et al.* (2007) investigated the effect of conditions required for orange juice processing on the stability of DNA from transgenic Pineapple sweet orange. Results showed that genomic DNA from orange juice suffered degradation within 2 days of storage, indicating that current standard industrial procedures to pasteurize orange juice as well as its acidic nature caused a strong degradation of genomic DNA below sizes reported to be suitable for horizontal gene transfer.

3.3 Expected Technologies

The primary function of genetic transformation in citrus is the development of new improved varieties and rootstocks based on otherwise genotypes of excellent quality but deficient in one or a few characteristics. However, genetic transformation has been usually attempted and applied to a restricted list of economically important genotypes. In this sense, there are a few or no reports on transgenic satsuma and clementine mandarins, navel and Pera sweet oranges, Eureka and Lisbon lemons, Rangpur lime or sour orange. More importantly, most of the work on citrus transformation has been performed with juvenile material, which will need many years to start flowering and fruiting and several years more to fully lose the juvenile characteristics. To gain a real profit from genetic transformation as a tool for citrus improvement, development of efficient transformation systems for mature tissues becomes an obligate task. Currently, Leandro Peña's group works almost

exclusively in mature citrus transformation, but few laboratories are using this approach. It could be claimed that juvenile material is highly responsive and that it could be used at least to investigate gene function, but as the main target of citrus genetic improvement for the industry of most citrus-growing areas is usually the fruit, transformation of mature plants is essential even for functional genomics studies. We developed a general basic procedure for genetic transformation of mature Pineapple sweet orange and are adopting it to many other citrus genotypes of interest. The mature transformation system highly relies on the use of plant material in excellent sanitary and ontological state, which is cultivated under extremely clean and very well controlled environmental conditions. Even with this, contamination of the plant material with saprophytes during the *in vitro* culture phase is still a limitation of the procedure. Other than this, transformation efficiency is still low for many genotypes of interest and more effort should be put in improving it.

Most transgenic improvement projects directed to fruit genetic modification depend on the use of tissue/organ-specific promoters. Moreover, appropriate transgene expression levels at specific developmental stages and under various environmental induction conditions could be ideal for certain improvement strategies aimed to increase disease resistance or higher abiotic stress tolerance. Rapid and reliable systems to evaluate regulatory sequences are required, especially for fruit tissues. Transient transformation procedures through either *A. tumefaciens* vectors or particle gun systems could be good alternatives to stable transgenic expression for testing promoter elements. Transient transformation could also be useful to test heterologous transit peptides that would direct the transgenically expressed protein to a given subcellular target site.

RNA interference-inducing hairpin vectors, in which target sequences are expressed simultaneously in sense and antisense (Watson *et al.*, 2005) are excellent tools to knock down putative genes of unknown function and thus decipher their possible role in citrus growth and/or development. They could be used for both genetic improvement projects and delineation of basic gene functions. At the same time, new transformation vectors are being designed, as those minimizing transgene

expression fluctuations through the use of transgene flanking matrix attachment regions (MARs) (Allen *et al.*, 2005), those precluding integration of plasmid vector sequences of prokaryotic origin that flank transgene expression cassettes (Kuraya *et al.*, 2004), or those allowing integration of large DNA inserts containing multiple genes, such as BIBAC (Binary-BAC) and TAC (transformation-competent artificial chromosome) vectors (Hamilton *et al.*, 1996; Liu *et al.*, 1999), to facilitate positional cloning and functional analysis of linked genes and for engineering complex metabolic pathways.

A European Union Directive forbids specifically the presence in transgenic plants of transgenes conferring resistance to antibiotics used for medical or veterinary purposes, due to concerns related to possible horizontal transfer of these genes to gastrointestinal bacteria. Although this is not affecting directly to *nptII*, it has been proposed its substitution by using only reporter marker genes or using herbicide resistance genes as alternatives for transgenic selection. In any case, the presence of marker genes in GMO foods prevents their public acceptance. In plants propagated by seeds, it is possible to remove the marker gene once transgenic plants have been recovered, by means of co-transformation with different transformation vectors and segregation of marker genes from the gene of interest in the transgenic sexual progeny. This is not feasible in vegetatively propagated plants and trees that possess very long juvenile periods.

An alternative comes from the use of the *manA* gene that encodes PMI (Haldrup *et al.*, 1998). When mannose is added to the plant tissue culture medium, it is transformed to mannose-6-phosphate, which cannot be metabolized by plant cells. Transgenic cells expressing *manA* could be able to transform mannose-6-phosphate into fructose-6-phosphate, which would be used by plant cells as carbon source. Therefore, transgenic cells accumulating PMI could have a clear advantage (positive selection) over nontransformed cells, when explants are exposed to a tissue culture medium containing mannose and deprived of any other carbon source. This system has already been successfully used for juvenile sweet orange and Carrizo citrange transformation (Boscariol *et al.*, 2003; Ballester *et al.*, 2008).

Another attractive possibility is offered by the multiautotransformation (MAT) vector system that combines positive selection, using the *ipt* gene, with a site-specific recombination and DNA removal system that generates marker-free plants (Ebinuma *et al.*, 1997; Sugita *et al.*, 1999). The *ipt* gene is located in the Ti plasmid of *A. tumefaciens* and encodes the enzyme *ipt*, which catalyzes the production of a precursor of several cytokinins (Ebinuma *et al.*, 1997). Cytokinins are widely used to stimulate organogenesis in many cultured plant tissues, including citrus. The second interesting element of the MAT vector is the site-specific recombination system *R/RS* from *Zygosaccharomyces rouxii* (Sugita *et al.*, 1999), in which the recombinase *R* removes the DNA fragment placed between two recognition *RS* sites from the transgenic cells after transformation. In this vector, *RS* sites flank both the *ipt* marker and the *R* recombinase transgenes. After site-specific recombination and excision of the DNA fragment between *RS* sites, *ipt* marker-free transgenic plants may be obtained. Ballester *et al.* (2007) have successfully used this system in sweet orange and Carrizo citrange and are currently further exploring their possibilities to generate marker-free transgenic citrus plants that carry only the transgene of interest (Ballester *et al.*, 2008).

A third possibility is regeneration without selection and screening of all regenerants through PCR analysis. Using this system in Mexican lime, more than 5% of the regenerants resulted to be transgenic (Dominguez *et al.*, 2002a, 2004). We are further exploring this procedure with other citrus genotypes.

Transgene stacking could have an outstanding interest in citrus genetic improvement programs because it would allow incorporating a second transgene in an already improved transgenic line. More transgenes could be pyramided into the transgenic line showing the desired phenotype for the first and second transgenes and so on. The main advantage of this approach is that it is possible to check individual expression and phenotype conferred by each transgene before proceeding to the next transformation round. However, because of the long juvenility of citrus, the process can take extremely long time if more than 2 or 3 transgenes have to be introduced. Alternatively, we have used *API*-transgenic plants for

evaluating the possibilities of transgene stacking in citrus. *API* overexpression extremely shortens the juvenile period in citrus (Peña *et al.*, 2001). *API*-Carrizo citrange nucellar seedlings were successfully retransformed with a *gfp* vector and GFP-positive transgenic plants flowered and set fruits within 1 year after sowing, as *API*-Carrizo citrange controls (Cervera *et al.*, 2006; Cervera *et al.*, unpublished results). This could be then a valuable material and strategy to attempt functional genomics studies related to aspects of fruit and flower development by using reverse genetics. Furthermore, it can be used as a tool to rapidly evaluate putative fruit-specific promoter elements by stable transgenic expression. In addition, it could be an excellent tool for genetic improvement because transgene expression and phenotype evaluation related to modification of fruit characteristics could be readily achieved in less than a couple of years. However, for this purpose the *API* strategy should be combined with a gene removal system, as the R recombinase/RS target sequence mentioned above or other similar recombinase system, to eliminate the *API* transgene once it has fulfilled its early fruiting promotive function.

Recombinase systems represent a very interesting option for many different purposes in citrus transformation, because they could allow removal of marker genes and removal of transgenes of interest once their presence is not needed anymore in the plant (Dale and Ow, 1991). Moreover, they have been used to site-specific insertion of transgenes at specific loci by homologous recombination (Srivastava and Ow, 2002). However, this system and others proposing homologous recombination in the nuclear genome have still important limitations, related for instance to unexpected chromosomal rearrangements, unintended recombination, and generally very low efficiency (Gilbertson, 2003). Alternatively, chloroplast transformation through homologous recombination represents a feasible technology not only to get stable and uniform transgene expression, but also to achieve very high transgene product accumulation since it permits the introduction of thousands of copies of foreign genes per plant cell. Furthermore, transgene dispersal through pollen is avoided since the chloroplast is maternally inherited in most plants. The main obstacle of this approach is that

efficient chloroplast transformation is restricted to very few plant species (Daniell *et al.*, 2005).

3.4 Intellectual Property Rights (IPR), Public Perceptions, Industrial Perspectives, Political, and Economic Consequences

Genetic improvement through transformation requires active research in many fronts, including plant biology and physiology, phytopathology, biochemistry, molecular biology, genetics, and genomics, plus a good knowledge of the crop and its problems and the support of the industry to be able to develop new genotypes of real importance.

Multinational companies are able to cover all these aspects and concentrate in a few crops and improvement in a few traits to develop new products so important as for instance insect-resistant and glyphosate-tolerant corn, cotton, and soybean varieties. Their research lines are not only planned to decipher biological problems and publish their results in scientific journals but mainly to discover new patentable tools for genetic improvement of genotypes that will become also patentable or registrable and consequently will be part of their property. Many strategies for transgenic plant improvement are based on genes or sequences and regulatory sequences as well as on procedures for transformation or for marker elimination already patented by big companies. In contrast, most of the research on citrus transformation is being performed by small laboratories from academic or agricultural institutions, depending basically on Ph.D. students, with low-funded 3-year projects, without any patent policy, with very low support from the local citrus industry, and requiring very expensive facilities. In this context, it is easy to speculate that any future important development on citrus transgenic improvement will come from a company interested in this technology or from a committed public institution involved in citrus research and with big support from the industry, able to support the elevated costs of maintaining international patents and of mandatory biosafety tests required by the current legislation of most countries.

However, it is difficult for private companies to invest in transgenic improvement of citrus

now, because public perception in developed countries (especially in Europe and Japan) about GM-food is in general very negative and there are no signs of change of this situation in the coming future. It could be proposed to work on transgenic rootstock improvement, until scion improvement is accepted by the consumer. In any case, it remains to be seen whether fruit from a tree composed of a transgenic rootstock and a nontransformed variety is considered transgenic or not by the legislators and, more important, by the consumers. It is sad to see that more than 40 years after the first use of genetic engineering and 20 years after the generation of the first transgenic crop plants, the general public is unable to understand the benefits of the technology, and prefers to believe in catastrophic and unreasonable opinions from people with clearly defined political interests than in commitments and integrity of scientists working in public institutions. It is a matter of time that people will realize that genetic transformation is an excellent technology for crop improvement, in many senses much better and safer than other commonly used strategies. It is hard to predict nowadays whether public will need years or decades to accept these GM foods. In the meantime, genetic transformation is probably the most efficient approach to make reverse genetics in citrus to investigate gene function and thus to gain better understanding in metabolic processes and plant–pathogen–environment interactions. This will be essential for the future of citrus genetic improvement. In addition, the transgenic technology will be necessarily improved and most of the current concerns and risks on pollen dispersal, presence of marker genes, unintended transgene silencing, etc., will likely be overcome by new scientific and technological developments.

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Grapefruit

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1. INTRODUCTION

1.1 History, Origin, Distribution, and Uses

The genus *Citrus* includes some of the most important fruit varieties in tropical and subtropical regions, and grapefruit, *Citrus paradisi* Macf., is one of the most important commercial groups of citrus cultivars. The original grapefruit, first discovered in Barbados, is believed to be derived from a backcross of sweet orange, *Citrus sinensis* (L.) Osbeck, and pummelo, *Citrus grandis* (Gmitter, 1995; Nicolosi *et al.*, 2000). However, the most current commercial grapefruit cultivars have originated from nonconventional breeding methods such as bud sport selections or induced mutagenesis (Hodgson, 1967; Gmitter, 1995), with recent emphasis on pigmented cultivars. The United States is the world leader in grapefruit production, producing approximately 2 million tons annually (about 40% of the world total). Other countries with significant grapefruit production include South Africa, Israel, Mexico, and Cuba. Grapefruit production and consumption have been declining in the United States, but production is increasing in South Africa (Economic Research Service, USDA, 2005). Florida accounts for about 75% of the US grapefruit production, but significant quantities are also produced in

Texas, California, and Arizona. Most grapefruit is targeted for the fresh market, but there is also a significant processing industry that primarily utilizes fruit not making fresh market grade. Grapefruit essential oil also has value in the flavor and fragrance industries.

1.2 Threats to Grapefruit Production

Like other citrus species, grapefruit cultivars are genetically homogenous, which provides high yields of uniform high quality fruit, characteristics that are appreciated by both consumers and farmers alike. However, this same genetic uniformity makes grapefruit more susceptible to emerging pests and diseases, resulting in economically significant reductions in fruit quality and production. The main pathogens affecting grapefruit worldwide include *Citrus tristeza virus* (CTV) (Bar-Joseph *et al.*, 1989; Gmitter, 1995), psorosis (Rostacher, 1993), and citrus bacterial canker (*Xanthomonas axanopodis* pv. *citri*) (Xac) (Yang *et al.*, 2000; Vilorio *et al.*, 2004). Citrus canker has been declared endemic in Florida and threatens the existence of the world's largest grapefruit industry there. Another emerging disease posing a severe threat to citrus production (also now endemic in Florida) is citrus greening

disease or huanglongbing (HLB) caused by *Candidatus Liberibacter* spp. This bacterial disease is caused by a phloem-restricted pathogen that is vectored by the insect *Diaphorina citri* Kuwayama, a citrus psyllid (Texeira *et al.*, 2005). Along with developing resistance to these diseases, control of insect pests vectoring the pathogens is also contemplated in the general strategy for genetically improving citrus cultivars. Improvement of current grapefruit cultivars is a necessary demand not only for pest and disease resistance but also for fruit quality and availability as necessary to sustain and grow markets.

1.3 Grapefruit Variety Improvement

Grapefruit improvement via conventional breeding procedures has been hampered by large tree size, nucellar polyembryony, seedlessness, high levels of heterozygosity, long juvenility, and the relatively large land area and high costs necessary to grow and evaluate segregating populations (Grosser and Gmitter, 1990; Moore *et al.*, 2005). Popular red-fleshed low-seeded cultivars including “Star Ruby”, “Flame”, and “Rio Red” are the result of mutation breeding (irradiation) (Gmitter, 1995). Biotechnological methods other than classical breeding protocols being utilized to improve citrus species, including grapefruit and grapefruitlike hybrids, include interploid hybridization that can involve embryo rescue for triploid plant recovery and somatic hybridization for parent development (Grosser *et al.*, 2000; Grosser and Gmitter, 2005) and genetic transformation as featured in this chapter (Kobayashi and Uchimiya, 1989; Moore *et al.*, 1992, 2005).

Genetic transformation has become the most attractive alternative method for improving existing grapefruit cultivars (and other citrus), as it allows the introduction of one or two specific traits while maintaining cultivar integrity (Gmitter, 1994; Bond and Roose, 1998). The first reports of citrus transformation began to appear more than a decade ago, utilizing protoplasts (Kobayashi and Uchimiya, 1989; Vardi *et al.*, 1990; Hidaka and Omura, 1993), and *Agrobacterium*-mediated transformation of citrus cells (Hidaka *et al.*, 1990) and stem segments (Moore *et al.*, 1992), and finally particle bombardment of citrus cells (Kayim *et al.*,

1996; Yao *et al.*, 1996) with low transformation efficiency. After Moore *et al.* (1992) reported the successful use of nucellar seedling stem explants for *Agrobacterium* inoculation, attempts to optimize *Agrobacterium*-mediated transformation of stem and epicotyl segments in several citrus species resulted in continuously increasing transformation efficiency (Kaneyoshi *et al.*, 1994; Peña *et al.*, 1995, 1997; Gutiérrez *et al.*, 1997; Bond and Roose, 1998; Cervera *et al.*, 1998; Luth and Moore, 1999; Domínguez *et al.*, 2000; Yang *et al.*, 2000; Costa *et al.*, 2002; Yu *et al.*, 2002; Almeida *et al.*, 2003; Kayim *et al.*, 2004; Kayim and Koc, 2005). Of these, Luth and Moore (1999) reported the first successful grapefruit transformation using epicotyl segments. A CTV untranslatable coat protein gene (*uncp*) and the *Galanthus nivalis* agglutinin gene (*Gna*), a plant-derived insecticidal gene, were the first agronomic traits to be successfully transferred into a commercially important grapefruit variety, “Rio Red”, with the aim of improving resistance to CTV and to the aphids that transmit the virus (Yang *et al.*, 2000). Since this time, hundreds of transgenic grapefruit plants have been generated by several research programs employing the stated protocol, utilizing several grapefruit cultivars and numerous target genes.

For the selection of transgenic tissue and buds, antibiotic resistance genes, most frequently neomycin phosphotransferase (*nptII*) as a selectable marker and the intron-inserted β -glucuronidase (UIDA) reporter gene as a scorable marker, have been used with *Agrobacterium* methodology. In *C. paradisi*, some of the commercially important grapefruit cultivars are seedless (zero to five seeds per fruit), including “White Marsh”, “Ruby Red”, “Rio Red”, and “Star Ruby” as well as some other commercial sweet oranges, which make it more difficult to obtain adequate nucellar seedling explants for *Agrobacterium*-mediated transformation (Fleming *et al.*, 2000). The *Agrobacterium*-mediated transformation system is best suited for highly nucellar seedy citrus genotypes, but also works for low-seeded nucellar cultivars if abundant fruit is available, including the mentioned low-seeded grapefruit cultivars (Bond and Roose, 1998; Gonzalez-Ramos *et al.*, 2005; Ananthakrishnan *et al.*, 2007). This limitation in citrus transformation can also be overcome for some cultivars

by using a protoplast transformation system with a nondestructive selection system (Niedz *et al.*, 1995; Fleming *et al.*, 2000). Currently, protoplast transformation systems with green fluorescent protein (GFP) reporter gene have been used by Grosser and his coworkers in a few different citrus species (Fleming *et al.*, 2000; Olivares-Fuster *et al.*, 2003; Albiach-Marti *et al.*, 2004; Guo *et al.*, 2005). The protoplast transformation methodology provides some advantage over other methods by eliminating antibiotic selection and the use of antibiotics, which is now considered by some consumer groups to be a negative trait, and may preclude marketing in some markets, particularly in Europe. This protocol may also be useful for cultivars not susceptible to *Agrobacterium* infection or that exhibit inefficient plant regeneration via adventitious shoot–bud induction from stem or epicotyl segments after antibiotic application (Fleming *et al.*, 2000). Although grapefruit regenerates from embryogenic cultures, successful protoplast transformation has not yet been demonstrated.

2. PROTOCOLS FOR PRODUCING TRANSGENIC GRAPEFRUITS

All examples of transgenic grapefruit in the literature were by standard *Agrobacterium*-mediated transformation of nucellar seedling stem pieces similar to the following protocols.

2.1 Plant Material

Seeds are obtained by extraction from harvested fruit (or from commercial supplier), the outer seed coat peeled, and surface sterilized for 15 minutes in a 20% solution of commercial bleach. These seeds are then rinsed with sterile water three times (5–10 min each rinse). Following the rinsing, two seeds are placed per glass tube containing seed germination medium. The tubes are sealed with Nescofilm and incubated in the dark at room temperature ($25 \pm 4^\circ\text{C}$) for 5 weeks. During this period, seedlings will germinate from the seeds and grow to be about 10–12 cm long. Stems of etiolated plants are cut into 15–20 mm segments, placed into liquid co-cultivation medium (CCM), and subsequently into *Agrobacterium* suspension.

Alternatively, stems can also be obtained from germinated *in vitro*-grown somatic embryos (Luth and Moore, 1999).

2.2 Transformation and Regeneration

The explants are incubated in *Agrobacterium* suspension of appropriate optical density (OD) for anywhere from 2 to 15 min, blot dried, and put on plates with agar-solidified CCM. Plates are sealed and placed in the incubator for 2 days ($25 \pm 4^\circ\text{C}$). After this period, explants are transferred from plates with CCM to either regeneration medium (RM) or DBA3 regeneration media supplemented with 333 mg l^{-1} Cefotaxime (and/or carbencillin) to kill *Agrobacterium*, and appropriate antibiotic for selection (usually $50\text{--}70 \text{ mg l}^{-1}$ kanamycin), and left in the incubator for about 5 weeks. Recently, the transformation efficiency of “Rio Red” grapefruit was improved by including a shoot elongation phase with a liquid medium overlay, which eliminated problematic nontransgenic escape shoots (Yang *et al.*, 2000). Thirty-five days after co-incubation with *Agrobacterium*, shoots are harvested and tested for the presence of the reporter gene. CCM contains $1\times$ Murashige and Skoog salts and vitamins with 30 g l^{-1} of sucrose plus 3 mg l^{-1} of 6-benzyl adenine (BA), 0.1 mg l^{-1} of α -naphthalene acetic acid (NAA) (0.1 ml of NAA stock solution), 0.5 mg l^{-1} of 2,4-D, and 19.6 mg l^{-1} of acetosyringone. RM includes $1\times$ Murashige and Skoog salts and vitamins with 30 g l^{-1} of sucrose plus 3 mg l^{-1} of BA, 0.5 mg l^{-1} of NAA, plus appropriate antibiotics (i.e., 333 mg l^{-1} of Cefotaxime). DBA3 medium consists of $1\times$ Murashige and Tucker salts and vitamins with 25 g l^{-1} of sucrose plus 3 mg l^{-1} of BA, 0.01 mg l^{-1} 2,4-dichlorophenoxy-acetic acid, 1.5 g l^{-1} malt extract, and 20 ml l^{-1} coconut water (then add appropriate antibiotics) (Deng *et al.*, 1992). Alternatively, though with lower efficiency, transformation can be accomplished with selection on media containing hygromycin (Costa *et al.*, 2002).

2.3 *Agrobacterium* Culture

Most frequently used strains of *Agrobacterium tumefaciens* for transformation of grapefruit are EHA101, EHA105, and C58C1. *Agrobacterium*

cultures are grown in yeast extract peptone (YEP) medium and suspensions used for incubation of explants set to an optical density OD_{620} of 0.5–1. These strains can also be independently transformed by electroporation with the different pBin35GUS constructs along with the helper plasmid pCH32 containing additional copies of the *virG* gene for enhancing transformation efficiency (Ghorbel *et al.*, 2001). *Agrobacterium* has a natural transformation system that produces mostly single insertions in the citrus plant genome, therefore, seemingly avoiding gene silencing defense mechanisms in the targeted plants. EHA105 was shown to be twice as efficient as C58C1 in transformation of “Ruby Red” grapefruit (and “Hamlin” sweet orange) by Eric Mirkov and coworkers (Eric Mirkov, pers. comm.). This specificity for transformation demonstrated that, among so many variables involved in transformation experiments, the bacterial genotype is a variable that can be optimized.

2.4 Tests for Reporter Genes

Most frequently used reporter genes are the gene for β -glucuronidase (GUS) and GFP. GUS tests are performed in 96 well microassay plates. A complete, thin cross section is cut from the bottom of the shoot and dipped in 50 μ l of GUS assay solution that was aliquoted into individual wells. The plate sealed with Nescofilm is left at 37 °C for at least 4 h. Addition of 50 μ l of fixing solution to the tested sample results in bleaching of the tissue making it easier to score the shoots exhibiting blue staining. An example of a GUS-stained transgenic grapefruit shoot is provided in Figure 1(a). Examples of typical transformation efficiencies for various grapefruit cultivars are presented in Table 1.

To test for the presence of *gfp* as a reporter gene for transformation, shoots are observed under the “fluorescent” microscope, such as a Zeiss SV11 epifluorescence stereomicroscope equipped with a 100 W mercury bulb light source and a FITC/GFP filter set with a 480/30 nm excitation filter and a 515-mm-long pass emission filter (Guo *et al.*, 2005). As a result of the presence of GFP in transgenic tissue, shoots appear completely chartreuse green (Figure 1b), light pink, or red with green patches of different size. Under the same

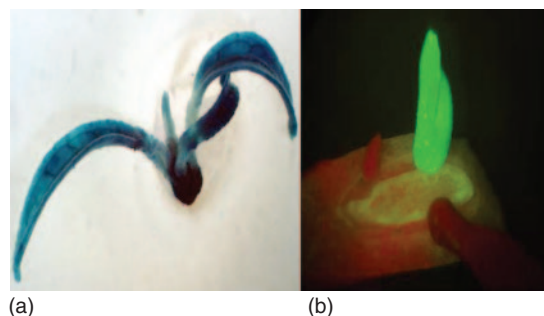


Figure 1 Reporter gene expression in transgenic grapefruit: (a) Histochemical assay of GUS constitutively expressed in a transgenic apical portion of a citrus shoot after infiltration with X-Gluc substrate. (b) Transgenic (chartreuse green) adventitious shoot expressing GFP and nontransgenic escape adventitious shoot growing from stem explant

conditions, wild-type shoots (escapes) appear red due to chlorophyll fluorescence.

2.5 Growth of Transgenic Seedlings

Once the shoots have tested “positive” for the presence of reporter gene they can be elongated on BG elongation medium without kanamycin (Yang *et al.*, 2000) (optional) and then either placed on root-induction medium or used in micrografting onto rootstocks (usually sour orange, rough lemon, or Carrizo). Some of the shoots do not respond to root-induction media that typically have a high concentration of auxins (1–3 mg l⁻¹ NAA). To overcome this problem, transgenic shoots can be micrografted on rootstock seedlings *in vitro* (Peña *et al.*, 1995), with Carrizo being a good choice as transgenic shoots can easily be differentiated from adventitious Carrizo shoots exhibiting trifoliate leaves. Rooted seedlings and micrografted plants are grown for an additional 2–4 weeks under conditions of high humidity and then acclimated to a greenhouse environment.

2.6 Molecular Analyses

2.6.1 Southern blot analysis

Plant genomics is rapidly moving toward a more integrationist, rather than reductionist perspective, thereby increasing emphasis on gene identification

Table 1 Average transformation efficiencies from various grapefruit (+) *Agrobacterium*-mediated transformation experiments, calculated as the total number of GUS positive regenerated shoots/total number of inoculated explants. The percentage of GUS (+) shoots is calculated as the number of shoots staining blue divided by the number of shoots tested

(A) Comparison of two *A. tumefaciens* strains in two cultivars (transformation efficiency percentage)

At strains	Number of experiments	“Ruby Red” (mean \pm s)	“Hamlin” (mean \pm s)
C58C1	4	2.6 \pm 1.1	1.4 \pm 0.4
EHA105	2	5.4 \pm 2.7	3.7 \pm 0.6

(B) Average citrus cultivar susceptibility to transformation by *A. tumefaciens*

Cultivar	Number of experiments	Transformation efficiency (%) (mean \pm s)
Ruby Red	6	3.5 \pm 2.1
Rio Red	3	1.8 \pm 0.9
Hamlin	5	2.8 \pm 1.3

(C) Transformation with CTV-392 sequence (EHA105 + pTLAB14)

Cultivar	Number of explants	Number of shoots	Percentage of GUS (+) shoots	Transformation efficiency (%)	Whole plants recovered
Flame	790	814	13.33	0.49	4
Marsh	4220	3470	12.62	1.35	47
Ruby Red	1845	661	18.18	3.78	25
Duncan	1838	1722	10.67	0.64	11
All	8693	6667	13.08	1.30	87

(D) Transformation with CTV p23 (hairpin) sequence (EHA101 + pTLAB10) for different cultivars of grapefruit

Cultivar	Number of explants	Number of shoots	Percentage of GUS (+) shoots	Transformation efficiency (%)	Whole plants recovered
Flame	2647	787	28.09	2.67	21
Marsh	2166	446	25.43	3.36	15
Ruby Red	675	69	38.89	11.59	8
Duncan	2186	928	28.92	3.66	34
All	7674	2230	28.31	3.50	78

and functional expression of novel proteins. To achieve this goal, genomic DNA from plants “positive” for the presence of the reporter gene (selected as an independent transgenic event) can be extracted, and 15 μ g of DNA is then digested with the appropriate restriction enzyme. A DNA-labeled probe can then be used in order to confirm the number of stable insertions of the transferred T-DNA fragment likely containing the gene of interest (see Figure 2 for an example). The number of insertions in this Southern blot ranged from one to about eight insertions; however,

more than 70% were single-insertion events. This has been a consistent trend in most of the independent *Agrobacterium*-mediated grapefruit transformations studied to date.

2.6.2 Northern blot analysis

To determine the accurate transcription of the genes, total RNA can be extracted from young leaves of selected transgenic plants. Ten micrograms of total single-stranded RNA are then

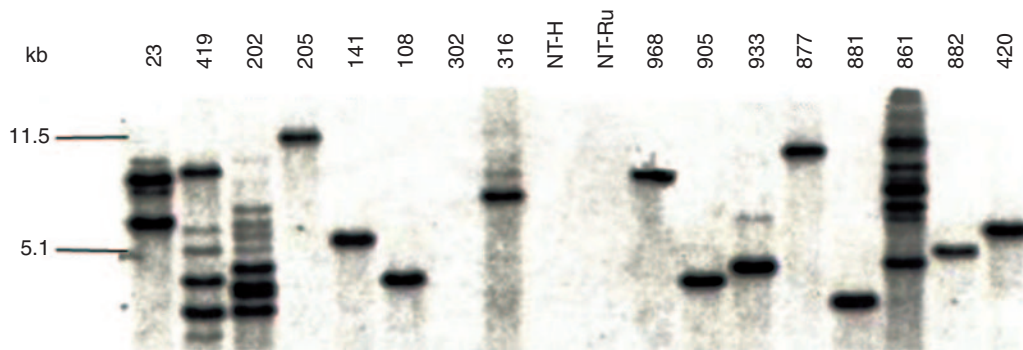


Figure 2 Southern blot hybridization of genomic DNA extracted from young leaves of two commercial citrus cultivars transformed with two antimicrobial genes. Genomic DNA was digested with *Hind*III and hybridized fragments showed the insertion number. From left to right: “Hamlin” sweet orange transformed with the lysozyme and the *SoD2* genes, lanes 1–4 and 5–8, respectively. Lanes 9 and 10 are nontransgenic “Hamlin” sweet orange and the “Ruby Red” grapefruit, respectively. Lanes 11–13 is the grapefruit cultivar “Ruby Red” transformed with the lysozyme gene. Lanes 14–18 is “Ruby Red” transformed with the spinach defensin gene

electrophoresed in formaldehyde gels and blotted onto nylon membranes (Amersham-GE Healthcare). The membranes can be hybridized with the complete DNA sequence of the transgenes of interest. The labeled probes are generally derived from the total DNA length of the structural genes of interest (see Figure 3 for an example).

3. DEVELOPMENT OF TRANSGENIC GRAPEFRUIT PLANTS

Due to its *in vitro* vigor and high potential for organogenesis (adventitious shoot production), grapefruit is probably the easiest commercially important citrus scion to transform using *Agrobacterium* and has, therefore, been used in many studies. Most of the early works was done with the seedy “Duncan” cultivar for convenience, but

now more research is shifting to other cultivars including “White Marsh”, “Rio Red”, “Ruby Red”, and “Flame”. One can expect to see many more reports on grapefruit transformation in the next few years, especially with antimicrobial target genes, as researchers try to develop solutions to citrus canker and HLB, true threats to the grapefruit and sweet orange industries worldwide. Genetic transformation in citrus, especially with grapefruit, has progressed tremendously during the past few years. Numerous transgenic plants can be produced within a year using genes coding for important agronomic traits or traits that will facilitate identification of gene function or characterization of a corresponding protein. In the future, grapefruit could be a model citrus scion for transformation with new genes discovered by citrus genome mapping projects and for functional genomics studies in citrus. Below are some



Figure 3 Northern blot of total RNA extracted from transgenic citrus leaves and nontransgenic controls. The hybridized transcripts of “Hamlin” sweet orange transformed with the spinach defensin and lysozyme genes are shown in lanes 1–3 and 11–15, respectively. While the transcripts of the spinach defensin gene in transformed “Ruby Red” grapefruit are shown in lanes 6–10. The nontransgenic (NT) controls of “Hamlin” and “Ruby Red” are shown in lanes 4 and 5, respectively

examples of target genes successfully transformed into grapefruit to date.

3.1 Virus-derived Genes for CTV Resistance

A tremendous effort by several laboratories has been focused on the development of transgenic CTV resistance in grapefruit, using candidate sequences derived directly from various CTV isolates. Coat protein- or RNA-mediated resistance has been the target of most of these studies. Although hundreds of transgenic grapefruit plants have been produced from several cultivars, only a few “Duncan” and “Rio Red” transgenic plants are showing potential for CTV resistance based on CTV graft challenges and subsequent enzyme-linked immunosorbent assay (ELISA) analysis (G.A. Moore and T.E. Mirkov, unpublished data). A few transgenic “Marsh” grapefruit plants containing the 392-CTV sequence (containing part of p23 and the untranslated region (UTR) of CTV genome) showed altered replication in protoplasts with CTV virions based on Northern blot analysis (Ananthakrishnan *et al.*, 2007), but it has yet to be determined if this result has any value at the whole-plant level. Information on transgenic grapefruit plants and the CTV-derived target genes is summarized in Table 2.

3.2 Trifoliolate Orange Derived CTV Resistance

Most citrus species and cultivars are susceptible to CTV infection. However, the trifoliolate orange (*Poncirus trifoliata* L. Raf.), a sexually compatible relative of *Citrus*, has a single gene, named *Ctv*, which confers virtual immunity to CTV (Yoshida, 1993; Gmitter *et al.*, 1996). This gene can prevent viral proliferation *in planta* by an undetermined mechanism (phenotypically, ELISA results are negative), essentially resulting in host immunity. Since the development of the first localized linkage map of the *Ctv* locus (Gmitter *et al.*, 1996), substantial efforts have been devoted to clone it for use in transformation experiments aimed at developing CTV-resistant cultivars (Deng *et al.*, 2000, 2001; Yang *et al.*, 2000). The susceptible allelic region, *Ctv*, has also been sequenced and analyzed by Fred Gmitter’s group (unpublished data) and compared with the resistance allelic region. Analysis of the full-length genetic sequence of *Ctv* has revealed seven disease resistance genes belonging to the CC-NBS-LRR class, of which two contain a frameshift or internal stop codon. When compared with the susceptible allelic sequence, three of the remaining five genes have nearly identical sequences, but two others are clearly distinct from their susceptible allelic sequences (F. Gmitter, unpublished data). These two genes

Table 2 Transgenic grapefruit plants with candidate CTV-derived sequences for potential virus resistance (*gus* reporter gene used in all experiments)

Sequence/construct	Cultivar	Reference
Coat protein gene from T36 (quick-decline isolate) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
Nontranslatable coat protein gene (T36) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
Coat protein gene from T30 (mild isolate) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
Coat protein gene from B249 (stem-pitting isolate) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
RNA-dependent RNA polymerase (T36) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
p20 protein gene (T36) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
p27 minor coat protein gene (T36) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
3' end (sense orientation) of DPI 3800 (isolate) (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
3' end (sense orientation) of DPI 3800 (FMV promoter and 35S terminator)	Duncan	Febres <i>et al.</i> , 2003
3' end (antisense orientation) of DPI 3800 (35S promoter and terminator)	Duncan	Febres <i>et al.</i> , 2003
p23 (hairpin construct) (FMV promoter and Rubisco terminator)	Duncan, White Marsh, Flame, Ruby Red	Ananthakrishnan <i>et al.</i> , 2007
392 bp sequence from 3' end of p23 ORF (FMV promoter and nopaline synthase term)	Duncan, White Marsh, Flame, Ruby Red	Ananthakrishnan <i>et al.</i> , 2007

are adjacent within 10kb and in opposite orientation. These two sequences have been their primary focus, as they were considered the most likely candidate CTV resistance gene(s) and have been designated as *Ctv.R5* and *Ctv.R6*. The ORF full length of *Ctv.R5* is 2700 bp, and *Ctv.R6* is 2670 bp.

3.2.1 Designing transgenes

Two binary vectors have been used for plasmid construction. The first binary Ti vector was derived from the assembly of pCAMBIA2301 with the *nptII* gene (under the 35S CaMV promoter and terminator) as the selectable marker and the 35S-*gus* gene (under the 35S CaMV promoter and nos terminator) as the reporter gene. *Ctv.R5* and *Ctv.R6* were inserted into the *Bam*HI site. The second binary Ti vector was derived from the assembly of pGreen0029 (provided by R.P. Hellens and P.M. Mullineaux) with the *nptII* gene (under the nos promoter and terminator) as the selectable marker (Hellens *et al.*, 2000) and the 35S-*gfp* gene (under the 35S CaMV promoter and terminator) as the reporter gene (Chen *et al.*, 2007). *Ctv.R5* and *Ctv.R6* were inserted in the *Spe*I site. These binary vectors were introduced into competent *Agrobacterium* AGL1 cells by electroporation.

3.2.2 Transformation and testing of regenerated plants

“Duncan” grapefruit was transformed as previously described using the *Agrobacterium* AGL1 strain harboring the binary vectors with *gus* as the reporter gene. Transgenic shoots regenerated from transformed epicotyl explants on selective medium were micrografted onto “Carrizo” seedlings *in vitro* (Peña *et al.*, 1995). After 6–8 weeks, these putative transgenic grafted plants were transferred to soil in liners. The leaves of the putative transgenic grafted plants were used for histochemical GUS assays or the *gfp* expression was observed with a fluorescent microscope Zeiss SV6. DNA was extracted from the putative transgenic grafted plants. Polymerase chain reaction (PCR) analysis was carried out using primers for *nptII*, *gus*, *gfp*, *Ctv.R5*, and *Ctv.R6* to verify the putative transgenic grafted plants. The transformation efficiency for pCAMBIA2301 containing *Ctv.R5*

and *Ctv.R6* genes was 1.88% and 1.54%, respectively. pGreen0029 containing *Ctv.R5* and *Ctv.R6* genes showed a transformation efficiency of 2.15% and 1.90%, respectively.

These transgenic plants have been grafted onto CTV-infected rootstocks to test the CTV replication by ELISA. However, the preliminary results have not been clear in confirming with certainty that either candidate alone is capable of inducing resistance to CTV. (Note: Similar results have been obtained via a collaborative effort by the groups of Eric Mirkov (Texas A&M) and Mikeal L. Roose (University of California, Riverside).

Hence, the possibility that both genes may be required for resistance is being explored. Studies have been carried out using two T-DNAs harbored in the same *Agrobacterium* strain either on different replicons (Daley *et al.*, 1998) or on the same replicon (Komari *et al.*, 1996) and used for co-transformation. Recently, a 1:1 mixture of AGL1 carrying the two binary vectors (one with *gus* as the reporter gene and the other with *gfp* as the reporter gene) separately was used to co-transform the epicotyl explants (Soneji *et al.*, 2007). This approach has resulted in some successful co-transformants, but they are still too small for graft inoculation challenges.

3.3 Virus-derived Genes for *Citrus* Psorosis Virus Resistance

In efforts to develop *Citrus* psorosis virus resistance, the entire viral coat protein (CP) gene was transferred into “Duncan” grapefruit, by the *Agrobacterium* method outlined above. Challenged independent transgenic lines did not show virus symptoms within the first 6 months. Some lines showed restricted symptoms around the graft inoculation site, suggesting there may be some level of resistance (Kayim *et al.*, 2005). However, a year later, transgenic plants developed characteristic symptoms of *Citrus psorosis virus* on the leaves of all challenged transgenic grapefruit plants (M. Kayim, unpublished data).

3.4 Spinach Defensin Gene (*SoD2*)

This gene construct encodes the tobacco PR-1 signal peptide for secretion, and *SoD2* is known

to be an antimicrobial peptide that affects the membranes of gram-positive and gram-negative bacteria. It is known to have broad-spectrum antimicrobial effects (Segura *et al.*, 1998; Lay and Anderson, 2005) and, therefore, has potential against both citrus canker and citrus greening.

3.4.1 Binary vector construction

SoD2 was cloned into binary plasmid pBin34SGUS, containing the selective *nptII* and *gus* genes for direct use in explant transformation (Yang *et al.*, 2000). The synthetic structural *SoD2* gene was obtained from W.O. Dawson's laboratory of the University of Florida's CREC. The structural gene sequence was optimized for citrus expression and contains the PR-1 signal peptide for protein secretion into the plant apoplast. This gene was cloned in pCR 2.1 TOPO vector (Invitrogen Corp., Carlsbad, CA) and digested with *EcoRI*. The 0.29 kb *EcoRI* fragment was inserted in the same restriction site of pRTL22 obtaining pRTL22/*SoD1* plasmid. Flanking the target gene were the double *Cauliflower mosaic virus* (CaMV) 35S promoter and the CaMV 35S terminus, upstream and downstream from the gene of interest, respectively. This functional gene sequence was digested with *HindIII* and inserted in the same unique site of pBin35SGUS plasmid vector. The newly produced DNA molecule was the vector pBin35SGUS/*SoD2* (Gonzalez-Ramos *et al.*, 2005).

3.4.2 Transgenic plant production

Populations of transgenic "Ruby Red" and "Rio Red" grapefruit plants containing the *SoD2* gene were produced. Transformation efficiency was approximately twofold higher for "Ruby Red" than "Rio Red", showing a genotype interaction.

3.4.3 Characterization of transgenic plants

3.4.3.1 Southern analysis to determine gene copy number

Genomic DNA was extracted from GUS positive plants, and 15 µg DNA from each independent

transgenic event was digested with the restriction enzyme *HindIII*. A GUS DNA labeled probe was used in order to confirm the number of stable insertions of the transferred T-DNA fragment likely containing the target gene (Figure 2). The number of insertions in these transgenic plants as determined by this Southern analysis ranged from about one to eight copies, with more than 70% being single insertions (a consistent trend in grapefruit transformation).

3.4.3.2 Northern analysis to verify transcription

Total RNA was extracted from young leaves of transgenic plants. Ten micrograms of total single-stranded RNA were electrophoresed in formaldehyde gels and blotted onto nylon membranes (Amersham-GE Healthcare). The membranes were hybridized with the complete DNA sequence of the target genes (*SoD2*, as well as Bovine Lysozyme gene described below). The labeled probes were derived from the total DNA length of the structural genes of interest. Results suggested that a large number of insertions in the plant genome increased the likelihood of a significantly reduced messenger RNA (mRNA) transcription or complete gene silencing. For example, a large number of insertions occurred in plants 419, 202, and 861, and the amount of mRNA accumulated in these same plants varied considerably (Figures 2 and 3). Thus, in Figure 3, it is shown that plant 419 was completely silenced; while plant 861, with a larger number of insertions compared to plant 419, produced some mRNA. On the other hand, plant 202, with a similar number of insertions as plant 861, produced substantially greater amounts of mRNA (Figure 3). In summary, large insertion numbers in the genome of transgenic plants make uncertain the products of plant transformation.

3.5 Bovine Lysozyme Gene (*BVLZ*)

This gene is a member of the bacteriolytic enzymes found in nature. It also provides broad-spectrum antimicrobial resistance and could be effective against citrus canker or greening. The construct maintains its native signal peptide while the AUG

start codon context has been optimized to improve expression levels in plants.

3.5.1 Binary vector construction

A similar strategy as outlined above for *SoD2* was utilized.

3.5.2 Transgenic plant production and analyses

As with *SoD2*, populations of transgenic “Ruby Red” and “Rio Red” were produced.

3.5.2.1 Southern and Northern analyses

Both were conducted as described above for *SoD2*.

3.5.2.2 Transgene expression

To investigate the level and stability of expression of *BVLZ*, total leaf proteins were isolated from transgenic plants. The complex mixture of proteins was separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Amersham) by electroblotting. The membrane was probed with a polyclonal primary antibody (rabbit anti-BVLZ serum), and goat antirabbit IgG (Fc) alkaline phosphatase conjugate as a secondary antibody. This system allowed the indirect visualization of the BVLZ protein (Figure 4). The secondary antibody was detected in about 90% of the transgenic plants tested for the level of protein

accumulation. The variation in the amount of protein expressed on the immunoblot appears to be associated with the amount of transcription of the gene of interest (data not shown).

3.5.2.3 Validating the antimicrobial recombinant proteins *SoD2* and *BVLZ* against asiatic citrus canker (*Xac*) in Florida

In the summer of 2005, under appropriate permits, transgenic budwood was shipped from Texas A&M to the laboratory of J.H. Graham at the University of Florida. Transgenic plants were assayed using a virulent isolate of *Xac*. Transgenic and nontransgenic plants were inoculum infiltrated on the abaxial surface of young expanding leaves with a 10^4 CFU ml⁻¹ bacterial suspension as explained by Vilorio *et al.* (2004). Preliminary results indicated that at least one of the transgenic events in the “Rio Red” cultivar, expressing the *SoD2* gene, has shown resistance to artificially inoculated *Xac* (Figure 5). The quantitative variables of bacterial count and lesion number per inoculation site are known to be correlated (Vilorio *et al.*, 2004). Therefore, based on lesion number, the susceptible nontransgenic “Rio Red” developed five times more lesions per inoculation site as compared to the resistant transgenic “Rio Red” counterpart expressing the *SoD2* gene (Figure 5a). It is shown in Figure 5(b) that only one insertion of the cassette containing the *SoD2* gene occurred in the genome of “Rio Red” transgenic resistant plant number 5. The Northern blot shown in Figure 5(c) provides evidence of the amount of mRNA produced by this resistant transgenic event in “Rio Red”.

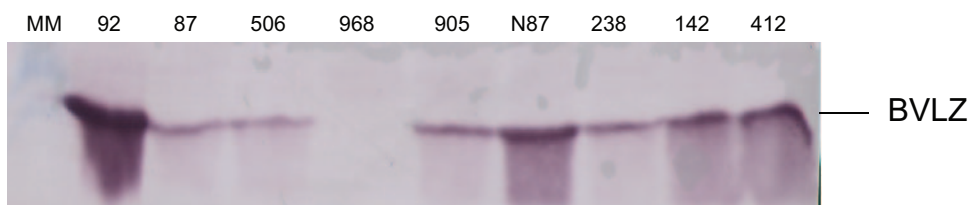


Figure 4 Western blot of total proteins extracted from young citrus leaves. From left to right: Lane 1 shows the protein size marker. Lanes 2–5 are transgenic “Ruby Red” expressing the BVLZ, except event 968. Event N87 is the citrange “Carrizo” used as a positive control. Lane 8 is a “Rio Red” transgenic event, and lines 9 and 10 are Hamlin events also expressing the lysozyme protein

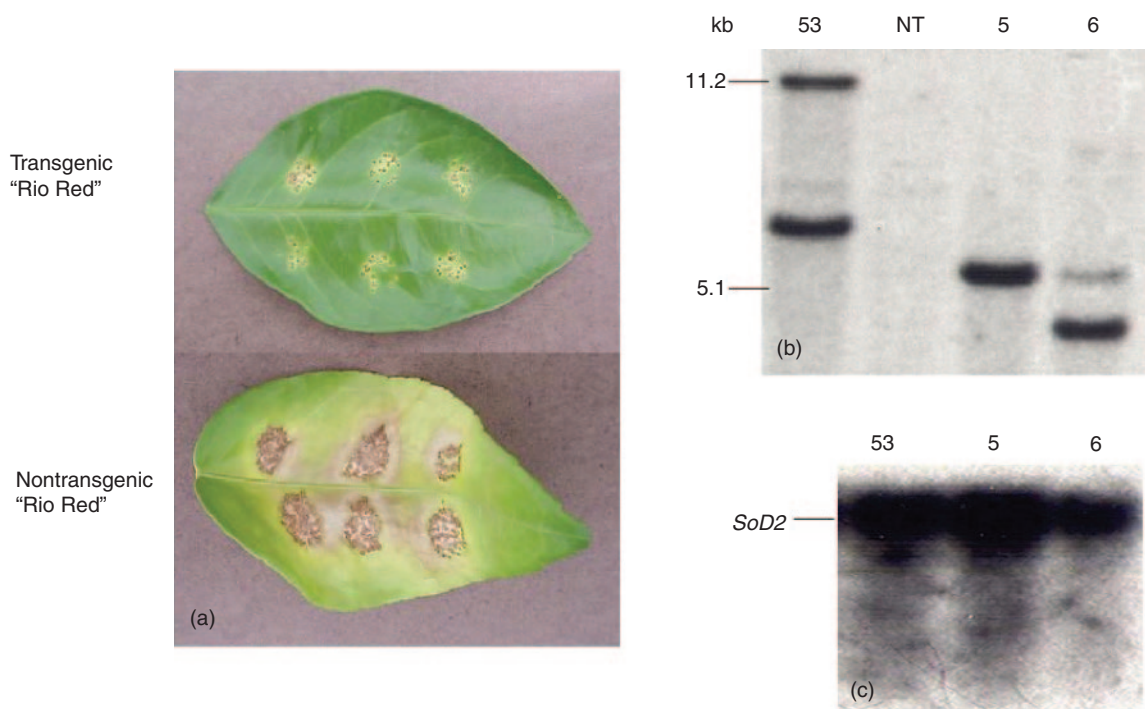


Figure 5 Artificially inoculated leaves of transgenic and nontransgenic "Rio Red", Northern and Western blots showing the insertion copy number and the constitutive mRNA expression of the *SoD2* gene. (a) Level of resistance produced by the transgenic plant event 5 containing the spinach defensin gene in the elite cultivar "Rio Red" compared to the nontransgenic control of the same grapefruit cultivar. (b) Southern blot analysis of the transgenic "Ruby Red" plant 53 and the transgenic "Rio Red" plants 5 and 6. (c) Western blot showing the amount of mRNA produced by the same plants with the transgenic events 53, 5, and 6

3.6 Biosynthetic Genes

Genes from the carotenoid biosynthetic pathway cloned into pCAMBIA2301 were successfully transferred to "Duncan" grapefruit using the standard *Agrobacterium*-mediated protocol described earlier. Transgenes included lycopene- β -cyclase, phytoene desaturase, and phytoene synthase, all under the control of constitutive promoters. All three vectors had the *nptII* selection gene and *gus* as a reporter. Transgenic plants were confirmed by PCR and Southern blot analysis (Costa *et al.*, 2002). Further characterization of these transgenic plants awaits field evaluation and fruiting after a long period of juvenility. This group also attempted transformation with BIBAC vectors in efforts to transform with larger T-DNA inserts. Unfortunately, these experiments resulted in poor shoot morphogenesis and no transgenic plant recovery.

4. FIELD EVALUATION OF TRANSGENIC TREES IN SOUTHERN TEXAS

Under appropriate USDA permits, the Texas A&M Agricultural Experiment Station in Weslaco (TAES) has the only field-grown transgenic grapefruit trees, which are expressing the *uncp* and *Gna* genes. These trees have been under observation, and the first preliminary data based on histological activity of the *gus* gene in endosperm and fruit rind, and fruit size and color, and quality as indicated by the total soluble solids in the fruit are shown in Table 3 and Figure 6.

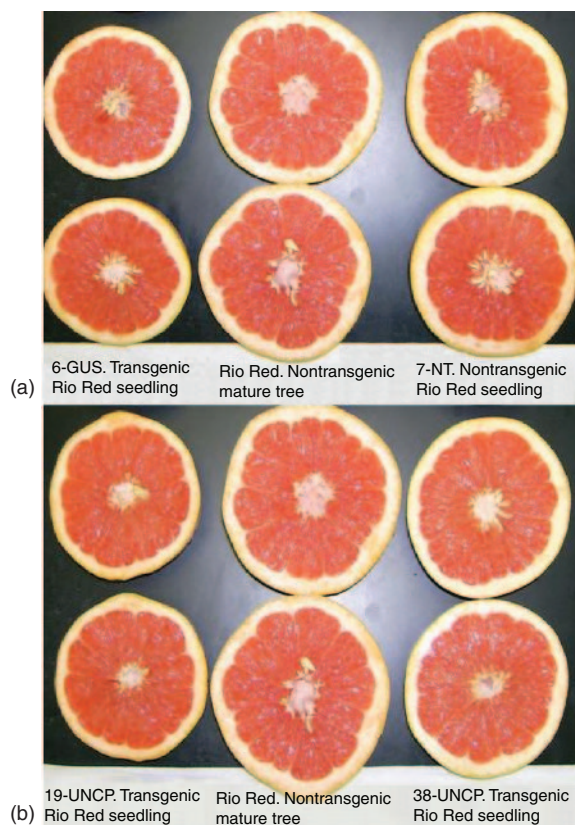
The Brix degrees were similar within the transgenic fruits and no different to the nontransgenic seedling fruits. GUS activity showed variations that probably could be explained in part by the natural level of expression and random variation. Within the transgenic group, fruits showed similar

Table 3 Number of fruits, seeds, and Brix degrees of transgenic “Rio Red” harvested at TAES-Weslaco 6 years after seedling trees were planted in the field

Tree and gene	Fruit number	Total seed number	Brix degrees (mean \pm s)	GUS activity
4-GUS	1	1	10.5	+
6-GUS	3	8	9.20 \pm 0.17	—
7-NT ^(a)	9	23	9.48 \pm 0.49	—
19-UNCP	8	20	9.45 \pm 0.40	+++++
27-UNCP	1	2	9.40	++++
38-UNCP	2	5	9.35 \pm 0.07	+++++
64-UNCP	3	5	9.83 \pm 0.38	+++
Rio Red-NT ^(b)	5	NA	11.6 \pm 0.05	—

^(a)Nontransgenic seedling^(b)Nontransgenic mature tree

size and color. The larger size of the nontransgenic fruit shown in Figures 6(a) and (b) is mainly due to the fact that this fruit was harvested from a mature tree.

**Figure 6** “Rio Red” fruits transgenic for GUS and UNCP. (a and b) Fruits 6-GUS, 19-UNCP, and 38-UNCP expressing their respective transgenes were harvested from transgenic seedlings. Fruit 7-NT is from a nontransgenic seedling. The fruit of nontransgenic “Rio Red” is from a mature tree

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Banana and Plantain

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1. INTRODUCTION

Bananas and plantains (*Musa* spp.) are the world's fourth most important food crop after rice, wheat, and maize in terms of gross value of production, with the vast majority of the crop grown and consumed in the tropical and subtropical zones. They provide a staple food for millions of people, particularly in Africa, an area where the green revolution has had little influence.

The performance of bananas and plantains can be severely affected by diseases and pests, including black Sigatoka (*Mycosphaerella fijiensis*), *Fusarium* wilt (*Fusarium oxysporum* f. sp. *cubense*), bacterial wilt (*Xanthomonas campestris* pv. *musacearum*), viruses (banana bunchy top virus, banana streak virus), nematodes, and weevils. These pest and diseases can cause economically significant food and income losses. Consequently, the largest single component in the cost of production of dessert banana and plantain in the tropics is disease control. Current control measures, which vary from cultural and biological to chemical control, comprise approximately 40% of total production cost (Ganry, 1993). Bananas and plantains are predominantly smallholders' crops; most growers cannot afford costly chemicals to control pests and diseases. This leaves host plant resistance as the most sustainable approach to counteracting pest and disease pressure, both under small-scale and

large-scale banana and plantain production schemes. Banana and plantain present many challenges for genetic improvement, notably associated with the reproductive biology, the complexity of trait expression, and the long generation time of the crop. Transgenic technology, together with conventional methods can assist in overcoming these problems in developing improved cultivars of banana and plantain.

1.1 History, Origin, and Distribution

Bananas and plantains are giant herbs that belong to the Musaceae, one of the six families of the order Zingiberales. The Musaceae have been associated with human history for a long time (Rowe and Rosales, 1996). The banana plant has been described as the "Tree of Paradise" in the Koran and as the "Tree of Knowledge" in the Book of Genesis in the Old Testament, hence the earlier taxonomic identification of bananas as *Musa paradisiaca* L. or *M. sapientum* L., which some authors still use nowadays.

The cultivation of bananas was first documented in ancient scriptures of India dating back to 500–600 BC (Reynolds, 1927). However, there is mounting archaeological evidence in support of earlier cultivation in Papua New Guinea, at least 7000 years ago and possibly as far back as 10 000 years ago (Denham *et al.*, 2003). Thus,

the New Guinean highlands would be the place where bananas were first domesticated. Organized plantation of bananas existed in China in 200 AD, four centuries before Islamic conquerors brought the banana to Palestine, and Arab merchants eventually spread the plant over much of Africa. The word *banana* is of West African origin, but it is derived from the Arabic *banaan* (finger), and passed into English via Spanish or Portuguese. However, the long debated when and by what routes *Musa* (bananas) were introduced to tropical Africa and South America remain unresolved.

Cultivated bananas and plantains are predominantly triploid ($2n = 3x = 33$) female-sterile varieties that evolved from two diploid ($2n = 2x = 22$) seminiferous species, *Musa acuminata* Colla (genome AA) and *Musa balbisiana* Colla (genome BB), originating from the tropical Malay region and the Northern Indian region, respectively (Simmonds, 1962).

It is hypothesized that the evolutionary pathway leading to the emergence of cultivated varieties from the seminiferous ancestors comprised two critical events, both of which occurred in Southeast Asia, the center of origin and primary diversity of bananas. Firstly, vegetative parthenocarpy and female sterility appeared in *M. acuminata*, allowing for production of pulp without seeds as evidenced by the occurrence of parthenocarpic and seedless diploid *M. acuminata*. Secondly, crosses within *M. acuminata* or between *M. acuminata* and *M. balbisiana* occurred, facilitated by female restitution and haploid fertilization, and produced two groups of natural hybrids. One group comprises autopolyploid and homogenomic hybrids that are essentially AAA dessert and East African Highland bananas. The other group contains allopolyploid and heterogenomic types: the AAB plantains and starchy bananas and the ABB cooking bananas.

The natural hybrids do not produce seeds and can only reproduce by vegetative propagation, implying that survival in nature and geographical dispersal could not occur without human intervention. Therefore, it is widely accepted that secondary diversification in areas devoid of wild *Musa* plants, e.g., Western and Central Africa, must be due to somatic mutations of a small number of introduced materials, rather than through multiple introductions (De Langhe *et al.*, 2005).

In Africa, the cultivars are usually known under a multitude of vernacular names, superimposing linguistic diversity to genetic and ecological diversity of the varieties in any given area (Rossel, 1998). Similar interactions between linguistics and genecology occur throughout Asia, the geographical origin and primary diversification center of the *Musaceae*. Thus, a good understanding of demographic history and dispersal mechanisms may help explain the structure of genetic diversity among populations of bananas and plantains across geographical regions.

1.2 Botanical Description

The taxonomy of bananas and plantains has attracted many excellent treatises that also describe the distinguishing features among cultivated forms (Simmonds, 1962; Stover and Simmonds, 1987; Robinson, 1996; De Langhe *et al.*, 2005). Baker (1893) was first to establish a formal classification of the genus *Musa*. He recognized three subgenera: *Physocaulis* (wild and inedible seeded bananas), *Eumusa* (species with edible fruits), and *Rhodochlamys* (inedible fruits and brightly colored bracts). Baker's classification was revised by Cheesman (1947) whose detailed taxonomic treatment was based primarily on chromosome number, pseudostem stature, inflorescence characters, and seed morphology. Cheesman adopted the concept of sections or series at the subgeneric level, dividing the genus *Musa* into *Eumusa* ($x = 11$), *Rhodochlamys* ($x = 11$), *Australimusa* ($x = 10$), and *Callimusa* ($x = 10$). The majority of the cultivated bananas belong to the section *Eumusa*. This section is the biggest in the genus and the most geographically widespread with species found throughout Southeast Asia from India to the Pacific Islands.

Cheesman's classification is still widely accepted, despite subsequent reports of basic chromosome numbers of 7 for *Musa ingens* and 9 for *Musa beccarii* (Shepherd, 1959) or the discovery of *M. ingens* that could not be fitted to any of the sections (Argent, 1976). In fact, several unresolved issues remain, including the evolutionary relationships among the four sections of the genus *Musa*, the separation of sections *Eumusa* and *Rhodochlamys* with apparently little taxonomic support, and the relationship between

the wild progenitors and the cultivated clones (Shepherd, 1999).

It is now accepted that the diversity of bananas in the wild has not been exhaustively described and classified, with new information prompting taxonomic revision from time to time at the species level, and more rarely, at the genus level (Valmayor and Danh, 2002; Häkkinen, 2006).

Ploidy and genome configurations have played a determinant role in the classification of bananas, with major groups including diploids (AA, BB, AB), triploids (AAA, AAB, ABB), and tetraploids (AAAA, AAAB, AABB, ABBB). There is wide consensus about the attributes conferred by A or B genomes in interspecific natural or artificial hybrids of *M. acuminata* and *M. balbisiana*. Hence, it is accepted that edibility of mature fruits arose from mutations causing parthenocarpy and female sterility in diploid *M. acuminata* (Simmonds, 1962). It is equally accepted that hardness is contributed by the B genome since *M. balbisiana* clones thrive abundantly in areas experiencing pronounced dry seasons alternating with monsoons. Also attributed to the B genome are fruit characteristics, such as starchiness and acid taste, causing AAB plantain to be starchier but less sweet and less palatable when raw than the AAA dessert bananas (Simmonds, 1962).

The African plantains (AAB) display greater variation than any other subgroup of triploid bananas in the world, with morphological variation occurring for inflorescence type, plant size, fruit orientation, fruit shape, pseudostem, and fruit color. Based on inflorescence morphology, four types have been distinguished, namely, French plantain, French Horn plantain, Horn, and False Horn plantain, which are further divided into giant, medium, and small types.

While various combinations of the A and B genomes predominate in most cultivars, two other genomes, denoted as S (from *M. schizocarpa*) and T (from *M. textilis*), occur albeit in a few accessions. Thus, genomic groups with the S genome include AS, AAS, and ABBS while those with the T genome are AAT, AAAT, and ABBT (Sharrock, 1990).

Pillay *et al.* (2004) provided a succinct review of studies on genome size and cytological features of bananas. Relatively few studies have been conducted in *Musa* to determine genome size, with large differences in the DNA content estimates

obtained by different authors (Lysak *et al.*, 1999; D'Hont *et al.*, 2000; Kamaté *et al.*, 2001). Thus, DNA content ranging from 1.11 pg $2C^{-1}$ to 1.33 pg $2C^{-1}$ was reported for diploid *M. acuminata* accessions. Likewise, nuclear DNA content ranging from 1.03 pg $2C^{-1}$ to 1.16 pg $2C^{-1}$ were found for diploid *M. balbisiana*, while S and T genomes were reported to contain 1.18 pg $2C^{-1}$ and 1.27 pg $2C^{-1}$ DNA, respectively. Clearly, additional research may be needed before robust comparisons of DNA content in the A, B, S, and T genomes can be made, prompting Pillay *et al.* (2004) to advise that caution be exercised in interpreting DNA content data in *Musa*.

1.3 Economic Importance

Banana and plantain are important cash and subsistence crops in most tropical and subtropical regions of the world, growing on production cycles of 12–18 months, essentially as perennial crops that can be harvested all year round (Robinson, 1996). Almost all banana and plantain cultivation falls within 30° latitude north and south of the equator (Stover and Simmonds, 1987). They require an average temperature of about 30 °C and a minimal rainfall of 100 mm per month (Swennen and Rosales, 1994). These crops are cultivated on approximately 10 million ha worldwide with an annual production exceeding 100 million metric tons, which are distributed among Africa (40%), Asia (30%), Latin America, and the Caribbean (30%).

As mentioned above, the existing cultivars are assigned to three genome groups, AAA, AAB, and ABB but varieties grouped in the same genomic category can be very dissimilar (Simmonds, 1962; Stover and Simmonds, 1987; Robinson, 1996). Thus, the AAA group contains sweet dessert bananas that are eaten raw when ripe and bananas of the East African highlands that require cooking before they can be eaten or brewing before drinking. Likewise, the West African and Asia Pacific (Maia maoli/Popoulou) plantains that are cooked before eating dominate the AAB group, which also contains dessert bananas of the Pome subgroup. Varieties in the ABB group are essentially used for cooking, but some may be eaten raw as dessert bananas usually when overripe.

Economically, bananas and plantains constitute major export crops in some countries

predominantly in Latin America and the Caribbean and a few countries in Western and Central Africa. However, only about 10% of world production, predominantly AAA dessert bananas of the Cavendish type, enters international trade. The bulk of the production provides an important staple food for rural and urban consumers in the production areas where bananas and plantains also constitute an important source of income for rural populations.

Bananas and plantains are the major staple food in the equatorial belt of Africa, where more than 70 million people derive in excess of 25% of their daily calorie intake from plantains in West and Central Africa (Robinson, 1996). Indeed bananas are rich in carbohydrates (about 35%) and fiber (6–7%), and have a relatively low protein and fat content (1–2%), and constitute a good source of major elements, such as potassium, magnesium, phosphorus, calcium, iron, and vitamins A, B6, and C (Marriott and Lancaster, 1983). Bananas play a vital role in the nutrition and well-being, and cultural life of millions of people in Central and Western Africa and South and Central America, often being the first solid food for infants (Price, 1995).

Bananas and plantains can be processed into a variety of secondary products from fibers to wrappings and are fermented to produce alcohol. However, these options are essentially practiced at rudimentary scales, despite their potential for industrial production.

1.4 Traditional Breeding

Banana and plantain were introduced in Africa only about 3000 years ago (De Langhe, 1995) and a remarkable diversity now exists for both groups, but all traditional varieties have become susceptible to a range of pests and diseases, the most serious of which are black Sigatoka (*Mycosphaerella fijiensis* Morelet), Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*), banana weevil (*Cosmopolites sordidus* Germar), and a complex of plant parasitic nematodes (*Pratylenchus goodeyi*, *Helicotylenchus multicinctus*, and *Radopholus similis*). Therefore, breeding efforts focus on developing new disease and pest resistant varieties that also retain the organoleptic properties of the traditional varieties.

There are two basic steps in plant breeding: (a) accessing natural variation or artificially creating

genetic diversity, and (b) selecting individuals with the desirable gene combinations from existing or artificially created populations. These principles have been applied for seed-propagated crop species using techniques that are often referred to as conventional. However, a commonly held view is that the most widely used so-called conventional or classical plant breeding techniques from sexual, seed-propagated crops are not appropriate for banana and plantain improvement. The distinction between “conventional” and “non-conventional” breeding lies in the methods of gene shuffling and in the origin of the genes being manipulated. Nevertheless, the methodological principles applied for sexually propagated crops can be, and have been, applied to banana and plantain breeding, with the aid of nonfield methods of recovering viable progeny and identifying those progenies with putatively desirable gene combinations. In this regard, there is nothing conventional about banana and plantain breeding (Tenkouano, 2005).

Banana and plantain breeders aim to produce seedless varieties, preferably in the triploid ($3x$) background common to the majority of existing cultivars. This usually involves crossing $3x$ cultivars to diploid ($2x$) accessions that are donors of resistance genes, selecting $4x$ and $2x$ primary hybrids from the $3x - 2x$ progenies, and crossing $4x - 2x$ hybrids to produce secondary $3x$ hybrids. Alternatively, secondary $3x$ hybrids may be produced via $2x - 2x$ crosses where one of the $2x$ parents produces $2n$ pollen. However, this requires screening of diploid lines for $2n$ pollen production, and setting crossing schemes that would allow pollination to coincide with periods that are favorable for $2n$ pollen production.

Other methods of producing triploid hybrids have been explored, such as tetraploidization of diploid accessions using colchicine prior to crossing with another diploid (Tézenas du Montcel *et al.*, 1996). Colchicine prevents the formation of mitotic spindles, resulting in mitotic restitution in treated cells. This technique may preserve advantageous linkages selected at diploid level, particularly in the tetraploid derived from the diploid, but recombination can occur when crossing the tetraploid with a diploid to produce a triploid, with the potential of disrupting linkages. Also, colchicine treatment may not affect uniformly all cells in multicellular meristems, causing

cytochimeras that may not be easy to dissociate (Roux *et al.*, 2001). Thus, efficient methods for *in vitro* dissociation of chimeras and selection of the desired cells are required, before such cells can be cultured to regenerate a plant that will now be used for crossbreeding. Furthermore, the use of colchicine can cause increased inbreeding, reduced vigor, and reduced genetic variability (Ortiz *et al.*, 1992). Thus, colchicine-mediated polyploidization is impractical for routine breeding operations in plantain and banana.

Hence, tetraploid \times diploid crosses remain the predominant triploid breeding scheme, although the multiploidy and heterogenomic structure of breeding populations results in unpredictable variation in genome size and structure across and within generations. This causes complex inheritance patterns and complicates phenotypic selection for most yield and growth traits (Ortiz and Vuylsteke, 1996).

Despite these limitations, many improved hybrids combining genetic resistance to black Sigatoka with appropriate agronomic characteristics have been developed (Swennen and Vuylsteke, 1993; Ortiz and vuylsteke, 1998). The improved hybrids are about two to five times more productive than the traditional plantain landraces, under natural conditions with no chemical control of black Sigatoka. These hybrids are now being introduced into farmers' fields in several countries, across Africa (Gallez *et al.*, 2004; Tenkouano and Swennen, 2004) with relatively high adoption prospects (Tenkouano *et al.*, 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

A good understanding of the structure of diversity in the existing materials is required to access natural variation or to artificially create genetic variation. Early breeders relied essentially on morphological and geographical information, which may not be adequate. Recent advances in the development of molecular or cytological tools stand to benefit modern breeders, notably by clarifying phylogenetic relationships, thus allowing for inferences about gene pools. For example, amplified fragment length polymorphism analysis of total DNA revealed that the *M. acuminata* complex of about 50 morphological subspecies

can be ascribed to only three genetic subspecies (*burmannica*, *malaccensis*, and *microcarpa*) while *M. balbisiana* was shown to contain two forms (Singapuri, I-63), suggesting that there might be at least three A genomes and two B genomes (Ude *et al.*, 2002a, b). Furthermore, it seems that the A genomes in the plantains come from the subspecies *microcarpa* while the B genome comes from the I-63 form of *M. balbisiana*. More recently, Ge *et al.* (2005) also traced variation in *M. balbisiana* to two main clades, based on simple sequence repeat length polymorphisms and CpDNA analysis. Such information becomes important when attempts are made to diversify and broaden the genetic foundation of breeding populations, particularly in the search for novel genes.

In fact, as new genomic resources become available, or are applied in conjunction with cytogenetic tools (Bartos *et al.*, 2005) or numerical tools (De Langhe *et al.*, 2005), the taxonomic description of the *Musaceae* will likely evolve. These findings have major implications for evolutionary or reconstructive breeding, aiming to re-enact existing landraces without some of their shortcomings such as disease susceptibility.

Despite these advances, many problems specific to the biology of plantains and bananas impede rapid breeding progress, including low reproductive fertility, triploidy, and slow propagation (Vuylsteke *et al.*, 1997). Thus, breeders may not be able to access all available variation, largely due to the high sterility of many cultivated varieties; the conundrum of banana breeding is that seed set is required to produce seedless varieties. Improving female or male sterile accessions can only be achieved by circumventing reproductive barriers, e.g., by deliberate mutagenesis or by direct gene transfer through genetic engineering.

The crossbreeding approach is time consuming, given the long generation time of the crop and it is also technically complex, since it requires both ploidy and genome selection. With the advent of flow cytometric analysis of nuclear DNA content (Dolezel, 1997) and molecular markers for the A and B genomes (Pillay *et al.*, 2000), ploidy and genome selection have become easier. However, predicting progeny performance remains a major challenge when ploidy and genome variation occurs within and across generations (Tenkouano *et al.*, 1999a, b). Ideally, genetic engineering would provide a more deterministic approach

for substituting undesirable genetic materials with those that would enhance the performance of the recipient line or cultivar with respect to a particular constraint.

Additionally, the available germplasm may not harbor the desired genes with respect to an emerging threat, which is clearly the case for the bacterial wilt caused by *X. campestris* pv. *musacearum* that is threatening livelihoods in the Great Lakes region of central and eastern Africa. There is little that crossbreeding can do to address such a threat in the near future, leaving genetic engineering as the best alternative to control the bacterial wilt epidemics through host resistance.

Genetic engineering could target the development of improved versions of existing cultivars for deployment or, alternatively, insert alien resistance genes into breeding lines for later use in crossbreeding schemes.

2. DEVELOPMENT OF TRANSGENIC BANANAS AND PLANTAINS

In view of the constraints of conventional breeding in banana and plantain already mentioned, the case for using transgenic approaches to improve these crops is particularly compelling. Because of the lack of cross-fertile wild relatives in many banana producing areas, as well as the male and female sterility of most edible bananas and plantains and the clonal mode of propagation, gene flow is not an issue for this crop, making a transgenic approach even more attractive.

Genetic transformation has become an important tool for crop improvement. Genetic engineering, i.e., the introduction and stable integration of genes into the nuclear genome and their expression in a transgenic plant offers a better alternative for the genetic improvement of cultivars not amenable to conventional crossbreeding, such as Cavendish bananas and False Horn plantains (Jones, 2000). The successful genetic transformation in plants requires the production of normal, fertile plants expressing the newly inserted gene(s). The process of genetic transformation involves several distinct steps, namely, identification of useful gene, the cloning of the gene into a suitable plasmid vector, delivery of the vector into plant cell (insertion and integration) followed by

expression, and inheritance of the foreign DNA encoding a polypeptide. With the advent of plant biotechnology and the rapid development of gene transfer techniques, the potential to introduce desirable traits is no longer restricted to those occurring in close relatives. In theory, genes, once identified, isolated, and cloned may be introduced to a plant from any organism.

Development of stable and reproducible transformation and regeneration technologies opened new horizons in banana and plantain breeding. Despite technical difficulties of transforming a monocot species, transformation protocols are now available for most *Musa* cultivars. Several transformation strategies have been published in the last 10 years by different banana biotechnologists (May *et al.*, 1995; Sagi *et al.*, 1995; Becker *et al.*, 2000; Ganapathi *et al.*, 2001; Khanna *et al.*, 2004; Tripathi *et al.*, 2005a).

2.1 Donor Gene, Promoter, Termination Sequence, and Selection Marker

The identification of resistance genes for the diseases facing banana and plantain is critical in order to create transgenic varieties resistant to these diseases. Upon identification of resistance genes, a method of introduction of these transgenes into banana and plantain will be necessary. Genetic transformation could be a useful tool for creating plants with improved characteristics. The status of research on genetic engineering of banana and plantain for disease resistance and future possibilities has also been reviewed (Sagi, 2000; Tripathi, 2003; Tripathi *et al.*, 2005b). Currently, the transgenes used for banana improvement have been exclusively isolated from heterologous sources like other plant species, insects, microbes, and animals (Tripathi, 2003; Tripathi *et al.*, 2004, 2005b).

2.1.1 Isolation and characterization of novel genes: targeted traits

2.1.1.1 Pest resistance

There are several possible approaches for developing transgenic plants with improved weevil and nematode resistance. A variety of genes

are available for genetic engineering for pest resistance (Sharma *et al.*, 2000). Among these are proteinase inhibitors (PIs), *Bacillus thuringiensis* (*Bt*) toxins, plant lectins, vegetative insecticidal proteins (VIPs), and α -amylase inhibitors (AI). PIs contribute to host plant resistance against pests and pathogens (Green and Ryan, 1972). They operate by disrupting protein digestion in the insect mid-gut via inhibition of proteinases. The two major proteinase classes in the digestive systems of phytophagous insects are the serine and cysteine proteinases. Coleopteran insects, including the banana weevil, mainly use cysteine proteinases (Murdock *et al.*, 1987) and recent studies indicate a combination of both serine and cysteine proteinase is useful (Gerald *et al.*, 1997). These inhibitors have already been used for insect control in genetically modified (GM) plants (Leple *et al.*, 1995). Presently, cysteine proteinase activity has been identified in the mid-gut of the banana weevil and *in vitro* studies have shown that cysteine proteinases are strongly inhibited by both a purified recombinant rice (*oryzacystatin-I* (OC-I)) and papaya cystatin (Abe *et al.*, 1987; Kiggundu *et al.*, 2003).

The use of PIs, as nematode antifeedants is an important element of natural plant defense strategies (Ryan, 1990). This approach offers prospects for novel plant resistance against nematodes and reduces use of nematicides. The potential of PIs for transgenic crop protection is enhanced by lack of harmful effects when humans consume them in seeds, such as rice and cowpea. Cysteine PIs (cystatins) are inhibitors of cysteine proteinases and have been isolated from seeds of a wide range of crop plants consumed by man including those of sunflower, cowpea, soybean, maize, and rice (Atkinson *et al.*, 1995). Transgenic expression of PIs provides effective control of both cyst and root-knot nematodes. The cystatins Oc-I and an engineered variant Oc-I Δ D86 were shown to mediate nematode resistance when expressed in tomato hairy root (Urwin *et al.*, 1995), *Arabidopsis* plants (Urwin *et al.*, 1997), rice (Vain *et al.*, 1998a), and pineapple (Urwin *et al.*, 2000). The partial resistance was conferred in a small-scale potato field trial on a susceptible cultivar by expressing cystatins under control of the cauliflower mosaic virus (CaMV) 35S promoter (Urwin *et al.*, 2001). The enhanced transgenic plant resistance to nematodes has

been demonstrated by using dual PI transgenes (Urwin *et al.*, 1998). Since cystatins have been shown to function in rice, which like *Musa* is a monocotyledon, and also have clear efficacy against a wide range of nematode species, their usefulness as transgenes for development of transgenic *Musa* for resistance to nematodes can be evaluated as having a high probability of success. Recently, Cavendish banana (Grand Nain) has been transformed using *Agrobacterium tumefaciens* to express a protein-engineered rice cystatin (Atkinson *et al.*, 2004) and tested in screen house for nematode resistance.

The expression and biological activity of the *Bt* toxins has been investigated in GM plants for insect control. *Bt* gene technology is currently the most widely used system for lepidopteran control in commercial GM crops (Krettiger, 1997). The expression of a selected *Bt* gene for weevil resistance may be a rather long-term strategy since no potential *Bt* gene with high toxic effects against the banana weevil has been identified as yet (Kiggundu *et al.*, 2003). Some *Bt* proteins are also effective against saprophagous nematodes (Borgonie *et al.*, 1996). The Cry5B protein is toxic to wild type *Caenorhabditis elegans*, other *C. elegans* mutants are resistant to Cry5B but susceptible to the Cry6A toxin (Marroquin *et al.*, 2000). The approach using *cry* genes has potential for plant nematode control (Wei *et al.*, 2003).

The other strategies for nematode resistance include the use of natural plant resistance genes (R genes) and lectins genes. Several R genes are targeted against nematodes. The *Hs1pro-1* from a wild species of sugar beet confers resistance to the cyst nematode *Heterodera schachlii* (Cai *et al.*, 1997). Plant lectins confer a protective role against a range of organisms (Sharma *et al.*, 2000). Lectins have been isolated from a wide range of plants including snowdrop, pea, wheat, rice, and soybean. Their carbohydrate binding capability renders them toxic to insects. Some lectins such as the snowdrop lectin (*Galanthus nivalis* agglutinin: GNA) have biological activity against nematodes (Burrows *et al.*, 1998). However, many lectins have toxic effects on insects and mammals, which raise concerns regarding toxicological safety and this, may prove a substantial limitation to the future commercial development of lectins.

AI and chitinase enzymes might also have a future potential for weevil control. AI operate by

inhibiting the enzyme α -amylase, which breaks down starch to glucose in the insect gut (Morton *et al.*, 2000). Transgenic adzuki beans are produced with enhanced resistance to bean bruchids, which are Coleopteran insects like weevils (Ishimoto *et al.*, 1996). Therefore, AI may be of interest for banana weevil control in GM banana. Chitinase enzymes are produced as a result of invasion either by fungal pathogens or insects. Transgenic expression of chitinase has shown improved resistance to Lepidopteran insect pests in tobacco (Ding *et al.*, 1998).

2.1.1.2 Bacterial and fungal resistance

Currently, no source of any banana germplasm exhibiting resistance to the disease has been identified. As an alternative, genetic transformation technology with fungicidal or bactericidal transgenes may offer an alternative solution to these problems.

The most attractive strategy for serious fungal disease like Black Sigatoka control in *Musa* is probably the production of disease resistant plants through the transgenic approach including the expression of genes encoding plant, fungal or bacterial hydrolytic enzymes, genes encoding elicitors of defense response, and antimicrobial peptides (AMPs) (Tripathi, 2005, 2006). AMPs have a broad-spectrum antimicrobial activity against fungi as well as bacteria and most are nontoxic to plant and mammalian cells. Examples of AMPs are magainin from the African clawed frog (Zasloff, 1987; Bevins and Zasloff, 1990), cecropins from the giant silk moth (Boman and Hultmark, 1987), and plant defensins (Broekaert *et al.*, 1995). The cecropin (Alan and Earle, 2002) and its derivatives (D4E1; Rajasekaran *et al.*, 2001) have been found to inhibit the *in vitro* growth of several important bacterial and fungal pathogens. Transgenic tobacco plants expressing cecropins have increased resistance to *Pseudomonas syringae* pv. *tabaci*, the cause of tobacco wildfire (Huang *et al.*, 1997).

Similarly, magainin is effective against the plant pathogenic fungi (Zasloff, 1987; Kristyanne *et al.*, 1997). Li *et al.* (2001) reported enhanced disease resistance in transgenic tobacco expressing Myp30, a magainin analog. Chakrabarti *et al.* (2003) reported successful expression of other

synthetic peptide, MSI-99 and enhanced disease resistance in transgenic tobacco and banana. A synthetic substitution analog of magainin, MSI-99, is employed in this study to impart disease resistance in transgenic tobacco (*Nicotiana tabacum* L.) and banana (*Musa* sp. cv. Rasthali). This peptide inhibited the growth and spore germination of *F. oxysporum* f. sp. *cubense* at 16 lg ml^{-1} . MSI-99 has been subcloned into plant expression vectors pMSI164 and pMSI168, targeting the peptide into the cytoplasm and extracellular spaces, respectively. Tobacco plants transformed with pMSI168 showed enhanced resistance against *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Botrytis cinerea*. Transgenic banana plants were obtained for both pMSI164 and pMSI168 transformations and showed resistance to *F. oxysporum* f. sp. *cubense* and *Mycosphaerella musicola*. On the basis of the broad-spectrum activity against fungal pathogens, individual or combined expression of cecropin, magainin, and their derivatives in banana may result in increased resistance to several pathogens.

Another source of antimicrobial proteins has been lysozyme, either from bacteriophage, hen eggs, or bovine. The lysozyme attacks the murein layer of bacterial peptidoglycan resulting in cell wall weakening and eventually leading to lysis of both gram-negative and gram-positive bacteria. The lysozyme genes have been used to confer disease resistance against plant pathogenic bacteria in transgenic tobacco (Trudel *et al.*, 1995), potato (Düring *et al.*, 1993), and apple (Ko, 1999).

There are many reports on the application of plant proteins with distinct antimicrobial activities (Broekaert *et al.*, 1997). Thionins are cysteine-rich low molecular weight proteins (about 5 kDa) and have been identified in various organs of a number of plant species. They show antimicrobial activity when tested *in vitro* against various bacteria and fungi (Florack and Stiekema, 1994). The antimicrobial action is based on the ability of thionins to form pores in cell membrane resulting in membrane disruption and cell death. Expression of thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens (Carmona *et al.*, 1993). Epplé *et al.* (1997) observed that constitutive over expression of thionin in transgenic *Arabidopsis* resulted in enhanced resistance against *F. oxysporum* f. sp.

matthiolae. The expression of thionin, viscotoxin A3, in transgenic *Arabidopsis* showed increased resistance to infection of clubroot pathogen *Plasmodiophora brassicae* (Holtorf *et al.*, 1998). Unfortunately, most thionins can be toxic to animal and plant cells and thus may not be ideal for developing transgenic plants (Reimann-Philipp *et al.*, 1989).

There are a number of known plant defensins, which are known to protect against plant pathogens. The radish defensin Rs-AFP1 and Rs-AFP2, have been shown to inhibit the growth of several pathogenic fungi *in vitro* (Terras *et al.*, 1992). The expression of Rs-AFP2 in transgenic tobacco confers partial resistance to attack by *Alternaria longipes* (Terras *et al.*, 1995). Two homologous peptides, Rs-AFP3 and Rs-AFP4, are also induced in radish leaves upon infection by *A. longipes*, thus further substantiating the role of defensins in plant defense. Two sugar beet leaf defensins, AX1 and AX2, homologs of the radish AFP2, have been isolated after infection with the fungal pathogen *Cercospora beticola* (Kragh *et al.*, 1995). The preliminary results indicate that the expression of these peptides in transgenic corn plants imparts significant resistance to Northern corn leaf blight caused by the fungal pathogen *Exserohilum turcicum*. A defensin from alfalfa seeds alfAFP (alfalfa antifungal peptide) when expressed under the control of 35S promoter in transgenic potato imparted resistance to *Verticillium dahliae*, *Alternaria solani*, and *Fusarium culmorum* but not to *Phytophthora infestans* (Gao *et al.*, 2000). It has shown resistance to fungal diseases in potato in the greenhouse as well as in the field. Kanzaki *et al.* (2002) reported the overexpression of the WTI defensin from wasabi (*Wasabia japonica*) conferring enhanced resistance to blast fungus in transgenic rice.

Several defensins isolated from radish and dahlia have been found toxic to *M. fijiensis* and *F. oxysporum* f. sp. *cubense*, two major fungal pathogens of *Musa* (Cammue *et al.*, 1993). They are nontoxic to human or banana cells. Large number of transgenic lines of *Musa* especially plantains expressing defensins have been developed at KULeuven (Remy, 2000). Many hundreds of transformed lines have been generated and screened under greenhouse conditions in Belgium for disease resistance and the most promising lines

of transgenic bananas and plantains are currently being evaluated in the greenhouse and field in Cuba and Costa Rica.

Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Pathosystem-specific plant resistance (R) genes have been cloned from several plant species against many different pathogens (Bent, 1996). These include R genes that mediate resistance to bacterial, fungal, viral, and nematode pathogens. Many of these R gene products share structural motifs, which indicate that disease resistance to diverse pathogens may operate through similar pathways. In tomato (*Lycopersicon esculentum*), the R gene *Pto* confers resistance against strains of *P. syringae* pv. *tomato* (Martin *et al.*, 1993; Kim *et al.*, 2002). *Pto*-overexpressing plants show resistance not only to *P. syringae* pv. *tomato* but also to *X. campestris* pv. *vesicatoria* and to the fungal pathogen *Cladosporium fulvum* (Mysore *et al.*, 2003). Similarly, the *Arabidopsis* *RPS4* gene specifies disease resistance to *P. syringae* pv. *tomato* expressing *avrRps4* (Gassmann *et al.*, 1999). The *Bs2* resistance gene of pepper specifically recognizes and confers resistance to strains of *X. campestris* pv. *vesicatoria* (Tai *et al.*, 1999). Transgenic tomato plants expressing the pepper *Bs2* gene suppress the growth of *Xcv*.

The *Xa21* gene isolated from rice has been shown to confer resistance against many isolates of *Xanthomonas oryzae* pv. *oryzae* (Song *et al.*, 1995; Wang *et al.*, 1996). Transgenic plants expressing *Xa21* under the control of the native promoter of the genomic fragment of the *Xa21* gene showed enhanced resistance to bacterial leaf blight caused by most *Xoo* races. The *Xa1* gene also isolated from rice confers resistance to Japanese race 1 of *X. oryzae* pv. *oryzae*, the causal pathogen of bacterial blight (Yoshimura *et al.*, 1998).

Successful transfer of resistance genes to heterologous plant species gives another new option to develop disease resistant plants. R gene-mediated resistance has several attractive features for disease control. When induced in a timely manner, the concerted responses can efficiently halt pathogen growth with minimal collateral damage to the plant. No input is required from the farmer and there are no adverse environmental effects. Unfortunately, R genes are often quickly

defeated by co-evolving pathogens (Pink, 2002). Many R genes recognize only a limited number of pathogen strains and therefore do not provide broad-spectrum resistance. Also efforts to transfer R genes from model species to crops, or between distantly related crops, could be hampered due to restricted taxonomic functionality.

Plants also employ a wide array of defense mechanisms against pathogen attack. Among those, hypersensitive response (HR) is an induced resistance mechanism, characterized by rapid, localized cell death upon their encounter with a microbial pathogen. Several defense genes have been shown to delay the HR induced by bacterial pathogen in nonhost plants through the release of the proteinaceous elicitor. Elicitor-induced resistance is not specific against particular pathogens. Hence, manipulation of such defense genes may be more ideal.

The plant ferredoxin like amphipathic protein (*pflp*, formerly called AP1) and HR-assisting protein (*hrap*), isolated from the sweet pepper, *Capsicum annuum*, are novel plant proteins that can intensify the harpinPSS-mediated HR (Chen *et al.*, 2000). These proteins have dual function: iron depletion antibiotic action and harpin triggered HR enhancing. The transgenes have been shown to delay the HR induced by various pathogens like *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* sp. in nonhost plants through the release of the proteinaceous elicitor, harpinPss in various crops including dicots like tobacco, potato, tomato, broccoli, orchids, and monocots like rice (Tang *et al.*, 2001; Lu *et al.*, 2003; Huang *et al.*, 2004). Also elicitor-induced resistance is not specific against particular pathogens, so it could be very useful strategy (Wei and Beer, 1996). The research is in progress at IITA for developing bacterial wilt (caused by *X. campestris* pv. *musacearum*) resistant banana varieties using *pflp* or *hrap* genes.

A series of resistance gene analogs have been isolated from banana, using degenerate polymerase chain reaction (PCR) primers targeting highly conserved regions in proven plant resistance genes (e.g., kinase or transmembrane-encoding domains, or leucine-rich repeat sequences). Plant disease resistance genes involved in signal transduction contain domains that are conserved throughout monocotyledons and dicotyledons. Primers have been designed to those domains in the *RPS2* gene of *Arabidopsis* and the *N* gene of tobacco. Using

these primers for PCR, candidate resistance genes have already been cloned from soybean, potato, rice, barley, and *Arabidopsis*. A similar strategy has been applied to clone candidate resistance genes from banana (Wiame *et al.*, 2000). A series of disease resistant genes were isolated from the somaclonal mutant CIEN-BTA-03 (resistant to both *M. fijiensis* and *M. musicola*) and the parent "Williams" that fall into two classes: nucleotide-binding site-leucine-rich repeat-containing kinases, and serine-threonine protein kinases of the *pto* type (Kahl, 2004). All the resistance genes were fully sequenced, and eight of them are also transcribed in the mutant, its parental genotype, "Pisang Mas" and a tetraploid *M. acuminata*. The researchers at Queensland University of Technology (QUT) have isolated the complete gene sequence of R gene candidate (*RGC-2*) from *M. acuminata* ssp. *malaccensis*, a wild diploid banana segregating for resistance to *F. oxysporum* f. sp. *cubense* (FOC) race 4. The development of *Fusarium* wilt resistant transgenic banana using this gene is in progress (Dale *et al.*, 2004).

2.1.1.3 Virus resistance

The most promising transgenic strategies for the ssDNA viruses like banana bunchy top virus (BBTV) is expressing a defective gene that encodes an essential virus life cycle activity, for instance, the replication of the virus is encoded in the replication gene or genes (*Rep*). Resultant Rep protein may retain the ability to bind to its target viral DNA but lack the functions of the *Rep* (Brunetti *et al.*, 2001). The defective Rep protein binds to the invading viral DNA and is thought to outcompete the native viral Rep protein, thus reducing or eliminating virus DNA replication. Lucioli *et al.* (2003) expressed the first 630 nucleotides of the *Rep* gene of tomato yellow leaf curl Sardinia virus (Acronymn) to generate resistance. The duration of the resistance was related to the ability of the invading virus to switch off transgene expression through post-transcriptional gene silencing (PTGS). Many researchers are trying to develop transgenic plants of *Musa* resistant to BBTV targeting the PTGS mechanism using mutated or antisense *Rep* genes.

Unfortunately, there appear to be no strategies that have been developed that generate high-level resistance to the plant dsDNA or pararetroviruses,

including the badnaviruses. Researchers at IITA, Nigeria in collaboration with the John Innes Centre (JIC), UK, are attempting to generate transgenic resistance to banana streak virus (BSV) based upon PTGS. The construct used for transformation is pC-BB with a BSV sequence from the viral reverse transcriptase-RNaseH domain under the regulation of CaMV 35S promoter. This approach involves the specific silencing of a viral gene or genes known to be involved with replication or pathogenesis.

2.1.1.4 Edible vaccine

Researchers are working to develop a banana that is an “edible vaccine” to fend off hepatitis, one of the world’s most widespread and devastating diseases. Charles Arntzen’s group at Boyce Thompson Institute for Plant Research, USA had successfully developed tobacco plants producing a vaccine against hepatitis B. It was found that the vaccine produced in plants is similar in form and function to that from human serum or recombinant yeast and provoked a strong immune response when injected into mice, while B and T-cell epitopes were preserved. Successful expression of antigens in plants was also achieved for Rabies virus G-protein in tomato (McGarvey *et al.*, 1995), Norwalk virus capsid protein in tobacco and potato (Mason *et al.*, 1996), Hepatitis B virus surface antigen in tobacco and potato (Thanavala *et al.*, 1995), *Escherichia coli* heat-labile enterotoxin B subunit (LT-B) in tobacco and potato (Hirst and Holmgren, 1987), Cholera toxin B subunit (CT-B) in potato (Arakawa, 1997). Foods under study include potatoes, bananas, lettuce, rice, wheat, soybean, corn, and legumes. Banana is a good candidate for edible vaccines since they were eaten raw, appealing to children, inexpensive to produce, native to many developing countries (Hassler, 1995). But the only limitation is the time from transformation to evaluation of fruit is 2 years or more.

Embryogenic cells of banana cv. Rasthali (AAB) have been transformed with the “s” gene of hepatitis B surface antigen (HBsAg, Kumar *et al.*, 2005). Higher monoclonal antibody binding of 67.87% of the antigen was observed when it was expressed with a C-terminal ER retention signal. HBsAg obtained from transgenic banana plants is

similar to human serum derived one in buoyant density properties. The transgenic plants were grown up to maturity in the green house and the expression of HBsAg in the fruits was confirmed by RT-PCR. These transgenic plants were multiplied under *in vitro* using floral apex cultures. Attempts were also made to enhance the expression of HBsAg in the leaves of transgenic banana plants by wounding and/or treatment with plant growth regulators. This is the only report on the expression of HBsAg in transgenic banana fruits.

2.1.2 Promoters

Plant genetic transformation is a potential tool in different areas such as manipulation and understanding of biochemical processes, knowledge of genome regulation, and integration of genes, which cannot be manipulated by classical breeding. In order to apply genetic engineering to both crop improvement and basic research, the availability of a variety of promoters that confer constitutive, i.e., expression in all cell types, tissue-specific, or developmental-specific transgene expression is required. Often a strong constitutive promoter is required to ensure sufficient expression of the transgene throughout the plant. Several such promoters have been isolated that direct strong constitutive expression in monocot plants. These include the maize ubiquitin promoter, the rice actin1 promoter, various enhanced CaMV 35S, and the synthetic pEmu promoter (Kay *et al.*, 1987; McElroy *et al.*, 1990; Christensen and Quail, 1996; Mitsuhashi *et al.*, 1996). However, the promoter activity can vary in different plant species and not all of these promoters can be used for high-level expression in all monocot species. Variation in transgene expression levels between different species and promoters may be due to different abundance of transcription factors, recognition of promoter sequences or intron splicing sites, or other factors (Wilmink *et al.*, 1995).

Most commonly used constitutive promoter is the CaMV 35S, a promoter active in most stages of development and in most plant tissues. However, it has been widely used for high-level constitutive expression in dicots, but confers lower levels of expression in monocots. Promoters isolated from monocots generally show higher activity in monocots, and adding an intron between the

promoter and the reporter gene can increase transcription levels (Rathus *et al.*, 1993). The rice actin promoter *Act1* (McElroy *et al.*, 1990) and the maize ubiquitin promoter *Ubi* (Christensen and Quail, 1996) achieved far better expression than the CaMV 35S in most monocots tested.

Effective applications of genetic engineering, however, are frequently likely to be dependent on the use of promoters specific to particular tissues or developmental events. It is desirable, for example, for nematode resistance to be expressed only after nematodes begin to feed and in the roots requiring the splicing to the resistance gene of a combination of suitable promoters. The approach for finding such promoters is to identify any gene expression with the required tissue or developmental specificity, and then to isolate the regulatory elements and test for specificity.

Currently, only a few promoters are widely used for plant transformation. Promoters can have different expression patterns even in closely related species; therefore information gathered from other transformation experiments may not be completely representative of the expression pattern of that specific promoter in banana. Therefore, the different promoters will have to be tested in banana in order to identify promoters that will be suitable for use in different transformation projects.

The production of transgenic bananas harboring genes encoding an improved genotype requires strong promoters for both constitutive and regulated high-level expression of the transgenes. Generally speaking, not enough promoters are available, though several groups are actively working to isolate useful promoter sequences, or at least fragments with promoter activity. Commonly used promoters in banana transformation are rice actin, CaMV 35S, and maize ubiquitin (Table 1). A number of monocot active promoters have been isolated from viruses infecting both dicot and monocot plants and are potential promoters for banana transformation. A promoter was isolated from sugarcane bacilliform badnavirus and showed expression in constitutive manner in banana and tobacco (Schenk *et al.*, 1999). This promoter represents a useful tool for the high-level expression of foreign genes in both monocot and dicot transgenic plants that could be used similarly to the CaMV 35S or maize ubiquitin promoter. Promoters from BSV were proved to drive high expression of *gusA* (β -glucuronidase) reporter

genes in transgenic monocot (banana, barley, maize, millet, sorghum) and dicot plants (canola, sunflower, tobacco), and also in *Pinus radiata* and *Nephrolepis cordifolia* (Schenk *et al.*, 2001). In greenhouse experiments with banana transgenics, the BSV promoter was found to be superior to the widely used maize ubiquitin and CaMV 35S promoters. Promoter regions of BBTv have been characterized. DNA segments incorporating the intergenic regions of BBTv DNA components S1 and S2 were isolated and fused to the *gusA* reporter gene to assess promoter activity in tobacco and banana (Hermann *et al.*, 2001). BBTv S1 and S2 promoters supported vascular tissue-associated reporter gene expression, and can certainly be exploited as tissue-specific promoters.

Up to date, the only constitutive heterologous promoters (the maize ubiquitin 1 and the rice actin 1 promoters) or viral promoters (derived from the BSV and BBTv) have been used for the production of transgenic banana plants (Table 1). For a number of future applications, transgenes will have to be expressed differentially or under specific abiotic (e.g., salt, wounding) and biotic stress conditions, which requires the use of a set of specific promoters to drive regulated gene expression. However, relatively few promoters are currently available for a specific or fine regulation of gene expression. It is expected that for these purposes homologous promoters will be more functional than heterologous ones, which should also raise less biosafety concerns. Many promoters have been identified directly from the banana genome by using a transfer DNA (T-DNA) tagging method with a promoterless codon-optimized luciferase (*luc*) gene (Remy *et al.*, 2005). A series of promoters has also been isolated from genes induced after challenge with a fungus. Isolation of promoters for banana transgenics, using modern technique like TAIL-PCR or other walking techniques is routine these days (Terauchi and Kahl, 2000). Commonly used terminators for banana transformation are the nopaline synthesis (NOS) and CaMV 35S.

2.1.3 Selection marker and reporter genes

Plant transformation is based on the delivery, integration, and expression of defined genes into plant cells, which can be grown to generate

Table 1 Genetic transformation of banana

Explant	Method	Constructs	Transgene	Selection marker	Reporter gene	Promoter	References
AM ^(d)	Agro ^(b) LBA4404	pBI141		<i>nptII</i> ^(l)	<i>gusA</i> ^(h)	Act1 ^(a)	May <i>et al.</i> , 1995
ECS ^(g)	MPB ^(j)	pWRG1515		<i>hpt</i> ⁽ⁱ⁾	<i>gusA</i> ^(h)	CaMV 35S	Sagi <i>et al.</i> , 1995
ECS ^(g)	MPB ^(j) Co-	pUbi-BTintORF1 ^(o) pBT6.3-Ubi-NPT ^(o)	BBTV ^(e)	<i>nptII</i> ^(l)		Ubi ^(m) BBTV ^(e)	Becker <i>et al.</i> , 2000
ECS ^(g)	MPB	pUbi-BTintORF5 ^(o) pBT6.3-Ubi-NPT ^(o)	BBTV ^(e)	<i>nptII</i> ^(l)		Ubi ^(m) BBTV ^(e)	Becker <i>et al.</i> , 2000
ECS ^(g)	MPB ^(j)	pUGR73 ^(o) pDHKan ^(o)		<i>nptII</i> ^(l)	<i>gusA</i> ^(h)	Ubi ^(m) CaMV 35S	Becker <i>et al.</i> , 2000
ECS ^(g)	Agro ^(b) EHA105	pGVSUN		<i>als</i> ^(c)	<i>gusA</i> ^(h)	Gelvin	Ganapathi <i>et al.</i> , 2001
ECS ^(g)	Agro ^(b) LBA4404 & AGL1	pCAMBIA1305.1		<i>hpt</i> ⁽ⁱ⁾	<i>gusA</i> ^(h)	CaMV 35S	Khanna <i>et al.</i> , 2004
ECS ^(g)	Agro ^(b) LBA4404 & AGL1	pART-Test7		<i>nptII</i> ^(l)	<i>mgfp</i>	CaMV 35S	Khanna <i>et al.</i> , 2004
ECS ^(g)	Agro ^(b) EHA105	pSAN164, 168	MSI-99 ^(k)	<i>nptII</i> ^(l)		Ubq3 ⁽ⁿ⁾	Chakrabarti <i>et al.</i> , 2003
AM ^(d)	Agro ^(b) EHA105	pCAMBIA1201		<i>hpt</i> ⁽ⁱ⁾	<i>gusA</i> ^(h)	CaMV 35S	Tripathi <i>et al.</i> , 2005a
AM ^(d)	Agro ^(b) EHA105	pC-BB	BSV ^(f)	<i>hpt</i> ⁽ⁱ⁾	<i>gusA</i> ^(h)	CaMV 35S	Author's lab
AM ^(d)	Agro ^(b) EHA105	pSPFLP	<i>pflp</i>	<i>hpt</i> ⁽ⁱ⁾	<i>gusA</i> ^(h)	CaMV 35S	Author's lab

^(a)Rice actin promoter^(b)*Agrobacterium*^(c)Confers resistance to sulfonylurea herbicide^(d)Apical meristem^(e)Banana bunchy top virus^(f)Banana streak virus^(g)Embryogenic cell suspension^(h) β -glucuronidase⁽ⁱ⁾Hygromycin phosphotransferase^(j)Microprojectile bombardment^(k)Magainin analog^(l)Neomycin phosphotransferase II^(m)Maize ubiquitin promoter⁽ⁿ⁾Promoter from *Arabidopsis*^(o)Plants were co-transformed with either a BBTV-derived construct or *GUS* reporter gene and a selectable marker constructs

transformed plants. Efficiency of stable gene transfer is not high even in the most successful transfer systems and only a fraction of the cells exposed integrate the DNA construct into their genomes. Moreover, a successful gene transfer does not guarantee expression, even by using signals for the regulation of transgene expression. Therefore, systems to select the transformed cells, tissues, or organisms from the nontransformed ones are

indispensable to regenerate the truly genetically transformed organisms.

The use of genes encoding for antibiotic or herbicide resistance has assisted the selection of transformants in the background of untransformed ones. The neomycin phosphotransferase II (*nptII*) gene that confers resistance to aminoglycoside antibiotics such as kanamycin and its analogs paromomycin and geneticin, and hygromycin

phosphotransferase (*hpt*) gene, which confers resistance to hygromycin have been successfully used as selectable marker in transformation of a variety of crop plants, including dicots and monocots. Similarly the *bar* (bialaphos resistance) and *pat* (phosphinothricin acetyltransferase) genes that confer resistance to the herbicide BASTA[®] and phosphinothricin respectively, have also provided an efficient selection of transgenics in a number of crops.

Herbicide or antibiotic assays, which kill or suppress nontransformed cells, while allowing proliferation of transformed tissues, are attractive, but sensitivity of tissues to the selecting agent is critical. A high incidence of untransformed “escapes” has been demonstrated in some studies; therefore, better selection markers are needed.

The *ipt* gene encodes the enzyme isopentenyl transferase and is located on Ti-plasmids of *A. tumefaciens*. This enzyme catalyzes the condensation of isopentenyl pyrophosphate with AMP to produce isopentenyl AMP, a precursor of several cytokinins (Akiyoshi *et al.*, 1984). Cytokinins stimulate organogenesis in many cultured plant tissues and are widely used to regenerate transgenic plants from cultured cells after transformation. When a chimeric *ipt* gene under the control of the CaMV 35S promoter was introduced into cells of potato, cucumber, and several *Nicotiana* species, transgenic cells proliferated and adventitious shoots differentiated in hormone-free medium (Smigocki and Owens, 1988). These transgenic plants exhibited loss of apical dominance. Therefore, it is easy to detect visually transgenic plants that carry a functional *ipt* gene. Chimeric *ipt* genes are not commonly used as selectable markers because the resulting transgenic plants lose apical dominance and are unable to root due to overproduction of cytokinins. Ebinuma *et al.* (1997) developed the multi-auto-transformation (MAT) vector system, in which the selectable marker is composed of a chimeric *ipt* gene inserted into the maize transposable element *Ac* to overcome some of the difficulties of the current transformation methods. It has been demonstrated that marker-free transgenic plants can be visually selected by using the chimeric *ipt* gene as a marker gene in tobacco plants and hybrid aspen. This method could be particularly valuable

for fruit like banana and forest trees, for which long generation times are a more significant barrier to breeding and genetic analysis.

There are several ways of determining where and when a particular gene is expressed in a plant. Most commonly used approach is the use of a reporter gene. A reporter gene produces a protein that is easily detectable in transformed organisms. Often, the protein possesses an enzymatic activity that can turn a colorless substrate into a colored product. Thus, one can see the location and amount of gene expression in a transformed organism by looking at the location and intensity of the colored product. The β -galactosidase (*lacZ*) and β -glucuronidase (*gusA*) genes are two examples of these reporter genes. When the reporter gene is fused to the promoter of the gene of interest, the reporter gene will be expressed only at the times and locations where the gene is expressed. This provides a method to detect a very limited expression of a gene, such as in small patches of cells (like root tips or pollen) or at certain times (such as after a certain stress or hormone treatment).

The green fluorescent protein (*gfp*) gene is an ideal selectable marker and reporter for gene expression analysis and plant transformation. The *gfp* gene, isolated from jellyfish, *Aequorea victoria*, encodes a small, barrel-shaped protein surrounding a fluorescent chromophore, which immediately emits green fluorescent light in the blue to ultraviolet range. Visual detection is possible at any time in living cells without their destruction and without the addition of any cofactor or external substrate. In addition, *gfp* gene product does not adversely affect cell growth, regeneration, and fertility of transformed plants. The availability of mutant forms of *gfp* differing in solubilities and emission spectra make it possible to simultaneously monitor multiple transformation events within an individual transformant (Stuber *et al.*, 1998). Green fluorescent protein (GFP) has been of great use when the organogenesis or conversion segments of transformation are inefficient under antibiotic and herbicide selection. In the beginning, GFP was used in conjunction with antibiotics and herbicides and has been shown to decrease the number of escapes for a number of tree species (Tian *et al.*, 1997). Recently, *gfp* has been used as a sole visual selectable marker in genetic transformation

of monocots like barley (Ahlandsberg *et al.*, 1999), oat (Kaeppler *et al.*, 2000), and rice (Vain *et al.*, 1998b). GFP selection system seems to be promising for tissue culture/transformation systems that are inefficient, for recalcitrant genotypes, and for plant species for which no system exists. Most commonly used reporter genes in banana transformation are *gusA* and *gfp* under the regulation of constitutive promoters Act1, CaMV 35S, and Ubi (Table 1).

2.2 Transformation Methods Employed

Genetic transformation offers an attractive means for introduction of agronomically important genes into banana cultivars. Some success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells. Gene insertion in plants can be achieved by direct gene transfer through particle bombardment or through biological vectors like a disarmed Ti (tumor inducing)-plasmid of *A. tumefaciens*. Genetic transformation using microprojectile bombardment of embryogenic cell suspension (ECS) is now routine (Sagi *et al.*, 1995; Becker *et al.*, 2000). An efficient method for direct gene transfer via particle bombardment of ECS has been reported in cooking banana cultivar Bluggoe and plantain Three Hand Plant (Sagi *et al.*, 1995). While Becker *et al.* (2000) reported the genetic transformation of Cavendish banana cv. Grand Nain.

Agrobacterium-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc.), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Shibata and Liu, 2000). Therefore, transformation of plants by *Agrobacterium*-mediated DNA transfer is currently the most commonly used phenomenon in accomplishing plant gene transfer (Lindsey, 1992). For quite some time banana was generally regarded as recalcitrant for *Agrobacterium*-mediated transformation. However, Hernandez *et al.* (1999) has reported that *A. tumefaciens* is compatible with banana indicating the potential

for genetic transformation. The recovery of transgenic plants of banana obtained by means of *Agrobacterium*-mediated transformation has been reported. The protocol has been developed for *Agrobacterium*-mediated transformation of ECS of the banana cultivars Rasthali, Cavendish, and Lady finger (Ganapathi *et al.*, 2001; Khanna *et al.*, 2004). Ganapathi *et al.* (2001) reported *Agrobacterium*-mediated transformation of shoot apex derived ECS of cultivar Rasthali (AAB) recording production of up to 40 plants per 0.5 ml packed cell volume in this cultivar. Banana functional genomics and plant improvement initiatives demand higher transformation frequencies and a standard protocol that can be used to transform all banana genomic groups including the high demand cultivars of AAA group, e.g., Cavendish. Khanna *et al.* (2004) described centrifugation-assisted *Agrobacterium*-mediated transformation protocol developed using banana cultivars from two economically important genomic groups (AAA and AAB) of cultivated *Musa*. This protocol resulted in 25–65 plants per 50 mg of settled cell volume of embryogenic suspension cells, depending upon the *Agrobacterium* strain used, and gave rise to hundreds of morphologically normal, transgenic plants in two banana cultivars from the two genomic groups.

At present most of the transformation protocols use cell suspension, however establishing cell suspension is a lengthy process and cultivar dependent. The protocol has also been established using shoot tips from various cultivars of *Musa* (May *et al.*, 1995; Tripathi *et al.*, 2005a). This technique is applicable to a wide range of *Musa* cultivars irrespective of ploidy or genotype (Tripathi *et al.*, 2003, 2005a). This process does not incorporate steps using disorganized cell cultures but uses micropropagation, which has the important advantage that it allows regeneration of homogeneous populations of plants in a short period of time. This procedure offers several potential advantages over the use of ECS as it allows for rapid transformation of *Musa* species and meristematic tissues have potential to regenerate plants from many different cultivars, unlike somatic embryogenesis, which is restricted to only a few cultivars. The transformation of meristematic cells may result in chimeric plants when only one or a few cells receive T-DNA. To

obtain uniformly transformed plants, two steps of selection and regeneration were performed to avoid regeneration of any nontransformed cells. The transformation of East African Highland bananas using meristematic tissues has also been established (Tripathi *et al.*, 2008).

Development of a highly efficient *Agrobacterium*-mediated transformation protocol for a recalcitrant species like banana requires the identification and optimization of the factors affecting T-DNA delivery and subsequent plant regeneration. Preinduction of *Agrobacterium* with acetosyringone (Khanna *et al.*, 2004; Tripathi *et al.*, 2005a) and use of surfactants like Pluronic F68 (Cheng *et al.*, 1997) have been reported to have a positive effect on *Agrobacterium*-mediated transformation. Hernandez *et al.* (1999) reported chemotactic movement and attachment of *A. tumefaciens* to wounded tissues of banana cultivars belonging to different genomic groups. Microprojectile bombardment is reported to be an effective method of wounding tissues to promote *Agrobacterium*-mediated transformation (Bidney *et al.*, 1992). We observed a significant increase in transformation efficiency measured as transient expression of reporter gene, in explants microwounded by microprojectile bombardment with naked gold particle prior to co-cultivation with *A. tumefaciens*. This improvement in transformation efficiency can be attributed to the initiation of active cell division upon wounding (Sangwan *et al.*, 1992), the improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites (Binns, 1991), and the production of *vir*-inducing compounds by the metabolically active cells (Stachel *et al.*, 1985).

Differences are known to exist between infection efficiencies of different Ti plasmid vectors and virulence of *Agrobacterium* strains. To date, the published reports of banana transformation via *Agrobacterium* described only three different strains, i.e., LBA4404, disarmed C58, and EHA101; and their derivatives (EHA105 from EHA101, AGL0 and AGL1 from EHA101) as mentioned in Table 1. *Agrobacterium* supervirulent strains EHA105 and AGL1 derivative of EHA101 were found to be better in comparison to the normal strain LBA4404 (Khanna *et al.*, 2004; Tripathi *et al.*, 2005a). Antibiotics such as cefotaxime, carbenicillin, and timentin have been used regularly in *Agrobacterium*-mediated

transformation of banana following coculture to suppress or eliminate *Agrobacterium*.

2.3 Selection of Transformed Tissue

A critical factor in transformation is the selection of transformed cells, since for each transformation event; the introduced gene(s) will be incorporated into only a fraction of the cells subjected to transformation. This selection is performed with the help of a selectable marker gene, which confers resistance to chemical agents, such as antibiotics, herbicides, which are otherwise toxic to plant cells. An effective selection process is needed to increase the efficiency of transformant recovery. For banana transformation, antibiotic resistance genes are widely used to enable this selection.

The most widely used selectable markers in monocot transformation are the genes encoding *hpt*, phosphinothricin acetyltransferase (*pat* or *bar*), and *nptII*. Use of these marker genes under the control of constitutive promoters such as the 35S promoter from CaMV, or the ubiquitin promoter from maize, works as efficiently for selection of *Agrobacterium*-transformed cells as for biolistic-mediated transformation. For banana, the *nptII* gene under the control of the NOS or CaMV 35S promoter has been used to successfully select stable transformants with kanamycin, similar to many standard dicot transformation protocols (Table 1). Kanamycin and hygromycin proved to be equally effective as selection agents (Khanna *et al.*, 2004).

Matsumoto *et al.* (2002) reported the use of *ahas* (acetohydroxyacid synthase) gene as a new selectable marker for banana transformation. Acetohydroxy acid synthase (AHAS) is the target enzyme for AHAS-inhibiting herbicides such as imidazolinones and sulfonylureas. Embryogenic cells of “Maçã” banana (*Musa* sp. AAB group, Silk) were bombarded with a plasmid vector containing the *ahas* gene, under control of the *ahas* promoter from *Arabidopsis*. The bombarded cells were regenerated on selected medium containing Imazapyr herbicide.

Dominant genes encoding either antibiotic or herbicide resistance are widely used as selectable markers in plant transformation (Yoder and Goldsbrough, 1994). The antibiotics and herbicides that select rare transgenic cells from non-

transgenic cells generally have negative effects on proliferation and differentiation. These agents may retard differentiation of adventitious shoots during the transformation process. Some plant species are insensitive to or tolerant of the selective agents and, therefore, it is difficult to separate the transformed and untransformed cells or tissues. Therefore, it is difficult to find appropriate selectable markers and to establish optimal conditions for transformation of such difficult species. Selectable marker genes remain in transgenic plants, and their gene products need to be assessed for safety and environmental impact (Flavell *et al.*, 1992).

It is difficult to introduce a second gene of interest into a transgenic plant that already contains a resistance gene as a selectable marker. There are a large number of desirable traits and genes worth incorporating into plants, but only a limited number of selectable marker genes are available for practical use. The problem becomes even more difficult if one wants to introduce a number of genes, and it is impossible to introduce them simultaneously (Goldsbrough, 1992; Yoder and Goldsbrough, 1994). It is desirable, therefore, to develop a system for the removal of selectable marker genes to produce environmentally safe transgenic plants and pyramid a number of transgenes by repeated transformation.

The positive selectable marker phosphomannose isomerase was first used for *Agrobacterium*-mediated transformation of dicot plants, i.e., sugarbeet (Joersbo *et al.*, 1998), and it has now been shown to be effective in transformation of monocots, such as rice and maize as well (Negrotto *et al.*, 2000; Lucca *et al.*, 2001). This can be a potential selection marker for banana transformation also.

2.4 Regeneration of Whole Plant

The introduction of transgene into a desired plant species for the development of stable transgenic plants requires an efficient regeneration system amenable to genetic transformation and stability of transgenes under field conditions. *In vitro* regeneration is the technique of growing plant cells, tissues, or organs isolated from a source plant. It has been found that plants can reproduce whole plants from fragments of plant material when given a nutrient media capable of supporting growth and

appropriate hormone control under laboratory condition. With the advent of gene insertion, plant cells with gene material inserted can be regenerated using *in vitro* culture to produce a whole plant. Plant regeneration of banana has been reported from various explant sources and from a variety of cultivars. Depending on different explants in combinations with various growth regulators, regeneration occurs via micropropagation or ECS.

2.4.1 Micropropagation

Micropropagation is the process by which explants having the pre-existing meristems lead to the development of multiple shoots. This is by far the most routinely applied technique used in plant tissue culture. Multiplication rate is several orders of magnitude larger than in the field. Indeed, while *in situ* a banana plant will produce about 10 shoots after 1 year, a shoot meristem *in vitro* can produce about 125–144 shoots within 8 weeks. Through repeated subculturing of proliferating shoots, an open-ended system can be maintained (Tripathi *et al.*, 2003). Propagation from existing meristems yields plants that are genetically identical to the donor plants. The shoot tip culture technique offers several additional advantages like clean planting material and *in vitro* preservation and exchange of banana germplasm.

The micropropagation technique for cultivated *Musa* is now well established (Cronauer and Krikorian, 1984; Banerjee and De Langhe, 1985). Shoot tip culture is easy and applicable to a wide range of *Musa* genotypes. An efficient regeneration protocol, which seems to be independent of ploidy level and genomic background, was developed for *Musa* species using apical meristems (Tripathi *et al.*, 2003). The selected species represent major groups of *Musa* including fertile diploid bananas (AA and BB genomes), the sterile triploid plantains (AAB), Cavendish bananas (AAA), and tetraploid hybrids (AAAA and AAAB).

Micropropagation has played a key role in banana improvement programs worldwide. Planting material derived from micropropagation performs equal to or superior to conventional material. Micropropagated plants establish faster, grow more vigorously, are taller, have a shorter and more uniform production cycle, and yield higher than conventional propagules.

2.4.2 Embryogenic cell suspension

Somatic embryogenesis is a process where a group of somatic cells/tissues leads to the formation of somatic embryos that resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable medium. ECS appear, as is the case with most monocotyledons, to be the material of choice for nonconventional *Musa* breeding.

Recently, much progress has been made in the establishment of embryogenic cell culture from banana explant sources and from a variety of cultivars. Novak *et al.* (1989) established embryogenic suspensions from somatic tissues such as leaf sheaths and corm sections of Grand Nain cultivar. For the cultivar Bluggoe, Dheda *et al.* (1991) cultured their sections from highly proliferated shoot tip cultures to produce ECS cultures. Embryogenic suspensions have also been established from immature zygotic embryos (Escalant and Teisson, 1989; Marroquin *et al.*, 1993). However, male flowers are the most responsive starting material for initiating embryogenic cultures of cv. Grand Nain (Escalant *et al.*, 1994; Cote *et al.*, 1996; Navarro *et al.*, 1997; Sagi *et al.*, 1998) and cultivar Rasthali (Ganapathi *et al.*, 2001). These suspension cultures can be regenerated into plantlets through somatic embryogenesis at high frequencies and grown in the field (Novak *et al.*, 1989; Dheda *et al.*, 1991).

Suspension culture promises a tool for faster multiplication rate than shoot tip cultures. Also, it allows for a quick response to demands and offers possibilities for automated multiplication. ECS cultures have successfully been used for cryopreservation (Panis *et al.*, 1996). The conservation of banana germplasm under cryopreserved conditions is an attractive alternative to the conservation of actively growing meristem tips. Plant regeneration from cell suspension cultures was investigated for its potential in mass propagation and as a tool in transformation using recombinant DNA technology. However, most of the procedures of developing ECS are still laborious, time consuming, and genotype dependent.

2.5 Testing

The proof of stable genetic transformation is the determination of integration of the foreign

DNA into the chromosome of the recipient plants. In early developing stages of a transformation system qualitative expression assays of the β -glucoronidase (GUS) or the GFP, encoded by the *gusA* and *gfp* reporter-genes, respectively, have been successfully used to monitor transgene delivery into banana tissues. The resulting banana transgenic plants are carefully evaluated for transgene integration, expression, and stability of transgene expression. A molecular and genetical screening is necessary to identify individual transformants with desired characteristics, such as single inserts, desired expression level, and single locus integration, etc.

Two molecular techniques, PCR and Southern blot analysis, are widely used to evaluate banana transgenic plants for transgene integration and integration patterns. Further, the expression of a transgene is evaluated at the RNA, protein, and bioassay levels. The presence of transgenic RNA demonstrates that the transgene is actively transcribed. Translation of transgene into the encoded protein can be demonstrated with Western blot or enzyme-linked immunosorbent assay (ELISA). Several bioassays have been developed for transgene expression for disease resistance in banana for example, leaf bioassay for testing gene expression for resistance to banana *Xanthomonas* wilt.

The transgenic plants are further tested for the desired agronomic performance in confined field trail. Rahan Meristem Ltd, recently conducted field trials of transgenic bananas growing plants in an area heavily infested with nematodes and the plants showed complete resistance (FreshPlaza, 2006). The nematodes could not reproduce on the transgenic banana plants developed by use of a special technology called RNA interference (RNAi).

During the last 10 years, after the public sector had developed the transformation technology, large-scale commercial producers of banana invested considerable additional resources in the research for transgenic solutions, especially to the problems of black Sigatoka disease and nematodes. Considerable progress has been made but failed to reach the stage of deploying commercially useful varieties. This effort has left a legacy of relevant technologies but the use of many of them, at least beyond the stage of experimentation, is severely limited by intellectual

property constraints, many of which remain unsolved in the absence of a clear policy on the use of transgenic banana by the private sector.

In the absence of commercial transgenic banana varieties, there has not been the level of private sector investment in biosafety testing. However, with the entry of countries such as Brazil and South Africa, which already have substantial areas of transgenic crops, into the field of genetic modification of *Musa*, more rapid progress is now expected. Meanwhile a number of developing countries are putting in place the necessary legal framework for testing and dissemination of GM crops, either specifically in order to facilitate the development of transgenic bananas, as in the case of Uganda, or in order to benefit from a range of transgenic crops that may in due course include banana and plantain.

2.6 Regulatory Measures Adopted

Regulations governing genetically modified organisms (GMOs) can potentially act at a number of key stages. Development of GM variety is a long process. Development of GM banana is in its initial stages in the laboratory, where the GMOs are produced and presence of the transgene is confirmed etc., and proceeds to field testing of the organisms produced to ensure they have the desired characteristics. Regulations at this stage may cover the conditions under which laboratory experiments take place; exchange of GM material between laboratories and conditions for testing GMOs in greenhouses, other contained facilities, or in the field. After the Research and Development stage, there may be interest in bringing the GM product to the market. Regulations here may cover assessment of the potential human health and environmental risks, to be carried out prior to eventual approval. If approval is granted, the next stage is the commercial release of the GM banana. Till date, there is no commercial release of transgenic banana and plantain.

Banana and plantain are important crops in most tropical and subtropical regions of the world. Many developing countries are beginning to develop regulations related to genetically engineered products (James, 1998). Furthermore, operational field-testing regulations have been implemented

in, for example, Argentina, Brazil, Mexico, Chile, Costa Rica, Cuba, India, Philippines, and Thailand. But still the majority of developing countries currently do not have a regulatory system for GMOs in place. Development of a regulatory framework may be a costly, time-consuming process involving extensive consultation and effort.

Many countries in sub-Saharan Africa are now establishing national biosafety committees and biosafety regulations for testing and dissemination of GM crops, specifically in order to facilitate the development of transgenic bananas in many countries like Uganda, Egypt. There are also initiatives to harmonize biosafety regulations at the regional level. The Food and Agricultural Organization (UN) is now focusing its biotechnology programme toward providing technical advice and capacity building regarding biosafety to its member governments. The Program for Biosafety Systems (PBS) is another important initiative that has been established to assist national governments in studying the policies and procedures necessary to evaluate and manage potential harmful effects of modern biotechnology on the environment and human health. The program's unique approach addresses biosafety as part of a sustainable development strategy, anchored by agriculture-led economic growth, trade, and environment objectives. Many countries have signed the Cartagena Protocol on Biosafety to the UN Convention on Biological Diversity agreed to put in place measures to ensure the safe handling of GMOs within countries and their transfer across national boundaries.

A number of existing international agreements have direct relevance to GMOs and they can be of assistance to developing countries in establishing appropriate regulatory structures that deal with potential concerns while, at the same time, promoting harmonization of national regulations at the international level.

3. FUTURE ROAD MAP

Bananas and plantains are seriously threatened by pests and numerous viral, bacterial, and fungal diseases. Thus, resistance to biotic stresses is an important part of regional or national efforts. Since all the cultivated varieties of banana are sterile

and, therefore, do not set seed, traditional breeding is more difficult than genetic transformation using molecular techniques. Although attempts to produce transgenic bananas and plantains are still proceeding too slowly, public acceptance of these novel plants and their products should already be prepared for through sound information and risk assessment, although the chances of transfer of transgenes from transgenic field material to wild species (the major public concern) are expected to be negligible in view of the sterility of many cultivars. The scope for further improvement of *Musa* species is large; along with other methods of crop improvements transgenic technologies should provide fast and effective methods of *Musa* improvement.

The number of transgenic bananas is continuously increasing. Transformation protocols, including tissue culture techniques, suitable transformation constructs with modified promoters driving one or more transgene(s), appropriate transformation techniques such as particle bombardment and *Agrobacterium*-mediated gene transfer, the detection of the transgenes and characterization of their insertion sites (copy number), are well developed. Transgenes have been used exclusively from heterologous sources rather than specifically from bananas.

3.1 Expected Products

The major research institutes/organizations and universities are concentrating on the development of two broad types of proprietary traits: (a) input traits such as pest or disease resistance and (b) output traits that improve the nutritional contents of foods or exhibit unique properties for very specific end uses or markets. Such input and output traits will be incorporated into existing elite varieties to provide material with further added value, which may offer to the farmers at lower costs or higher yield, and increased value of the end product. Initially, it is likely that transgenic banana varieties brought to the market will focus on diseases (bacterial and fungal) and pest (nematode) resistance as the technologies are in advanced stage in many laboratories. However, the long-term commercial potential of plant biotechnology is considered to be in the development of value-added output traits that will

address a wide range of specific needs or market niches.

Genetic modification of banana has also been considered as a means toward increasing the value of this crop to health and nutrition in developing countries. As a crop that is widely consumed as a weaning food by children and as a starchy staple by all sectors of the community in some countries, banana has been advocated as a carrier for vaccines and as a source of carotenoids that can counteract debilitating vitamin A deficiency. However, although much of the necessary technology is now available, these applications have yet to advance to the stage of practical evaluation.

Nutritionally enhanced crops could make a significant contribution to the reduction of micronutrient malnutrition in developing countries. Biofortification (the development of nutritionally enhanced foods) can be advanced through the application of several biotechnologies in combination. Genomic analysis and genetic linkage mapping are needed to identify the genes responsible for natural variation in nutrient levels of common foods. These genes can then be transferred into popular cultivars through breeding or, if sufficient natural variation does not occur within a single species, through genetic engineering. Vitamin and mineral deficiencies, which contribute to the deaths of millions of children each year, can be easily prevented by adding just a few key nutrients to staple food.

Transgenic plants that produce medicinal compounds such as subunit oral vaccines have already been developed, but experts concede that application of this technology is at least a decade away. There are several technical problems, which need to be addressed before plant edible vaccines become a reality in practice. Most inserted genes are expressed in very low levels in plants, which need to be enhanced. Arntzen *et al.* (2005) recently reported that a synthetic cholera vaccine gene that was more "plant" like in its sequence is four times more productive than the original gene. The stability of vaccine proteins when transgenic fruits or leaves are stored at ambient conditions is another concern. There are also concerns about oral tolerance and the dosage of oral vaccine. Only further collaborative research between plant and medical scientists may resolve these and other issues. In the near term,

the edible-vaccine technology might be better targeted at animals. In fact, such an approach may benefit agriculture as billions of dollars are spent presently on vaccinating farm animals and poultry. Transgenic plants supplying feedstock containing edible vaccines may represent the first commercial application of this intriguing technology.

The next future product can be banana varieties with longer shelf life. Banana is the most widely consumed fruit worldwide. Fruits are picked before they are allowed to ripen. They are then transported to their final destination under controlled atmospheric conditions where they are gassed with a plant hormone, ethylene, to induce ripening. Once ripening has been artificially triggered, the fruit has to be eaten or sold immediately before they spoil. First round of banana field trials by Senesco Technologies, Inc. and Rahan Meristem show that using Senesco's delayed ripening technology significantly extends the shelf life of banana. Senesco claims that banana fruit lasted twice as long as the control (nonenhanced) fruit (Crop Biotech Update, 2003). The Senesco bananas ripened normally, but the onset of spoilage and blackening that follows ripening was significantly delayed. The banana field trials indicate that the delayed ripening technology slows the process of cell death once ripening has occurred, without affecting normal growth of the plant and its fruit. This ensures that bananas are the same size, shape, weight, and color as non-enhanced bananas, with the same taste and nutritional characteristics.

Global Agricultural production has been seriously threatened with the continuing deterioration of arable land, scarcity of water, and increasing environmental stress. Over the recent decades, rainfall has seemingly become less reliable, which is putting pressure on the food security and livelihood status of smallholder farmers in sub-Saharan Africa. Drought does not only affect typical dry land but increasingly affects the more semi-humid areas where crops such as cassava and banana dominate. For example, East African highland bananas would need an annual precipitation of at least 1200 mm, but in some recent years annual precipitation in many banana growing areas dropped to below 800 mm rainfall, resulting in yield losses of more than 50% (P. Van Asten, IITA, personal communication). Conventional plant breeding

and crop physiology have had limited success in building and deploying tolerance to climatic stresses in the developing world. Recent advances in transgenic approaches to provide enhanced drought tolerance hold promise to move forward. The stress-regulated expression of *DREB1A* gene by the *rd29A* promoter has shown to confer tolerance to drought, low temperatures, and salinity in *Arabidopsis*, rice, and wheat (Dubouzet *et al.*, 2003; Pellegrineschi *et al.*, 2004). Other gene *XVSAP1* derived from the resurrection plant *Xerophyta viscosa* Baker is implicated in resistance to abiotic stress resistance (Garwe *et al.*, 2003). We anticipate that this will be a useful strategy to adopt for banana. Drought resistant banana varieties will extend the geographic spread of banana production in Africa.

3.2 Addressing Risks and Concerns

Before release, any new banana GM varieties will go through an environment and food safety analysis, in addition to variety registration procedures. Several African countries, where GM banana research is in progress, are signatories to the Cartagena Protocol on Biosafety, which deals with the conservation of biological diversity and the equitable sharing of benefits from the use of genetic resources. The Protocol seeks to protect biological diversity from potential environmental risks posed by living modified organisms (LMOs) and GMOs resulting from modern biotechnology, taking into account risks to human health and focusing on trans-boundary movement of LMOs.

There is currently no scientifically accepted evidence to suggest that transgenic crops *per se* are any more or less toxic or allergenic than their conventionally bred counterparts (Ruibal-Mendieta and Lints, 1998). Since banana is an important food and fodder crop in Africa, any effort to genetically modify the crop with eventual goal of producing food and feed, novel genes ought to be made in the context of social, economic, and political considerations of the new technology and proceed in a safe and highly responsible manner. Accordingly, several studies will be conducted on the transgenic banana to guarantee that the product placed on the market as food or feed is safe. In particular, the compositional analysis to ascertain that

transformed banana is substantially equivalent to isogenic nontransformed plants, nutritional trials for digestibility in model animals to confirm nonallergenicity using standard protocols, toxicity of transgenic banana to confirm nontoxicity in model animals and indicator species and finally acute gavage mouse studies to confirm nontoxicity will be performed before release of the GM varieties.

There are potential benefits to human health and well-being by using transgenic bananas. The insertion of genes for vitamins into staple crops such as banana can enhance their nutritional value. Genetic engineering could be used to develop pharmaceuticals and vaccines in plants, decreasing the risk of adverse reactions, and enabling faster vaccination of large populations.

A variety of concerns have been expressed regarding the impact of transgenic crops on the environment. The major potential risks of GM crop to the environment and wildlife is the gene flow. Banana is a sterile, vegetatively propagated plant lacking wild relatives in Africa meaning that the potential for outcrossing transgenic banana with other banana cultivars or other crops is negligible.

Commercial banana production requires application of large amounts of pesticides that pollute the environment, and whose residues accumulate in plantation workers. Therefore, it would be ethically justifiable to produce a transgenic banana variety that would allow for a reduction in pesticide application and a subsequent improvement in human health. Genetically engineered pest and disease resistance could reduce the need for pesticides and other chemicals, thereby decreasing the environmental load and farmers' exposure to toxins. The development of bananas resistant to pests, disease, and extreme weather, decreasing the risk of devastating crop failure will be potentially useful contributions to sustainable farming. Although weighing risks and benefits is necessary, it is neither easy nor the sole concern in considering the ethics of agricultural biotechnology.

In Western countries where food supplies are abundant and incomes are high, people can afford to be critical about the introduction of new agricultural technologies and production processes about which they are unsure. In developing economies, by contrast, the benefit/cost ratio is very different. Many food-insecure people in

developing countries live in rural areas, earn a significant share of their income from agriculture, and meet a substantial share of their food needs from their own production. For them, increasing agricultural productivity and thereby real income is a high priority. And for the urban poor in those countries, anything that lowers the effective price of basic foods is highly desirable.

Bananas and plantains are staple food crops for over 100 million people in sub-Saharan Africa and over half a billion worldwide. Despite its importance, there are many diseases and pests severely effecting banana production and threatening livelihood of millions of farmers. The production of nematode, fungus, bacterial, and virus-resistant transgenic will be a major benefit for farmers. It may also be possible to incorporate other characteristics such as drought tolerance, thus extending the geographic spread of banana and plantain production, and thus contributing significantly to food security and poverty alleviation in developing countries.

3.3 Expected Technologies

Research in the field of transgenic improvement and functional genomics in banana is constrained by low efficiency and cultivar-specific transformation systems and therefore, an efficient transformation protocol is crucial. Currently, the transformation system based on cell suspension cultures is most commonly used but development of cell suspension is time consuming and cultivar specific. Several labs are trying to develop an efficient and rapid transformation system that can be applied to broad range of cultivars.

Successful genetic transformation requires not only efficient gene delivery, but also an efficient selection system to distinguish transgenic from nontransgenic events. Several different selection strategies have been used in bananas, mainly using antibiotic or herbicide resistance genes. However, there have been concerns over the use of these selective markers. Concerns have been raised that the selectable marker genes could be transferred into microbes and increase the number of resistant pathogens, or that horizontal transmission of the marker gene into wild relatives may result in weedy pests (Nap *et al.*, 1992). Several strategies to eliminate marker genes have been employed.

Co-transformation using binary vectors carrying two separate T-DNAs was used for rice transformation and the segregation of the transgenes in the progeny allowed the retrieval of marker-free plants (Komari *et al.*, 1996). Becker *et al.* (2000) has demonstrated co-transformation in banana, which should be used further. Transient expression of the *cre/loxP* site-specific recombination system (Gleave *et al.*, 1999) together with a conditional dominant lethal gene *codA* (Perera *et al.*, 1993) resulted in the elimination of marker genes from transgenic plants without sexual crossing.

Mannose has been reported as a novel selectable agent and the phosphomannose isomerase gene (*pmi*) as its suitable selectable marker (Joersbo and Okkels, 1996). Recently, transgene removal systems denoted as MAT vectors have been developed to generate marker-free transgenic plants through a single step transformation (Ebinuma *et al.*, 1997). The introduction of the *pmi* during transformation makes this an ideal system for positive selection whereby plants containing the gene are able to survive on a medium containing mannose. This can be potential selection marker for banana transformation.

Currently, the strong constitutive promoters are routinely used for banana transformation but tissue-specific promoters might be better especially for developing pest resistant varieties. The most useful types of promoters for nematode control strategies are those providing root-specific gene expressions since roots are not consumed in bananas. There is a value in limiting spatial and temporal patterns of expression of an effector to the locale of the parasites and away from food parts of the plant. Root-specific promoters would serve this ideal and drive effective resistance against a number of nematode species. Feeding site-specific promoters would be of more specialized value against particular nematode species. Several root-specific promoters of value for nematode control have been identified at the University of Leeds, UK. Ideally the optimal promoter for nematode control would exhibit the correct spatial and temporal expression pattern only within roots and throughout parasitism.

In order to accelerate efforts in producing improved varieties of banana and plantain, a Global *Musa* Genomics Consortium was established in 2001 with the goal of assuring the sustainability of banana as a staple food crop by developing

an integrated genetic and genomic understanding, allowing targeted breeding, transformation, and more efficient use of *Musa* biodiversity. Basically, the consortium aims to apply genomics to the sustainable improvement of banana and plantain. The consortium believes that genomic technologies such as analysis and sequencing of the banana genome, identification of its genes and their expression, recombination and diversity can be applied for the genetic improvement of the crop (Frison *et al.*, 2004). Genetic and physical mapping of the *Musa* genome will make it possible to isolate genes that can be used in genetic transformation.

Currently, no transgenic bananas and plantains are commercially available; however there is enormous potential for genetic manipulation of *Musa* species for disease and pest resistance using the existing transformation systems. Using molecular techniques, novel genes encoding agronomically important traits can be identified, isolated, characterized, and introduced into cultivars via genetic transformation. The use of appropriate gene constructs may allow the production of nematode, fungus, bacteria, and virus-resistant plants in a significantly shorter period of time than using conventional breeding, especially if several traits can be introduced at the same time. Long-term and multiple disease resistance can be achieved by integrating several genes with different targets or modes of action into the plant genome. Technically, this can be done either in several consecutive steps or simultaneously. Plant biotechnology has the potential to play a key role in the sustainable production of *Musa*.

3.4 Intellectual Property Rights (IPR), Public Perceptions, Industrial Perspectives, Political and Economic Consequences

Most innovations in biotechnology are developed using the knowledge or technologies generated from previous innovations. Many plant biotechnology products or techniques are “modular” in that they are assembled from a number of previously developed technologies/transgenes, each of which may be subject to a separate patent. The commercialization of many proprietary biotechnology products is typically contingent on other proprietary biotechnology products or

processes and in particular on agreements between IPR holders regarding the relative contributions of different proprietary technologies to the product in question. Many biotechnology products (e.g. transgenic seeds or transgene constructs) now have a complex IPR pedigree because a large number of proprietary products or processes are involved in developing the product.

The current generation of transgenic bananas and their testing, however, highlights some problems that need to be avoided in future. Some genes of agronomic interest were owned by the industry, and it took much effort by the Catholic University of Leuven before these genes could be used freely for plantain and cooking bananas. Therefore, it is urgent that a mechanism is put in place whereby an authority at the global level will interact with the industry to negotiate access to protected technologies for developing countries. However, in the case of food production by smallholders like banana and plantain, it is absolutely necessary that technologies are royalty free and that the farmers should have privilege to save seed (or planting material) for subsequent replanting and distributed from farmer to farmer without any financial return to the industry. It is clear that delivery of GM products to smallholder farmers in Africa will be expensive. Remington and Bramel (2004) had described handling of GM seeds for small farmers in Africa through partnership with NGOs.

Currently, a number of institutions in Africa are focused on developing and promoting biotechnology for farmers (CBI, 2003). Foundations, international organizations like CGIAR, NGOs, and national governments are promoting the potential benefits of biotechnology in addressing the critical need to increase food production in Africa. Several research networks have been established to promote biotechnology research in crops such as cassava, banana, rice, sweetpotato, and cowpea that can benefit the poorest farmers in Africa.

Donor initiatives are promoting research and development of biotechnology products with public/private partnerships, and donors and international organizations initiatives will strengthen biosafety regulation and technical expertise in developing countries to enhance the application of biotechnology products. Novel partnerships are being formed between the private sector, donors,

and nonprofit organizations to find common and acceptable grounds. For example, many donor agencies are providing ways for North–South partnerships to open up African markets in a mutually beneficial and sustainable manner. African Agricultural Technology Foundation (AATF) was launched in 2003, whose mission is to acquire technologies through royalty-free licenses along with associated materials and know-how for use on behalf of SSA's resource-poor farmers, while complying with all laws associated with the use of these technologies. AATF has been instrumental in facilitating technology transfer negotiations whereby proprietary biotechnologies have been made available to Africa. Recently, IITA has negotiated royalty-free license from Academia Sinica, Taiwan through AATF for access of technology for bacterial wilt resistance. AATF has signed the licensing agreement with Academia Sinica, Taiwan for access to the *pflp* gene for use in banana for the commercial production of varieties resistant to banana bacterial wilt in all of sub-Saharan Africa and granted a sublicense to IITA for developing the improved varieties.

In view of the tremendous losses incurred by smallholder *Musa* farmers due to pest and disease attack, and the economic, environmental, and health costs associated with plant protection measures in large-scale commercial plantations of banana, it is very much to be hoped that the remaining technical, intellectual property, and regulatory obstacles to the deployment of transgenic bananas and plantain can soon be overcome. It is not anticipated that transgenic varieties will threaten the diversity of existing varieties grown by banana farmers. Rather, studies in East Africa suggest that varieties modified for pest or disease resistance will be incorporated into the range of varieties already grown as part of a strategy to reduce risk, provide multiple products, and satisfy varying tastes. In the meantime, various biotechnology techniques are already contributing to conventional breeding efforts and are expected to become even more effective in this area as genetic maps and markers are refined. The use of tissue culture plants is already contributing to the development of novel production systems for smallholder farmers and, as part of a balanced program of deploying biotechnology techniques cost effectively in the developing countries, tissue culture is expected to be much more widely used

in increasing the productivity and sustainability of such systems in the future.

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Pineapple

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Pineapple, *Ananas comosus* (L.) Merr., is native to South America and was first seen by Europeans when Christopher Columbus landed on an inhabited island in the Caribbean that he named Guadalupe on his second voyage in 1493 (Collins, 1948). Little is known regarding the domestication of the pineapple before 1493 except that at that time the plant was already in cultivation throughout the West Indies, and most likely on the mainland of Brazil (Collins, 1951). Although widespread in cultivation and in the wild, work by Duval *et al.* (2005) with restriction fragment length polymorphism markers of chloroplast DNA supports the hypothesis of a center of diversity and hence origin of the domesticated pineapple in Guyana, north of Brazil. The *Ananas* genus is, however, generally considered indigenous to Brazil and northern Paraguay (Duval *et al.*, 2003). In early times, the pineapple was used extensively by the Amerindians of these regions as a fresh fruit, for making wine and twine, and for medicinal purposes (Collins, 1949, 1951; Leal and Amaya, 1991; Leal and Coppens d'Eeckenbrugge, 1996). From the Americas, pineapples were taken back to Europe to be grown in the hothouses of the wealthy gentry but eventually they spread around the globe predominately by seafaring Portuguese traders. Pineapple is now grown in most tropical and subtropical countries.

Extensive collections of wild germplasm are held in Brazil, Martinique, and Venezuela (Ferreira and Cabral, 1993; Coppens d'Eeckenbrugge *et al.*, 1997).

1.2 Botanical Description

The pineapple is a monocotyledonous, perennial, herbaceous plant with crassulacean acid metabolism (CAM) photosynthesis. The CAM system of photosynthesis gives the plant good water-use efficiency and the ability to withstand extreme water deficit (Malezieux *et al.*, 2003). The fruit, a sorose of multiple fused fruitlets, is borne on the terminal. New shoots capable of producing another fruit arise from axillary buds (Okimoto, 1948; Coppens d'Eeckenbrugge and Leal, 2003).

1.2.1 Taxonomy

There are three subfamilies within the family Bromeliaceae—Bromelioideae, Pitcairnioideae, and Tillandsioideae. The subfamily Bromelioideae contains 27 genera and 536 species (Brown and Gilmartin, 1986) including the pineapple genus *Ananas*. The closely related genus, *Pseudananas*, contains the single species *P. sagenarius*. The taxonomy of pineapple has been reviewed several times but the most enduring is the current system based on the key of Smith and Downes (1979). In this system, the genus *Ananas* contains seven

species—*A. comosus*, *A. ananassoides*, *A. nanus*, *A. bracteatus*, *A. parguazensis*, *A. fritzmuelleri*, and *A. lucidus*. This classification is, however, based largely on traits that are governed by only a few genes or that are continuous and strongly influenced by the environment (Leal and Coppens d'Eeckenbrugge, 1996). Molecular studies (Duval *et al.*, 2001, 2005) indicate gene flow and continuous variation between the species with no clear indicators of speciation. All these species are also interfertile. It has been proposed by Coppens d'Eeckenbrugge and Leal (2003) and supported by Duval *et al.* (2005) that all the seven species of *Ananas* should be reclassified as varieties within the one species, *A. comosus*, and that *P. saganarius* should be reclassified as a species of *Ananas* called *A. macrodontes*.

The wild species, *A. ananassoides*, shows the greatest level of polymorphism and is considered the ancestor of all domesticated genotypes. *A. fritzmuelleri* appears closely related to *A. macrodontes*, and *A. lucidus* appears closely related to *A. ananassoides*. *A. bracteatus* appears close to *A. fritzmuelleri* and *A. macrodontes*.

1.2.2 Genetics

A. comosus has a diploid chromosome number of 50 with small chromosomes (Collins and Kerns, 1931; Marchant, 1967; Brown and Gilmartin, 1986; Brown *et al.*, 1997). Within *Ananas*, triploid, tetraploid, and heteroploid varieties exist (Capinin and Rotor, 1937; Collins, 1960). Triploids arise when unreduced egg gametes are fertilized by normal haploid pollen (Collins, 1933). *P. saganarius* is a naturally occurring tetraploid (autopolyploid) with 100 chromosomes (Collins, 1960; Duval *et al.*, 2005). Triploids are generally more vigorous than diploids (Collins, 1933) and can be commercially acceptable (Leal and Coppens d'Eeckenbrugge, 1996), but tetraploids are generally inferior to triploids and diploids. While very vigorous in growth, tetraploids produce small fruit with lower sugar content (Kerns and Collins, 1947).

Collins and Kerns (1931) reported that meiosis was regular in *A. comosus* and only rarely were there irregularities, such as lagging chromosomes or abnormal tetrads. However, Coppens d'Eeckenbrugge *et al.* (1993) demonstrated a low

level of pollen fertility (low production and poor viability) in *A. comosus* that could be at least partly attributed to meiotic irregularities.

Differences in size of mature pollen grains are common (Collins and Kerns, 1931). This size variation has been associated with meiotic irregularities (Bhowmik and Bhargabati, 1975; Bhowmik, 1977). Large or giant pollen cells are thought to contain the diploid number of 50 chromosomes (Collins, 1960).

The number of ovules per flower varies considerably within *A. comosus* from 16–27 in the Queen group to a maximum of 71 in the Perolera group. Rao and Wee (1979) reported 24–84 ovules per flower for the different *A. comosus* types. Few of these ovules develop into seed (Coppens d'Eeckenbrugge *et al.*, 1993). Even where highly compatible varieties are manually hand pollinated, there are usually no more than approximately 20 seeds per flower ever obtained in *A. comosus* types (Sanewski, unpublished data). Cleistogamy can occur in some varieties of *A. comosus* (Chan *et al.*, 2003).

Most varieties of *A. comosus* are self-incompatible due to the inhibition of pollen tube growth in the upper third of the style (Kerns, 1932), which is gametophytically controlled by a single locus with multiple alleles (Brewbaker and Gorrez, 1967). Some cultivars exhibit partial incompatibility (Cabral *et al.*, 2000). The primitive types, *A. ananassoides*, *A. bracteatus*, and *P. saganarius*, are either partially or completely self-compatible.

1.3 Economic Importance

The pineapple is a versatile plant and yields many products. In addition to the familiar canned, dehydrated, and juice products, the fruit can also be minimally processed into a fresh but peeled, cored, and sliced product. The peel and core refuse from processing are often used as cattle feed. The leaf has long been used for its fiber, which can be made into a range of products from high-quality cloth and paper to composite plastics (Hepton and Hodgson, 2003). The plant is occasionally used for ornamental purposes and a small market exists for the flowers of some genotypes.

Crude extracts from the fruit, stem, and leaves yield, among other compounds, several

proteinases, most notably bromelain, which has a range of pharmaceutical uses based only partly on its proteolytic activity. Activities of bromelain include antiedematous, anti-inflammatory, antithrombotic, fibrinolytic, and immunomodulatory (Maurer, 2001).

World production of pineapple was estimated at over 18.2 million tonnes in 2006 (FAOSTAT, 2008). While much of the world trade in pineapple is as processed product, approximately 70% of production is consumed fresh in the countries of origin (Rohrbach *et al.*, 2003). The leading producers are Thailand, the Philippines, Costa Rica, China, Brazil, and India. The export value of pineapple and pineapple products from the producing countries was over US\$665 million in 2004 (FAOSTAT, 2008). Most of the world's export production (about 70%), and most of the canned pineapple (about 95%), comes from a single cultivar, Smooth Cayenne (Rohrbach *et al.*, 2003).

1.4 Traditional Breeding

It is generally recognized that the indigenous people of South America contributed substantially to the domestication of the pineapple (Leal and Coppens d'Eeckenbrugge, 1996) probably through the selection of spontaneous mutations expressing desirable traits. The types found growing in and around villages usually exhibit desirable traits such as improved palatability, improved fruit size, seedlessness, smooth leaves, and, in some cases, improved leaf fiber properties, which are not commonly found in wild types (Collins, 1951; Coppens d'Eeckenbrugge *et al.*, 1997).

The first major hybridization program was conducted by the Pineapple Research Institute (PRI) in Hawaii in several phases from 1917 to 1972 (Wortman and Kerns, 1959; Williams and Fleisch, 1993). The objectives initially were to replace Smooth Cayenne as a processing cultivar but eventually expanded to include fresh fruit. Specific objectives included varieties more suited to summer, winter, tropical conditions, high-density plantings, mechanical harvesting, and heart and root rot resistance (Smith, 1965). Both intra- and interspecific crosses were conducted and selection encompassed many aspects of productivity, fruit quality, and pest and disease

resistance. The PRI program developed cultivars more resistant to root and heart rot, pink disease, fruit marbling, fruitlet core rot, and with some tolerance to mealybug wilt and root knot and reniform nematodes. Resistance to the physiological disorder, blackheart, was also demonstrated as well as improvements in ascorbic acid content, yield, canned product quality, fibrosity, and higher or lower acidity (Dull, 1965; Rohrbach and Pfeiffer, 1975; Williams and Fleisch, 1993; Rohrbach and Schmidt, 1994). Despite the considerable effort that was expended in this breeding effort, few varieties were commercialized at that time. Most varieties eventually exhibited a fault that precluded large-scale commercialization (Williams and Fleisch, 1993). In more recent years, the declining margins in processed pineapple have seen an increase in interest in the fresh fruit sector. There has consequently been a reappraisal of PRI varieties and at least four varieties have now been commercialized to some extent for the fresh fruit market.

Many pineapple-producing countries now conduct hybridization and selection programs. The most notable programs exist in Australia, Brazil, Martinique, Malaysia, and Taiwan. Most breeding has utilized the different varieties of *A. comosus* such as Cayenne, Queen, Mordilona, Spanish, and Pernambuco or hybrids thereof to produce segregating populations of seedlings for screening and selection. Objectives are usually aimed at suitability for the fresh fruit market and, in some cases, include additional characteristics such as resistance to Fusariose caused by *Fusarium moniliforme* var. *subglutinans* (Cabral *et al.*, 1993, 1996; Hidalgo *et al.*, 1998), improved eating quality (Sanewski, 1998), reduced incidence of translucency (Leal and Coppens d'Eeckenbrugge, 1996), and a shorter cropping cycle (Chan and Lee, 2000).

Most domesticated varieties of the pineapple are considered highly heterozygous (Chan, 1989; Sanewski, 1998). Most pineapple hybridization programs have, therefore, achieved only limited success despite considerable effort (Chan *et al.*, 2003). Selfing has been proposed as a strategy to increase the level of homozygosity and purge undesirable recessive alleles from the breeding gene pool (Coppens d'Eeckenbrugge *et al.*, 2005). However, the low level of self-compatibility in most commercial varieties makes this somewhat

impractical. Lower levels of inbreeding may, therefore, be more appropriate.

Clonal selection has also been used to improve varieties, particularly Smooth Cayenne (Collins and Kerns, 1938; Chan *et al.*, 2003).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

The high level of heterozygosity in domesticated pineapple has been the main cause of the very limited success in hybridization breeding programs worldwide. Sexual recombination of the many chromosomes coupled with a large number of selectable characteristics has meant that improvements in some characters usually come at the expense of a decline in others. Nevertheless, new hybrids specifically aimed for the domestic fresh fruit markets have been developed. A first result of these efforts has been the successful introduction of a low-acid cultivar by Del Monte from Costa Rica into European and American markets (Rohrbach *et al.*, 2003). However, sufficiently significant improvements in some characteristics, such as resistance to nematodes or mealybug wilt, have not been possible, because the resistance systems do not exist at a useful level within the *Ananas* gene pool.

Smooth Cayenne is the main variety used for processing and has been grown on a large-scale commercial basis throughout the world since the early 1900s. Production and processing practices are, therefore, highly developed for Smooth Cayenne. For new cultivars to be acceptable to growers and processors, new cultivars must be very similar to Smooth Cayenne in agronomic and processing attributes. Because Smooth Cayenne is highly heterozygous and imparts considerable transgressive recombination, conventional hybridization is unsuitable for developing new processing types if the outcomes are to be achieved in a reasonable time frame. Transformation with engineered genes is ideally suited to improving Smooth Cayenne. It should allow specific changes to be made to targeted genes without rearranging the entire genome.

Although the length of time and costs of the initial transformation research necessary to clone the suitable genes and produce the transgenic

plants can be high, once the plants have been obtained the process is relatively cheaper and more straightforward than conventional breeding. Extensive field trials at multilocal sites and factory evaluation will still be necessary to obtain the best plant for commercial release.

2. DEVELOPMENT OF TRANSGENIC PINEAPPLES

Methods involving the introduction of recombinant DNA to pineapple cells and tissues are via *Agrobacterium tumefaciens*-mediated transformation and direct gene transfer through microprojectile bombardment. Reviews including sections on genetic engineering and molecular genetic improvement of pineapple have been published (Smith *et al.*, 2002b, 2005; Botella and Smith, 2008). However, 2006 saw the publication of a number of important studies on pineapple transformation. For instance, two groups employed biolistics technology to introduce economically important traits, i.e., herbicide resistance (Sripaoraya *et al.*, 2006a, b) and blackheart resistance (Ko *et al.*, 2006). Other groups focussed on using *Agrobacterium* infection techniques (Yabor *et al.*, 2006), also Firoozabady *et al.* (2006) and Trusov and Botella (2006) who reported on further evaluation of the expression of a useful 1-aminocyclopropane-1-carboxylate (ACC) synthase gene for fruit ripening.

2.1 Donor Genes

2.1.1 Source

A limited range of donor genes have been employed for pineapple transformation. To optimize transformation conditions, usually the β -glucuronidase (*gus* or *uidA*) reporter gene, originating from *Escherichia coli*, has been used. The green fluorescent protein (*gfp*) gene from *Aequorea victoria* has also been used to monitor transformation (Graham *et al.*, 2000a; Ko *et al.*, 2006), with the advantage that analysis of expression of this gene can be monitored over time in the same tissues as it is nondestructive.

As selectable marker genes, both herbicide-resistant genes and antibiotic-resistant genes are used. The most common herbicide-resistant genes introduced to pineapples are the *bar* gene (Sripaoraya *et al.*, 2001, 2006a, b; Yabor *et al.*, 2006) and the *surB* gene. The *bar* gene is obtained from *Streptomyces hygroscopicus* and inactivates phosphinothricin (PPT), conferring resistance to the herbicide bialaphos, but it also converts glufosinate ammonium, the active component of the commercially manufactured herbicide BastaTM, to an inactive form. The acetolactate synthase *surB* gene is derived from tobacco and confers resistance to chlorsulfuron, a sulfonylurea (Firoozabady *et al.*, 2006). The neomycin phosphotransferase (*nptII*) gene, conferring resistance to the antibiotics kanamycin, paromomycine, and geneticin (G418), is also extensively used.

Only a few economically important transgenes have been introduced into pineapple thus far. One of the most studied are the ACC synthase genes (*acacs1*, *acacs2*, and *acacs3*; Botella *et al.*, 2000; Trusov and Botella, 2006) to control natural flowering and to delay ripening. These genes have been isolated from the pineapple plant itself. The ACC synthase gene from pineapple is expressed in meristematic cells and activated to induce flowering under certain environmental conditions (low temperatures and photoperiod). Although pineapple is a nonclimacteric fruit, ethylene is still believed to play a significant role during ripening. Considering harvesting costs take up 34% of the total production costs, synchronized flowering will minimize harvesting and production costs and hence improves the competitiveness of the industry. To enhance *acacs* gene expression, a complex promoter–leader–intron structure was inserted at the 5' end of the transgenes, described by Firoozabady *et al.* (2006) and Trusov and Botella (2006). A *waxy* gene leader derived from *Zea mays* was fused to the *acacs* gene and an intron derived from the *CHS-A* gene of *Petunia hybrida* was inserted into the central region of the *waxy* leader.

Polyphenol oxidase (PPO) genes to control blackheart (*PINPPOI*; Stewart *et al.*, 2001) have also been isolated from blackheart-affected fruit. Blackheart occurs when temperatures in subtropical pineapple growing areas reach below 20 °C combined with lower light intensities in the

winter months. Alternatively, this occurs during cold transport of fruit followed by higher storage temperatures, of relevance in export of fresh fruit. The injury is evident around the core of the fruit and is, therefore, not detectable on intact fruit, resulting in wastage and decreased consumer confidence in the fruit. The *PINPPOI* gene has been used in sense, antisense, and hairpin constructs in an effort to silence PPO expression in pineapple fruit (Ko *et al.*, 2006).

Espinosa *et al.* (2002) and Yabor *et al.* (2006) included two antifungal genes (*chi* and *ap24*) in their studies, in an attempt to reduce losses caused by *Phytophthora nicotianae* var. *parasitica*. The class-I chitinase gene originates from *Phaseolus vulgaris*, while the *ap24* gene is derived from *Nicotiana tabacum*. Their studies are based on degrading fungal cell wall compounds and destabilizing fungal membrane potential. The *gluc* gene is a class-I tobacco β -1,3-glucanase gene.

2.1.2 Designing transgenes

A study on promoters was reported by Graham *et al.* (2000a), whereby transient assays using the biolistic gene delivery technique were monitored to define the activity of a number of promoters in various pineapple tissues. Promoter-*gus* gene fusions were delivered into callus, leaf, and fruit tissues and β -glucuronidase (GUS) activity determined after 48 h. Constructs containing the *Ubi-1* promoter showed strong activity in all tissues, as did the cauliflower mosaic virus (CaMV) 35S promoter. Other promoters to drive selectable marker genes were also examined and subclover stunt virus (SCSV) and banana bunchy top virus promoters were found to drive high-level marker gene expression, particularly in callus tissue.

A summary of genes, promoters and terminators used in published pineapple transformation studies is given in Table 1.

Firoozabady and Gutterson (1998) and Firoozabady *et al.* (2006) described the use of a disarmed *Agrobacterium* strain, C58, which was superior for carrying a binary vector containing the *surB* gene with a number of different plasmids tested. *Agrobacterium* strains LBA4404 and EHA101 were also used, but these yielded a

Table 1 Summary of published transgenes introduced into transformed pineapple plants

Reference	Promoter	Transgene	Selectable marker	Terminator	Comment
Nan <i>et al.</i> , 1996	Various	<i>gus</i>		Not reported	Biolistics
Graham <i>et al.</i> , 2000a, b	nos or SCSV4		<i>nptII</i>	nos/SCSV5	<i>Agrobacterium</i>
	CaMV 35S	<i>gus</i>		ocs	
	CaMV 35S	<i>gfp</i>		nos	
Sripaoraya <i>et al.</i> , 2001	Ubi-1	<i>gus (uidA)</i>	<i>bar</i>	Not reported	Biolistics
Espinosa <i>et al.</i> , 2002	CaMV 35S	<i>uidA (gus)</i>	<i>hph</i>	nos	<i>Agrobacterium</i> ;
	nos		<i>nptII</i>	nos	combined antibiotics resistance
	ocs-35S	<i>chi</i>		nos	Hybrid promoter for
	CaMV -pA5	<i>gluc</i>	<i>bar</i>	nos	<i>chi</i> ; pA5 = rice actin
	35S CaMV			nos	I promoter
Also Yabor <i>et al.</i> , 2006	Hybrid pA5	<i>chi</i>		nos	
	35S CaMV	<i>ap24</i>	<i>bar</i>	nos	
	Ubi-1			nos	
Firoozabady <i>et al.</i> , 2006	Ubi-1		<i>surB</i> and <i>nptII</i>	nos	<i>Agrobacterium</i>
	Smas	<i>gus</i>		nos	
	Ubi-1		<i>surB</i>	ocs	
	d35S	<i>acacs3</i>		ocs	d35S = enhanced
			<i>surB</i>		CaMV35S promoter
Also Trusov and Botella, 2006	Smas	<i>acacs2</i>		ocs	
	Ubi-1		<i>surB</i>		
Ko <i>et al.</i> , 2005, 2006	CaMV 35S	<i>gus</i>		ocs	Biolistics
	SCSV4	<i>gus</i>		SCSV5	
	Ubi-1	<i>gfp</i>		nos	
	CaMV 35S	<i>ppo</i>		ocs	
	CaMV 35S and Ubi-1	<i>opp.i.ppo</i>		nos and ocs	CaMV35S-OPP-nos.i. Ubi-1-PPO-ocs
	CaMV 35S		<i>nptII</i>	CaMV 35S	Co-bombardment with
	SCSV4		<i>nptII</i>	SCSV7	transgene

much lower level of GUS activity. The plasmids contained the following constructs:

- *nptII* gene and *surB* gene both driven by ubiquitin (*Ubi-1*) promoter from *Z. mays* allowed selection of transgenic tissue by two agents,
- *gus* gene driven by *Smas* promoter and *surB* gene driven by *Ubi-1* promoter were constructed to monitor DNA delivery and transformation by GUS expression and to apply selection using chlorsulfuron,
- *surB* gene driven by *Ubi-1* promoter and ACC synthase gene induced in fruit during ripening (*acacs2*) driven by *Smas* promoter to introduce fruit ripening control gene into pineapple and to apply chlorsulfuron selection,
- ACC synthase gene expressed in meristem (*acacs3*) driven by an enhanced CaMV 35S promoter to control precocious flowering.

Agrobacterium strains LBA4404 and AT2260 were used by Espinosa *et al.* (2002). The plasmids contained the following constructs:

- *nptII* gene under control of *nos* promoter, as well as *gus (uidA)* gene and *hph* gene conferring resistance to hygromycin, both driven by CaMV 35S promoter,
- *chi* gene under control of a hybrid *ocs*-CaMV 35S-*rice actin I (pA5)* promoter, plus the *ap24* gene driven by CaMV 35S promoter for fungal tolerance, and the *bar* gene under control of *Ubi-1* promoter,
- *chi* gene under control of *pA5* promoter, plus *gluc* gene under control of CaMV 35S promoter, and the *bar* gene driven by *Ubi-1* promoter.

Graham *et al.* (2000a, b) used *Agrobacterium* strain AGL0. This strain was transformed with

binary vectors containing constructs designed to express a number of genes. The following constructs were used in their studies:

- selectable marker gene *nptII* driven by *nos* or SCSV4 promoter and SCSV5 terminator,
- *gus* reporter gene driven by CaMV 35S promoter,
- *gfp* gene, with the CaMV 35S promoter operably linked to the gene.

Based on findings of Graham *et al.* (2000a) and taking intellectual property (IP) issues with available promoters into consideration, the following constructs for biolistics transformation were then prepared and used by Ko *et al.* (2005, 2006):

- two plasmids expressing the *nptII* selectable marker gene driven by CaMV 35S promoter and terminator, or controlled by segment 4 promoter and segment 7 terminator coding sequences from SCSV, cloned into the polylinker site of the expression caset pART7 (Gleave, 1992),
- two *gus* marker gene constructs, one driven by CaMV 35S constitutive promoter and the octopine synthase region of *A. tumefaciens* (*ocs*) terminator, the other controlled by SCSV4 promoter and SCSV5 terminator,
- *gfp* reporter gene under control of the maize *Ubi-1* promoter and the nopaline synthase region of *A. tumefaciens* (*nos*) terminator,
- pineapple *PINPP01* gene in a sense orientation (*ppo*) controlled by CaMV 35S promoter and *ocs* terminator, or in a sense–antisense orientation including a hairpin RNA interference (RNAi) intron (*opp.i.ppo*), whereby CaMV 35S promoter and *nos* terminator are driving the *opp* gene, and *Ubi-1* promoter and *ocs* terminator controlling the *ppo* gene.

According to Smith *et al.* (2000) using the hairpin RNAi strategies greatly increases inactivation of gene expression.

Target genes were co-transformed with the selectable marker gene in a 3:1 molar ratio.

For the development of herbicide resistant pineapple plants, Sripaoraya *et al.* (2001) transformed pAHC25 containing the *gus* (*uidA*) reporter gene and the *bar* selectable marker gene, both driven by maize *Ubi-1* promoter with its first

exon and first intron. Transgenic plants were tested by treatment with Basta.

2.2 Methods Employed

Transformation efficiencies of pineapple through either particle bombardment or *Agrobacterium*-mediated gene delivery are generally around or below 5%, based on the number of transgenic events per number of explants subjected to the transformation process. Firoozabady *et al.* (2006) reported a transformation efficiency of up to 60% for transgenic lines of the Smooth Cayenne cultivar “Champaka” containing the *acacs* genes expressed in the ripening of pineapple fruit. This transformation efficiency was based on the number of transgenic lines produced per weight of cells subjected to treatment.

2.2.1 *Agrobacterium*-mediated transformation

Firoozabady and Gutterson (1998) and Firoozabady *et al.* (2006) described the use of two embryogenic systems for transformation of pineapple—a friable embryogenic cell cluster (ECC), with potential for somatic embryo production, and a chunky nondispersible embryogenic tissue (ET), which consists of a cluster of immature somatic embryos. After 2 to 3 days of co-cultivation in the dark, explant material was placed on a recovery medium without selection pressure for 5–12 days. ET needs sonication and vacuum infiltration to allow the bacteria to penetrate into the tissue. Embryogenic tissue was then subjected to a sequence of treatments on maturation media containing 1 mg l⁻¹ BA (6-benzyl adenine) and an increasing concentration of chlorsulfuron for 3–4 months until globular embryos were produced or germination took place. Shoot clusters were then produced, followed by shoot multiplication. Up to 90% of treated tissue produced shoots within 4 months. Shoot elongation occurred on a medium containing 0.2 mg l⁻¹ BA, followed by root initiation on 1/2 MS + 0.5 mg l⁻¹ each of α -naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA), and activated charcoal. Shoot clusters were maintained indefinitely in liquid medium containing 1.5 mg l⁻¹ each of BA and NAA, with monthly subcultures.

Selection was continued throughout the regeneration process to eliminate chimeric plants.

Ko *et al.* (2005), on the other hand, co-cultivated *in vitro*-grown leaf bases with *Agrobacterium* bacterial suspension under 5 min of vacuum infiltration at 27–29" Hg, to assist with penetration into the plant cells. Co-cultivation occurred for a period of 3 weeks under a natural daylight cycle, followed by selection on a medium containing 50 mg l⁻¹ G418 with monthly subcultures twice, then increasing the G418 concentration to 100 mg l⁻¹ (Graham *et al.*, 2000b). After 3 to 5 months, transformed organogenic callus was produced, and after a further 3 to 4 months, shoots were regenerated from the transgenic callus. In total 63 transformed lines were recovered, with an average transformation efficiency of 2.4%. Compared to a biolistics technique (Ko *et al.*, 2006) this method only requires tissue to go through one callus phase for 4 to 5 months, thereby minimizing the chance for somaclonal variation. Furthermore, to produce sufficient explant callus to achieve similar numbers of transgenic lines produced through the microprojectile bombardment strategy (see Section 2.2.2), it would take an additional 12 months of *in vitro* culture.

The introduction of antifungal genes *chi* and *ap24* into callus tissue has been achieved by Espinosa *et al.* (2002) and Yabor *et al.* (2006), with up to 6.6% efficiencies of transgene plant recovery reported. Espinosa *et al.* (2002) introduced the application of temporary immersion bioreactors (TIBs) in the selection and micropropagation phase of the process. Following the initial studies, Yabor *et al.* (2006) applied the system to study biochemical side effects of *Agrobacterium*-mediated pineapple genetic transformation on the plants. The presence and amounts of compounds related to the pathways describing responses to stress and photosynthesis were measured.

In all *Agrobacterium*-mediated experiments, care should be taken to prevent the bacteria from overgrowing the tissue sample during co-cultivation, while after the relevant co-cultivation time, the bacteria needs to be killed without the explants being affected. Shackelford and Chlan (1996) and Singh Gill *et al.* (2004) reported on experiments performed to determine which antibiotics are most efficient for this purpose. Different strains of *Agrobacterium* appear to have different sensitivities to different antibiotics, while

some antibiotics were found to be detrimental to callus growth by decreasing transformation efficiencies or interfering with the regeneration process (Firoozabady *et al.*, 2006). Cefotaxime appeared to be the most popular antibiotic used in the reported studies.

2.2.2 Biolistics transformation

Biolistics has also been used to good effect with pineapple transformation. Early studies by Nan *et al.* (1996) reported on transient expression experiments using the *gus* reporter gene and various promoters on a variety of tissues (parts from crowns, slips, and lateral buds of field-grown plants); however, no further reports of production and evaluation of transformed plants have been published. Recent studies by Ko *et al.* (2006) have described stable integration of genes introduced by microprojectile bombardment of callus. The *PINPP01* gene was isolated and used in a construct that was delivered to 17–35-weeks-old "Smooth Cayenne" callus cultures by way of co-bombardment with a plasmid conferring *nptII* resistance, using G418 as the selective agent. From four experiments, each bombarded with the *gus* or *gfp* constructs, 15 independent transgenic events each were produced, equaling to an effective stable transformation efficiency of 0.21–0.85% for *gus* and a comparable 0.34–1.5% for *gfp*. The average transformation efficiency for the *ppo* construct was 0.86% (ranging from 0.2% to 2%), while that for the *opp.i.ppo* construct was 1.7% (ranging from 0.35% to 3.5%). Furthermore, each experiment using the hairpin construct produced transgenic lines, which was not the case with the various *ppo* constructs. These transformation efficiencies are acceptable, considering the high regeneration potential of 80–90% from callus cultures (Ko *et al.*, 2005).

Successful transformation of pineapple plants through the introduction of the *bar* gene into the cultivar "Phuket" was reported by Sripaoraya *et al.* (2001, 2006a, b). The target material was leaf bases arranged on an agar medium containing mannitol as a pretreatment to the plant tissue 2 h prior to bombardment. The authors argue that since introduction of transgene was achieved into cells that directly produce shoots, there are no problems with multiple transgene integration sites.

Although a transformation efficiency of up to 2.6% was achieved, all experiments were based on clones from five transformed plants, of which four were found to originate from the same transgenic event. Herbicide resistance in the crop improves weed control in the field, thereby reducing competition for available nutrients, resulting in increased fruit yields and economic returns. Furthermore, improved weed control also aids in disease-pest control, as weeds can harbor populations of mealybugs, ants, and aphids, which are known vectors of viruses causing mealybug wilt disease.

2.3 Selection of Transformed Tissue

To screen transformed tissue from nontransformed tissue after co-cultivation or bombardment, either antibiotic-resistant or herbicide-resistant cells are produced for selection purposes, while the cells also contain the gene of interest. Kill curves, based on lethal or sublethal dosage, are established by incorporating various concentrations of antibiotics or herbicides in the *in vitro* culture medium. The inhibitory or lethal effects on nontransformed tissue are then monitored.

By co-bombarding a construct containing *nptII* for kanamycin (100–500 mg l⁻¹) or G418 (50–200 mg l⁻¹) resistance and containing *hph* for hygromycin (10–50 mg l⁻¹) resistance, Graham *et al.* (2000a) found that G418 in a concentration of 100 mg l⁻¹ and hygromycin in a concentration of 50 mg l⁻¹ both inhibited callus growth. However, kanamycin was not an effective selection agent even at the highest concentration. Kanamycin tolerance of transformed pineapple tissue was also reported by Espinosa *et al.* (2002) and Firoozabady *et al.* (2006) at up to 125 mg l⁻¹ and 1000 mg l⁻¹, respectively.

A stepwise or delayed selection on the transformed tissue is usually preferred as it allows the bombarded or co-cultivated tissue to recover from the treatment. This was described by Sriporaya *et al.* (2001), where no selection pressure was applied for the first 4 weeks after bombardment, followed by the addition of 0.5 mg l⁻¹ PPT and then 1 mg l⁻¹ after 56 days postbombardment. Firoozabady *et al.* (2006), on the other hand, used 10–20 µg l⁻¹ chlorsulfuron for the first 3 weeks post-co-cultivation and then increased the concentration to 30–50 µg l⁻¹,

depending on which type of explant material was treated. They found that applying sublethal levels of selection immediately after co-cultivation reduced the rate of stable transformation events by 85–95% and application of lethal levels of selection immediately after co-cultivation, or within 20 days of a sublethal selection period, resulted in no transformed tissue being produced. Ko *et al.* (2006) elected to use 50 mg l⁻¹ G418 for two fortnightly subcultures, followed by 100 mg l⁻¹ G418 for subsequent monthly subcultures. Finally, Espinosa *et al.* (2002) and Yabor *et al.* (2006) co-cultivated tissue placed on callus proliferation media for 4 weeks before selection on 2.5 mg l⁻¹ PPT. They found that 10–20 mg l⁻¹ hygromycin suppressed shoot multiplication after 45 days in their liquid TIB system as well as on solid medium. On the other hand, the herbicide PPT inhibited multiplication of plant tissue in the liquid system at a concentration of 2.5 mg l⁻¹, while on solid medium the plant tissue was able to tolerate up to 7.5 mg l⁻¹ PPT. By treating putative transgenic plants in the TIB system with 2.5 mg l⁻¹ PPT, where the shoots were immersed for 2 min every 3 h, PPT-resistant shoots were obtained after 30 days.

2.4 Regeneration of Whole Plants

Regeneration of shoots and plants through either organogenesis or embryogenesis is a prerequisite for successful genetic engineering, but depends heavily on the original explant material used (Firoozabady and Moy, 2004), cultivar response (Firoozabady *et al.*, 2006; Sriporaya *et al.*, 2006b), and type of transgene construct introduced (Ko *et al.*, 2006). Firoozabady and Moy (2004) produced five types of embryogenic tissues and found that only one, described as ECC, was suitable for pineapple transformation. Other types of cells gave rise to pineapple plants via organogenesis or embryogenesis but produced mainly chimeric shoots, containing both transformed and untransformed cells.

Many alternative combinations of growth hormones for induction of plant regeneration have been reported. The addition of different components in different concentrations in successive developmental stages described by Firoozabady and Gutterson (1998), Firoozabady and Moy (2004), and Firoozabady *et al.* (2006) is one

of the most complex growth and regeneration media reported for pineapple transformation. The authors described the emergence of a yellowish tissue with globular structures after 4 weeks of culture of ECC in selection medium. This was followed by 4 weeks of culture on selective agent-free medium, then to medium containing 5 mg l^{-1} BA for 8 weeks, when embryo formation was induced. Maturation and germination was achieved by subculture of embryos onto medium containing 1 mg l^{-1} BA. To produce shoots embryos were transferred onto agar-solidified medium containing 1 mg l^{-1} BA for 4 weeks. Transgenic shoot clusters were then transferred back into liquid medium containing 1 mg l^{-1} BA, followed by subculture onto medium containing 0.2 mg l^{-1} BA for 4 to 10 weeks to encourage multiplication and elongation. Further maintenance and multiplication for an indefinite period of time occurred on MS medium (Murashige and Skoog, 1962) containing 1.5 mg l^{-1} BA and 0.5 mg l^{-1} NAA. Individual rooting of shoots was achieved by culturing shoots on $1/2$ strength MS to which 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} IAA are added. After 2 to 4 weeks complete plants were obtained. Activated charcoal, $0.5\text{--}3 \text{ g l}^{-1}$, was added to improve growth of the transgenic plants.

In summary Firoozabady *et al.* (2006) stated that the critical factors for transformation of embryogenic cell clusters are 2 months of culture on high-sucrose medium, producing globular embryos, then a 2- to 3-month culture period on BA containing medium, which produced transgenic embryos and shoots. The authors reported that 70–90% of transgenic events produced shoots. In contrast, the regeneration media developed by Sripaoraya *et al.* (2001, 2006b) and Ko *et al.* (2005, 2006) for regeneration of transformed pineapple plants involved a simple two-step subculture.

Ko *et al.* (2005, 2006) used a method based on the regeneration studies of Rangan (1982). After transgenic callus pieces were allowed to initiate and proliferate on MS selection medium containing 10 mg l^{-1} each of BA and NAA, the tissue was subcultured onto growth hormone-free MS medium containing 5% (v/v) coconut water and 400 mg l^{-1} casein hydrolysate and the selective agent G418. Within 2 months shoot regeneration can be observed, whereby 70–90% of the transformed callus proved to be highly regenerable (Figure 1). Large-scale

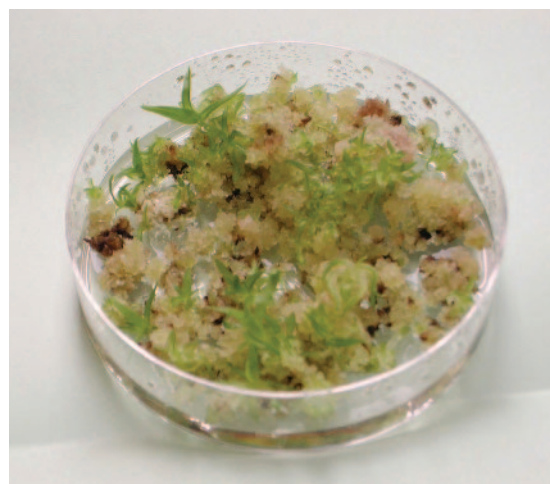


Figure 1 Transgenic pineapple plants regenerating from callus via organogenesis

shoot regeneration continued within the following 4 to 8 months, whereby some independent transgenic lines regenerated more readily than others. Subsequent root production occurred on medium without plant growth hormones. Within 3 weeks of transgenic plants being cultured in individual tubes, 80% of the shoots produced long, thin, single roots, whereas inclusion of growth hormones in the medium induced the production of thick roots, which were easily damaged during transplanting to pots.

Sripaoraya *et al.* (2001, 2006b) subcultured regenerated plants emerging on the leaf bases on selection medium containing 1 mg l^{-1} each of 2,4-D and BA onto a medium containing twice the amount of the selective agent PPT (2 mg l^{-1}) and 2 mg l^{-1} BA for further multiplication. Subsequent rooting occurred on growth regulator-free medium under selection.

A novel approach to transgenic plant regeneration was described by Espinosa *et al.* (2002) and also used by Yabor *et al.* (2006), in which transformed cells were allowed to multiply initially by culturing callus on solid medium containing 0.5 mg l^{-1} BA, without any selection pressure. After 30–45 days putative regenerated transgenic plants were recovered, which were then micropropagated in their TIB liquid system using the selection treatment. However, most of the emerging shoots were reportedly not well developed, being pale yellow in color, and possibly chimeric.

2.5 Testing of Inheritance of Transgene and Effects on Transgenic Plants

2.5.1 GUS assay

By performing a standard histochemical GUS assay (Jefferson *et al.*, 1987) 24–48 h postbombardment, Sripaoraya *et al.* (2001) and Ko *et al.* (2006) were able to count the number of blue spots on the treated explant tissue, which was indicative of the number of cells that had been transformed with the *gus* marker gene. However, transient transformation frequencies are not necessarily correlated with stable transformation frequencies and much depends on the tissue type and even variety being transformed (Sripaoraya *et al.*, 2001). GUS activity was also detected in plant parts of *gus* containing transgenics *in vitro*, in the glasshouse or in the field. Although the GUS assay has been regarded as a lethal, once only test of confirming transgene expression, Firoozabady *et al.* (2006) reported that when stained pieces of calli, somatic embryos, leaf sections, roots, or fruit sections from transformed plants were washed and cultured on recovery medium for 1–2 months, followed by subculture onto selection medium, transgenic plants were produced and the assay was repeated on the same pieces for up to 6 years posttransformation.

2.5.2 Polymerase chain reaction analysis

For molecular analysis of transformed plants the most popular method for screening large numbers of samples is polymerase chain reaction (PCR) analysis. Total genomic DNA is required to amplify the targeted gene fragments and specific primers for the targeted sequences are designed to detect the presence or absence of the transgene fragment. *Bar* and *gus* gene fragments amplified with specific primers yielded 460- and 380-bp products, respectively, for Sripaoraya *et al.* (2001). Ko *et al.* (2006), on the other hand, designed primers to amplify a 750-bp product to confirm stable *gus* transformation. They also employed specific primers designed to recognize *gfp*, *ppo* and *nptII* coding regions, giving a 710-bp fragment for the *gfp* product, a 765-bp fragment for *ppo*, and an 800-bp fragment for *nptII*.

Primers designed to amplify the region of the *bar* gene and the *nos* terminator were used by Espinosa *et al.* (2002), while internal primers for the *surB* gene were used for PCR analysis by Firoozabady *et al.* (2006) and Trusov and Botella (2006).

2.5.3 Southern blot analysis

Southern hybridization analysis confirms that the sequences between the primers are as expected and it gives an estimation of copy numbers. Differing integration patterns, as seen on the gel, verify independent transformation events and, because it is possible for several copies of transgene to insert at the same site, the copy number and the number of insertion sites are not necessarily the same.

Small amounts of genomic DNA are required for the analysis: from 5 µg (Ko *et al.*, 2006) to 8 µg (Sripaoraya *et al.*, 2001) or 10 µg (Espinosa *et al.*, 2002). In contrast, Firoozabady *et al.* (2006) digested 50 µg of DNA for hybridization.

Genomic DNA can be digested with a number of restriction enzymes. The ones reportedly used for pineapple were *SacI* (Sripaoraya *et al.*, 2001), *BamHI* (Espinosa *et al.*, 2002; Ko *et al.*, 2006 for the *gus*, *gfp* and *ppo* constructs), *HindIII* (Ko *et al.*, 2006 for the *nptII* construct), and *NcoI* (Firoozabady *et al.*, 2006).

Hybridization probes are usually specific, such as digoxigenin (DIG)-labeled *bar* and *gus* probes (Sripaoraya *et al.*, 2001), *bar* gene from the PPT-resistant plants digested with *PstI* (Espinosa *et al.*, 2002), and ³²P-labeled DNA fragments of *gus*, *gfp*, and *nptII* genes generated from PCR reactions (Ko *et al.*, 2006). However, less-specific probes have also been successfully applied, e.g., a complementary DNA (cDNA) fragment of the *Smas* promoter sequence by Firoozabady *et al.* (2006) and the CaMV 35S promoter sequence for transgenic plants carrying the *ppo* construct by Ko *et al.* (2006), thereby avoiding background detection of endogenous *ppo*.

A summary of copy numbers found in transgenic pineapple plants are listed in Table 2. Table 2 indicates that *Agrobacterium*-mediated transformation has a lower risk of integration of multiple copies, compared to the biolistics method.

Table 2 Copy numbers in transgenic pineapple lines

	Transgene	Number of copies	Reference
Agrobacterium	<i>Bar</i>	1	Espinosa <i>et al.</i> , 2002
	<i>ppo</i>	2–5	Ko <i>et al.</i> , 2005
	<i>acacs2</i>	1–6	Firoozabady <i>et al.</i> , 2006
	<i>acacs2</i>	1	Trusov and Botella, 2006
Biolistics	<i>bar</i>	4–8	Sripaoraya <i>et al.</i> , 2001
	<i>gus</i>	5–8	Ko <i>et al.</i> , 2006
	<i>gfp</i>	3–10	Ko <i>et al.</i> , 2006
	<i>ppo</i>	1–23	Ko <i>et al.</i> , 2006
	<i>nptII</i>	3–13	Ko <i>et al.</i> , 2006

2.5.4 Other analyses

An advantage of *gfp*-transformed tissue sections and shoots is that they can be monitored continuously under the stereo microscope using a filter that detects bright green fluorescence starting 4 days posttransformation treatment.

When using herbicide resistance as a selectable marker, several *in vitro* and transgene product activity assays based on herbicide resistance expression, can be performed for confirmation of stable incorporation of the transgene (Sripaoraya *et al.*, 2001, 2006a, b):

- Reverse transcriptase-PCR analysis for detection of the accumulation of *bar* transcripts, whereby total RNA was extracted from the plant and the expression of the *bar* and *gus* genes in transgenic plants was confirmed,
- Basta tolerance of regenerated micropropagated plants, by adding 0–20 mg l⁻¹ Basta (glufosinate ammonium) to the agar medium *in vitro*. After 28 days nontransformed plants were either dead on treatments above 3 mg l⁻¹ or were chlorotic/necrotic, while transformed, resistant plants had green leaves even at the highest levels of treatment,
- The same test was repeated for Basta tolerance of plants under glasshouse conditions. After acclimatization in the glasshouse for 75 days, plants were sprayed with a concentration of 100–1400 mg l⁻¹ glufosinate ammonium and scored after 14 days. All nontransformed plants died within 14 days of spraying at the 100 mg l⁻¹

rate, while PPT-tolerant plants remained green and continued to grow,

- Six-months-old glasshouse plants were planted in a field trial, where after 210 days of establishment, plants were sprayed with 0–4 g l⁻¹ glufosinate ammonium. After 28 days plants were assessed and green plants vs. chlorotic/necrotic plants regarded as an indication of survival as a result of transformed versus nontransformed status of the plants. All transgenic plants were tolerant to all herbicide concentrations evaluated, while nontransformed plants died following treatment with 2–4 g l⁻¹ glufosinate ammonium.

Firoozabady *et al.* (2006) developed a rooting assay by adding 10–100 µg l⁻¹ chlorsulfuron to the rooting medium. Transformed shoots produced roots, while nontransformed shoots did not produce roots.

2.5.5 Somaclonal variation

Plants that have been regenerated from tissue culture usually have higher rates of somaclonal variation, especially if plants were regenerated from callus tissue. Plant material used for transformation is often maintained for months, even years, under *in vitro* conditions. In addition, the stresses and cellular differentiation processes accompanying the transformation treatment can be expected to add to the *in vitro* stresses and further increase the incidence of somaclonal variation.

Leaf spininess is one of the most common variants encountered in Smooth Cayenne and the trait is relatively easy to observe and record, even at early stages of propagation. Smooth Cayenne carries the gene for spininess in a recessive form and reversion to spininess happen naturally as a result of stressed environmental conditions. Smith *et al.* (2002a) examined Smooth Cayenne plants that had been regenerated from callus cultures, those that had been micropropagated and those that had been transformed, and found that the incidence of spiny-leaved off-types were comparable. Leaf spininess varied from 0–14% for transgenic lines, 0–12% for callus lines while 11% of the micropropagated plants produced spiny leaves.

Others have encountered higher percentages of spininess in their transgenic population. Firoozabady and Moy (2004) found that plants regenerated from somatic embryo-derived cultures and from organogenic-derived cultures only had one noticeable abnormal phenotype, i.e., spininess in 21% and 4% of the plants, respectively. Whereas Firoozabady *et al.* (2006) reported 37% spininess amongst transformed plants grown in the greenhouse, which previously had been in tissue culture for 12 months. In comparison, 3–5% spininess was recorded for control micropropagated and field-propagated plants. Trusov and Botella (2006) reported 59.5% spininess in their transformed pineapple population, observed over two successive asexual generations. To determine whether the high incidences of spininess found in some of the above studies are transitory or heritable, several generations of asexual propagation are required.

Increased vegetativeness and changes in the phenotype of fruits were also observed (Smith *et al.*, 2002a) (Figure 2) and typical of changes seen in pineapples propagated directly from tissue culture derived material. Sripaoraya *et al.* (2006b) also observed increased production of slips. Many of these changes seen in the first generation of plants *ex vitro* are often not encountered in a second somatic generation derived from planting

crowns from these plants (Smith, unpublished data). This serves to reinforce the view that production of transgenic pineapples will require several cycles of selection in the field for those plants that carry the desired transgene in a stable and superior genotype.

2.5.6 Yield and quality

Reports from agronomic studies of transgenic pineapples are limited, and in many cases transgenic plants are still undergoing evaluation. Sripaoraya *et al.* (2006a, b) have evaluated their cloned transgenic field-grown plants for time to flowering, fruit size, yield, and resistance to heart and root rot diseases at time of harvest. Fruit quality, in terms of sugar and citric acid content, fiber content, aroma, and texture, have also been evaluated. They found that agronomic characteristics were not significantly different between transformed and nontransformed plants. Neither was time to flowering and fruit harvest, which was not unexpected, considering all plants were treated with ethephon to induce flowering. Aroma and texture comparisons were performed by a taste panel and transgenic plants and controls were comparable.

Similar assessments were made by O'Hare *et al.* (2003) from plants that had been engineered for blackheart resistance. They attempted to establish a correlation between variability in fruit shape in transgenic and nontransgenic fruits and its impact on organoleptic characteristics. Harvested fruits were assessed for size, shape, flesh, and skin color (hue angle), followed by removal and juicing of the pulp and a representative sample of each individual fruit measured for total soluble solids (TSS) and titratable acidity (TA). Preliminary results show that the range of fruit shapes and sizes, particularly in the first generation of plants posttissue culture, has a significant impact on both the flavor (TSS and TA) and internal flesh color. Normal-shaped fruits from different transformation events and sources had comparable TSS, TA, and internal color. In comparison, conical or bottle-shaped fruits (with "restricted necks") had significantly lower TSS and TA, which may indicate retarded fruit development. Large fruits on the other hand, had low TA but normal TSS, possibly indicating



Figure 2 Transgenic pineapple fruit; transgenic line producing normal fruit (left); variant fruit types from other transgenic lines (right); it demonstrates the need for field evaluation of each transgenic line to test for distinctness, uniformity, and stability



Figure 3 Transgenic pineapples with fruit ready for assessment

advanced maturity. Further trials are required to determine the relative stability of the observed traits in the subsequent generations (Figure 3).

Measurements of plant heights and weights of transformed versus nontransformed plants were taken by Yabor *et al.* (2006) drawing the conclusion that plant characteristics and peroxidase activity are not affected by transformation. However, this group was more interested in biochemical changes occurring in plants having undergone genetic transformation and found that levels of malondialdehyde had increased, while levels of other aldehydes, chlorophyll b, and total chlorophyll pigments within the first 30 days of establishment in the greenhouse decreased. This indicated that integration of the foreign DNA took place in the genomic region involved in synthesis of the mentioned compounds. Whether these changes affect the quality of the fruit, which is linked to public concerns regarding safety of genetically

modified (GM) food, remains to be fully examined (Sripaoraya *et al.*, 2006b).

2.6 Biosafety

A. comosus is not found naturally. It is found only under cultivation (Collins, 1960). Furthermore, Smooth Cayenne pineapple is not a competitive colonizer of natural ecosystems, and this is a stable characteristic, as evidenced by the absence of Smooth Cayenne in a natural state throughout the tropics/subtropics, where it is grown or in its center of origin in South America.

Two mechanisms of dispersal, namely, dispersal of clonally propagated plants or pollen escape need to be considered.

Pineapple is usually propagated vegetatively. All shoots produced by the plant including the crown, slips, and suckers are capable of forming a new

plant. All these vegetative structures can survive detached from the parent plant for extended periods, possibly up to 6 months depending on the prevailing conditions. In addition, the stem of the parent plant is capable of reshooting. However, while the pineapple plant is highly adapted to vegetative reproduction, it is not a likely avenue of dispersal if the site can be fenced to prevent entry of feral animals that are capable of removing fruit and slips. In addition, the *A. comosus* plant is not capable of producing underground shoots such as stolons. This is true for all the species of pineapple including the noncultivated types, with the exception of *P. saganarius*. Dispersal would, therefore, need to be by the assisted movement of vegetative parts by humans or large animals.

Cultivated pineapples can survive as volunteers in a commercial setting, where plants regrow from stem sections that are not removed when a crop is destroyed. These pose little risk in this instance as plots are usually monitored after the trials and any remaining plants destroyed by application of a suitable herbicide followed by rotary hoeing. Regulatory agencies usually require test sites to be monitored for volunteer plants for at least 2 years after the completion of the trial. If volunteers are encountered, they should be treated with herbicide or removed and destroyed by autoclaving or deep burial. No nontransformed pineapple plants should be grown at the trial site for at least 2 years following the completion of the trial.

Issues associated with the potential for pollen spread are more complex. Three scenarios, namely, cross-pollination of Smooth Cayenne pineapples, cross-pollination of other commercial types of pineapples (Queen, Mordilona, Pernambuco, Spanish groups or hybrids), and interspecific hybridization events must be considered.

Smooth Cayenne pineapples are self-incompatible and poorly fertile and autogamy is rare (Coppens d'Eeckenbrugge *et al.*, 1993). Seeds are extremely rarely seen in commercially produced Smooth Cayenne and the likelihood of transgenic pollen fertilizing Smooth Cayenne pineapples is therefore extremely low.

Hybridization of the other *A. comosus* types listed above could theoretically occur since these types are sexually compatible with Smooth Cayenne. Smooth Cayenne pineapples are also sexually compatible with the related *Ananas* species—*A. ananassoides*, *A. bracteatus*, *A. lucidus*,

A. nanus, and *A. parguazensis*. Where clones and species that are sexually compatible with Smooth Cayenne are present, it is recommended to bag transgenic flowers at test sites.

Pineapple pollen is not dispersed by wind (Kerns *et al.*, 1968). In the Americas, humming birds are considered to be the principal pollinating agents (Purseglove, 1972). Honeybees and pineapple beetles (*Nitidulid* spp.) will occasionally affect cross-pollination between different compatible cultivars if they are grown near each other (Wee and Rao, 1974). While honeybees seen visiting pineapple flowers are feeding on the nectar, which has seeped out onto the sepal and bract surfaces (Okimoto, 1948; Purseglove, 1972), they will enter the flower and collect pollen.

Pollen viability varies widely between different cultivars and species of *Ananas*. Nayar *et al.* (1981) studied seven cultivars and found viability varied from 2.5% to 55%. Smooth Cayenne's pollen viability is in the low to moderate range, however, fertility as rated by pollen quantity, viability, and seeds/flower is extremely low (Coppens d'Eeckenbrugge *et al.*, 1993).

Seed production in *A. comosus* is also very low (Coppens d'Eeckenbrugge and Duval, 1994). Moreover, there are no detailed studies on the longevity of seed survival of which we are aware. Williams (1969) reports that pineapple seed can be stored satisfactorily for one, and probably 2 years, at 10% RH and 7.2 °C. However, Miles Thomas and Holmes (1930) reported that some seeds could be viable after approximately 2.5 years. In any case, seeds are not normally found in pineapple fruits (Coppens d'Eeckenbrugge and Duval, 1994).

Since pineapple survives poorly, if at all, in the natural environment, and there seems to be no real risk associated with pollen escape, we believe pineapples are one of the safer transgenic plants to be released into the environment. However, regulators are also concerned with the transgenes and their stability of expression, whether they pose a risk to the environment, whether it is possible to identify and track the movement of transgenic plants and their parts, as well as food safety and labeling issues.

Pineapples have been transformed by both biolistics and through *Agrobacterium*-mediated methods. For biolistics transformation, vector sequences are not usually removed from the final construct. In these instances vector sequences can

integrate into the genome of transformed plants in a quasi-random fashion. For *Agrobacterium*-mediated transformation, only the recombinant transfer DNA (T-DNA) element (contained between the right and the left borders of the Ti plasmid) is transferred into plant cells.

Some components of the constructs used for transformation are derived from agents known to cause disease in plants and animals, however, none are in a form that might be considered pathogenic by themselves and most are commonly used sequences in transgenic plants. For instance, DNA segments can be derived from *E. coli*, *A. tumefaciens*, or CaMV. Therefore, even though some strains of *E. coli* are pathogenic to humans and animals, none of these segments are known to be pathogenic in the constructs used for transformation. Likewise, *A. tumefaciens* is pathogenic to plants, however, none of the sequences used in gene constructs are themselves pathogenic and while CMV is a plant pathogen and the 35S promoter is widely used in plant transformation, the sequence itself is not pathogenic.

PCR techniques have been developed for the detection of transgenic material and discussed in Section 2.5.2. One possibility for detecting GM pineapples is to use oligonucleotides that recognize the *nptII* coding region or the *bar* gene, both used as selectable marker genes. This test is highly specific and sensitive.

Transgenic phenotypes are sometimes incomplete and can behave in an unstable fashion. The aim of the field testing of transgenic lines is, therefore, to select lines with a stable predictable phenotype.

Approximately 30 mutations have been recorded in Smooth Cayenne pineapple since the early 1920s. These include white flowers (as opposed to purple), foliar floret proliferation, multiple sepals and bracts, enlarged fruit segments, protruding fruit segments, increased or decreased trichome density, seediness, multiple crowns, a lower harvest index, misshapen fruits, smaller diameter fruits, leaf spininess, reduced chlorophyll or anthocyanin levels, dwarfed characteristics, translucent fruits, multiple crowns, and fruits with increased incidence of basal knobs. Only one mutation, nonporous fruit, is considered progressive (Collins and Kerns, 1938; Groszmann, 1939). This high level of somatic mutation and high level of polymorphism within germplasm collections, as indicated by amplified fragment

length polymorphism (AFLP) markers, points to a high level of genome instability in pineapple (Kato *et al.*, 2004). Characters, such as leaf spininess, are considered extremely unstable and the frequency of mutation is high (Collins and Kerns, 1938).

Another potential source of instability is somaclonal variation, phenotypic alterations that are frequently observed in tissue culture derived plants, and this has been described in Section 2.5.5.

Because pineapple is consumed as both fresh and processed fruit, transgenic products will also fall under the scrutiny of a national food regulatory agency. In this case, data must be provided on toxicology, allergenicity, and other possible adverse effects of each transgenic line developed. In addition, nutritional quality of the food should not be adversely affected by the genetic modification. In the case of some genetic modifications, such as blackheart resistance, it is reasonable to assume that the nutritional quality will be improved. The fruit produced on plants resistant to blackheart could be left on the plant for a longer period during production in the winter months, instead of harvesting immature fruit, allowing enhanced development of sugars and other compounds normally associated with maturation of fruit. In this case, it is anticipated it may be possible to improve eating quality.

3. FUTURE ROAD MAP

3.1 Expected Products and Technologies

Transformation will undoubtedly play an important role in pineapple improvement in the not-too-distant future and could take advantage of new developments in genomics (Botella and Smith, 2008). Targeted traits for pineapple improvement through genetic engineering are: manipulation of ethylene biosynthesis to modify flowering and fruit ripening in the crop (Botella *et al.*, 2000; Firoozabady *et al.*, 2006; Trusov and Botella, 2006), suppression of the expression of the PPO gene to improve fruit quality (blackheart resistance; Graham *et al.*, 2000a; Smith *et al.*, 2002a; Ko *et al.*, 2005, 2006), pathogen resistance (nematodes; Rohrbach *et al.*, 2000; Atkinson *et al.*, 2003; suppression of mealybug populations associated with pineapple closterovirus causing pineapple wilt disease; Melzer *et al.*, 2001; *Phytophthora* fungal infection; Espinosa *et al.*,

2002), and herbicide resistance (Sripaoraya *et al.*, 2001, 2006a, b). The involvement in pineapple biotechnology of several groups around the world was discussed by Botella and Fairbairn (2005). It was indicated that although there are relatively few pineapple genes available on the public DNA databases (51 sequence entries in the GenBank nucleotide sequence database in 2004), useful genes can also be extracted from other species-independent sources. In addition, Moyle *et al.* (2005, 2006) have developed a database containing pineapple EST sequence information of genes expressed as a result of nematode-plant interaction during root infection and of genes involved in the molecular basis of nonclimacteric fruit ripening, to which pineapple belongs. This first reported collection of EST sequences isolated from pineapple contains over 5500 EST cloned sequences and aids toward understanding, isolating, and identifying genes involved in the described processes. The database can be accessed at <http://www.pgcl.com.au>.

Differences in tissue culture, regeneration and transformation efficiencies occur between pineapple cultivars and explant material. Each laboratory will employ their preferred transformation technique but should aim for reliable recovery of transgenic plants from proven, field-tested mother stock and with freedom to operate with plants and their products. More research is needed to develop an assay to screen for desired phenotypes in transgenic *in vitro* or glasshouse material, so phenotypes can be predicted prior to field planting (Wang and Waterhouse, 1997). Furthermore, as co-suppression and probably antisense constructs function at the level of messenger RNA (mRNA) degradation and involve activity of an endogenous system capable of sequence-specific degradation of mRNAs, applying these technologies to plants causes unpredictability of the nature of phenotypes which occur in individual transgenic lines. Therefore, the frequency of lines that show complete, stable gene inactivation for particular genes vary and cannot be predicted in advance (M. Graham, personal communication). In practice, this requires screening of large numbers of independent transgenic lines, involving extensive phenotypic analysis and field trials. In pineapples with cycling times of up to 22 months to produce fruit, a complete cycle of field trials will take 8–10 years. By introducing novel approaches to using new types of gene constructs, e.g., the hairpin construct as

proposed by Smith *et al.* (2000), the frequency and stability of desired phenotypes can be increased. Future assessments also require an inclusion of food-related (edible fruit quality) and environmental safety aspects of genetically modified plants (Sripaoraya *et al.*, 2006a; Yabor *et al.*, 2006).

New genes conferring important agronomic traits are still required. Among the multiple possible targets, resistance to disease and nematode attack will have the biggest effect on production. New developments in RNAi silencing are being developed to engineer durable resistance to root-knot nematodes (Huang *et al.*, 2006) and should find application in pineapple improvement. Genetic modifications leading to increased fruit nutritional quality and taste will appeal to consumers, helping to decrease the skepticism that is currently experienced towards genetically modified foods.

3.2 Addressing Risks and Concerns

A great deal of the research done on the development of transgenic pineapples has been proprietary and little has been published until recently. There are good reasons for this and much has to do with the size of the export industry and the involvement of large multinational companies in the production and marketing of the product. However, before countries will have freedom to operate with genetically engineered lines, intellectual property ownership must be ascertained and strategies put in place to ensure the plants produced are free from encumbrance, which could otherwise restrict the sale of product. Scientists and research managers need to be aware that an audit of third party intellectual property ownership is as important to their research approach as a search of the published literature. The advice of IP consultants and patent attorneys are to be valued as highly as that of scientific colleagues. A greater appreciation of the strategies that need to be put in place so that new transformed plant varieties can be grown commercially, free from any encumbrances, is mandatory for commercialization. Equally important is the need to develop and protect intellectual property.

Public acceptance is currently a major issue with all transgenic crops. The perceived safety of transgenic foods and the ownership of genetic

material are at the center of these concerns. It is difficult to anticipate changes in acceptance, but advances in transformation techniques, such as the development of selectable markers to replace antibiotic resistance markers, and the use of the technology for more consumer-orientated product improvements might improve public acceptance of GM foods. Incorporation of only native genes with expression only in plant parts not intended for consumption are also approaches worth consideration. Irrespective of changes in acceptance, it is evident that food-labeling laws in many countries will demand the identification of genetically modified contents. These laws will then require identification and segregation of fields of genetically modified plants. It is important for the pineapple industry to embrace protocols at this early stage to enable identification and segregation of genetically modified plants and thus ensure consumer confidence.

Modern tools of genomics and *trans/cis*-genics offer the possibility of completely reinventing pineapple production systems, based on the use of productive new varieties, with durable resistance to biotic and abiotic stresses. Investment is needed now to develop these improved technologies and ensure a stream of innovations that will progressively come on line to sustain the improvement in production systems. In the future, both the tools for genetic improvement and the knowledge of the ecology of pineapple production—generated in an effort to reinvent commercial production of processing pineapples—can be applied for the sustainable production of the 70% of pineapples that are currently grown for fresh fruit and local consumption around the world. These are subject to essentially the same pest and disease threats as Smooth Cayenne production but public concerns about GM and lack of information currently constrain the use of inputs or the adoption of technologies that offer sustainable increases in productivity and the development of “functional” foods (Kern, 2002).

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Papaya

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1. INTRODUCTION

Papaya (*Carica papaya* L.) is a large herbaceous plant native to tropical America. It is a rapidly growing, hollow stemmed, short-lived perennial of the family Caricaceae. Papaya has a long history of cultivation and use in tropical and subtropical regions between 32 degrees latitude, north and south of the equator. It is cultivated largely for consumption of fresh fruit, but in addition has several industrial, pharmaceutical, and medicinal applications. Additionally, papaya is one of only six transgenic crops that are being commercially cultivated in the United States. In 2005, the total area under transgenic crops in the United States was 49.8 hectares (James, 2005). The transgenic maize, soybean, canola, and cotton exhibit herbicide tolerance and *Bacillus thuringiensis* (*Bt*) resistance in combination or alone comprise nearly all of the transgenic acreage planted, while a very small fraction of a percentage of the biotech acreage are planted with virus-resistant transgenic squash and papaya.

This review covers the development, commercialization, and properties of transgenic papaya that have been reported in the literature. The Hawaiian transgenic papaya story is described and analyzed because it has all the elements that are needed to finally get a commercialized product, and because it represents a case in which the product was commercialized through the efforts of researchers from public institutions. All other

commercialized transgenic products have so far been commercialized by private companies. It is hoped that lessons learned in developing and commercializing transgenic papaya may serve to enhance commercial deployment of transgenic plants of other specialty crops. Several reviews have been written on the Hawaiian transgenic papaya story (Gonsalves, 1998, 2006; Gonsalves *et al.*, 1998b, 2004a, b, 2006; Fermin and Gonsalves, 2004; Fermin *et al.*, 2004b; Gonsalves and Fermin, 2004; Tripathi *et al.*, 2006; Suzuki *et al.*, 2007).

Much of the Hawaii papaya story in Section 2.1 is derived from several reviews that we have written on the subject (Gonsalves, 1998, 2006; Gonsalves *et al.*, 1998b, 2004a, b, 2006; Fermin and Gonsalves, 2004; Fermin *et al.*, 2004b; Gonsalves and Fermin, 2004; Tripathi *et al.*, 2006).

1.1 Botany of Papaya

Although its origin is not known, papaya is believed to have been domesticated in the lowlands of eastern central America, probably in the region between southern Mexico and northern Honduras (Badillo, 1971; Manshardt and Zee, 1994) by ancestors of the Maya civilization of the region (Storey, 1976; Manshardt, 1992). Following the conquest and colonial period of Spain, papaya spread rapidly along tropical trade routes. One

account suggests that papaya was taken from the Spanish Indies (West Indies) to the Malaccas and on to India and the Philippines. From the Malaccas or the Philippines, it is thought to have spread into the South Pacific Islands. The Portuguese moved papaya from Brazil to their colonies in Africa, India, and the Far East (Purseglove, 1968). By 1800, papaya was being grown throughout most of the tropical world, even in some of the most recently discovered remote islands of the South Pacific.

The Caricaceae is a small family that currently includes 6 genera and 31 species. Papaya (*C. papaya*) is the only member of the genus *Carica*. Originally, the first major monograph (Badillo, 1971) of the family Caricaceae listed 4 genera. The genus *Carica* had 22 species that were placed in two sections. Section *Carica* contained only papaya (*C. papaya*), while section *Vasconcellea* held the remaining 21 species. Two major revisions followed in 1993 and 2000 in which Badillo (2000, 2001) elevated the sections to generic level. Papaya continued to be the only member of the genus *Carica*, while the remaining 21 species were all moved to the new genus, *Vasconcellea*.

While papaya is morphologically similar to other species of the family (now recognized as species of the genus *Vasconcellea*), numerous reports indicate that papaya is genetically and reproductively isolated from the other species of the family supporting the current classification. Comparative studies of DNA species diversity utilizing amplified fragment length polymorphism (AFLP) (Kim *et al.*, 2002; Van Droogenbroeck *et al.*, 2002) and chloroplast DNA diversity utilizing restriction fragment length polymorphism (RFLP) (Aradhya *et al.*, 1999) suggest that papaya is genetically distinct from all other species of the family. Further, interspecific hybridizations have been largely unsuccessful unless tissue culture methods are used to rescue abortive embryos (Manshardt and Wenslaff, 1989a, b) thus confirming the reproductive isolation of papaya. In contrast, cross-pollination between the other species, other than papaya, is often successful, resulting in some fertile progeny (Manshardt and Wenslaff, 1989a, b; Drew *et al.*, 1998; Gonsalves *et al.*, 2006).

Papaya is a large herbaceous, dicotyledonous tree that is sparingly branched, hollow stemmed and grows up to 3–8 m in height. Plants usually produce a single erect stem with a crown of

15–20 alternate, large, palmately lobed leaves supported by smooth, hollow petioles. When grown for 4 or 5 years, trees often produce several branches.

Plants are polygamous, with male, female, and hermaphrodite flowers. Flowers are borne in leaf axils. Wild plants are frequently dioecious with female or male plant types. In contrast, domesticated plants or selections usually produce flowers that are hermaphroditic, but which can display degrees of maleness described by Storey (1941, 1976). Male or staminate flowers are long, pendulous, multiflowered cymose inflorescences. The corolla of the male flower forms a slender tube about 2/3 of its length and terminates in five free petals. The flower is unisexual, having functional stamens, but lacking a functional pistil, which is greatly reduced or vestigial. Female or pistillate flowers are inflorescences with few flowers. These flowers have large functional pistils, but are entirely lacking stamens or their vestiges. Petals are free from one another. Hermaphrodite or andromonoecious flowers are bisexual with pistil and stamens on a relatively short inflorescence. The petals are fused for one-half to three-fourths of their lengths, forming a rigid tube (Giacometti, 1987).

Papaya is a polygamous diploid with nine pairs of chromosomes and sex is determined by a single locus with multiple alleles that determines sexuality (Hofmeyr, 1967; Storey, 1976). There are two basic alleles that determine sex type. The dominant or male allele (*M*) occurs in two forms, *M_h* or *M_m*, while the female allele is recessive (*m*). Thus, staminate plants are designated *M_mm*, andro monoecious or hermaphrodite plants are designated *M_hm*, and pistillate plants are designated *mm*. An apparent lethal factor is associated with double dominant alleles (*M_hM_h*, *M_mM_m*, *M_hM_m*) since about 25% of mature seeds are aborted or empty.

The female tree is extremely stable, always producing pistillate flowers. Conversely, the staminate and hermaphrodite trees can be sensitive to fluctuations of environmental factors, and can go through seasonal sex reversals (Hofmeyr, 1939; Storey, 1941). Warm night temperatures, water stress, and high nitrogen levels can cause a sex reversal toward maleness in hermaphrodite plants (Nakasone and Paull, 1998), resulting in problems in fruit set and harvest skips during the year. These conditions often last only a few weeks, during

cooler months of the year, but contribute to less predictable yields.

Papaya fruit produced on female and hermaphrodite trees resemble melons in a superficial way. Fruit from female trees are distinctly spherical or melonlike in shape, while those from hermaphroditic trees are pyriform, oval, or cylindrical, depending on the variety. Similarly, fruit size ranges widely from 255 g to 5–6 kg. Fruit color ranges from pale to bright yellow-orange to red. Dioecious cultivars are more stable and vigorous, and grow better in the subtropical areas of Florida, Australia, and South Africa when compared to hermaphrodite cultivars, which are not adapted to cool winters (Giacometti, 1987). In most tropical countries, where production is designed for export, the Hawaiian Solo types are grown. The small fruit (450–500 g) produced by these cultivars are easier to pack and ship.

1.2 Origin of Hawaiian Solo Papaya

Little is known about the introduction of papayas into Hawaii. Some authorities credit Don Francisco de Paula Marín with bringing seeds from the Marquesas Islands to Hawaii between 1800 and 1823. Others believe that papayas came to the islands via Asia and the South Pacific islands before contact by the Europeans. Whatever its origins, it is clear the first papayas in Hawaii were the large-fruited types, typically grown in most of the papaya growing areas.

However, on October 7, 1911 Gerritt P. Wilder introduced a small-fruited papaya from Barbados to Hawaii. This introduction would completely transform the Hawaii papaya industry. These smaller papaya lines were formally named “Solo” in 1919 because Puerto Rican laborers referred to the fruit of these lines as solo, or fruits appropriately consumed by a single individual or “solo” in their language. By 1936, Solo was the only papaya variety being grown commercially in Hawaii. Subsequently, additional breeding and selection to improve the different solo varieties became the breeding objective of horticulturists at the University of Hawaii, resulting in the solo lines commonly grown in Hawaii today. The best known of these varieties are “Kapoho” and “Sunrise”, a sibling selection of Sunrise known as “Sunset” and “Waimanalo”, a deep orange-fleshed variety

prized in local markets. These solo varieties are all small-fruited varieties less than 0.65 kg in size. They are prized for their sweetness (brix levels higher than 12%), subtle nuances in flavor, and except for “Waimanalo” are firm enough to be readily handled and treated for export from Hawaii. Two of these have also become important varieties exported from Brazil, namely Sunrise and Sunset.

1.3 Economic Importance

Papaya fruit is most commonly eaten fresh, peeled, and seeded, or it is processed for making fruit salad, juice, jam, jelly, pie, or ice cream flavoring. Unripe fruit can be eaten raw in salad, cooked in syrup and eaten as a dessert, and the boiled leaves are used as vegetable. The fruit has digestive properties due to the presence of a digestive enzyme papain (Sturrock, 1940) and has nutritional value with high contents of vitamin A, vitamin C, calcium, potassium, and iron. The latex of unripe fruit is a fluid of milky appearance that contains proteinases, including papain. Papain is extracted by making incisions on the surface of green fruit, and is used to tenderize food and in pharmaceutical industries. Approximately 3501t of crude papain are exported annually from production areas (Jones and Mercier, 1974). For the majority of producer countries, papain is a by-product of fruit production (Madrigal *et al.*, 1980) from about 0.2% of the total harvested area.

Papaya is widely planted in home gardens because it is relatively easy to grow from seed. The first mature fruit can be harvested 9 months after sowing seeds, and fruit is produced year round. Commercially, when trees are grown at a density of 1500 to 2500 per hectare, annual production can range from 84 to 140 metric tons per hectare. Fruit are harvested for 1–2 years, after which the trees are usually too tall for efficient harvesting.

Papaya is now produced commercially in many of the tropical and subtropical areas of the world for both domestic consumption and for export. According to 2005 FAO statistics, the top exporting countries, in order, were: Mexico, Malaysia, Brazil, Belize, the United States (Hawaii), and the Netherlands. A significant amount of fruit is also being exported from China, India, Ecuador, Guatemala, the Philippines, France, and

Thailand. Countries with the most significant domestic production, in order, were: Brazil, Nigeria, Mexico, India, Indonesia, Ethiopia, Democratic Republic of the Congo, Peru, China, Colombia, the Philippines, Thailand, Venezuela, and Cuba.

Papaya is currently the fastest growing tropical fruit crop in the world, increasing more than 13% in 1 year (since 2004–2005). In 2005, world papaya production was estimated at 6.62 million metric tons. The two largest producers of papaya are Brazil and Nigeria with 1.7 and 0.75 million metric tons of fruit, respectively. Two largest exporters of papaya are Mexico and Malaysia with 86 260 and 44 600 metric tons of fruit, respectively. Solo type fruit produced in Hawaii and the Philippines are marketed in Japan. The major producers competing in the US market are Brazil, Mexico, Belize, and Hawaii. In the European market, Brazil is the major supplier of fruit.

1.4 Papaya Ringspot Virus

Papaya ringspot virus (PRSV) was discovered by Jensen (1949) who named the virus due to the ringspot symptoms it causes on infected fruit. It is by far the most widespread and damaging virus that infects papaya (Purcifull *et al.*, 1984). Trees infected with PRSV develop a range of symptoms: mosaic and chlorosis of leaf lamina, water-soaked oily streaks on the petiole and upper part of the trunk, and distortion of young leaves that resembles mite damage (Figure 1). Infected plants loose vigor and become stunted. When infected at the seedling stage or before flowering, trees do not normally produce mature fruit. Production of fruits by trees infected at progressively later stages is severely reduced and of poor quality, due to the presence of ringspots and lower sugar concentrations.

PRSV is a potyvirus that is transmitted in a nonpersistent manner by a number of aphid species, such as *Myzus persicae*, and has a systemic host range that is limited to papaya and cucurbits. The virus also produces local lesions on *Chenopodium quinoa* and *Chenopodium amaranticolor*. Seed transmission is not a significant means for spreading the virus, although there has been one report that described 2 of 1355 papaya seedlings from PRSV-infected fruits that



Figure 1 Symptoms of papaya ringspot virus

showed PRSV symptoms (Bayot *et al.*, 1990). As far as we know, this observation has not been confirmed. PRSV is grouped into type P (PRSV-p), which infects cucurbits and papaya, and type W (PRSV-w), which infects cucurbits but not papaya (Purcifull *et al.*, 1984). The latter type was previously referred to as watermelon mosaic virus 1 (WMV-1). Although both types are serologically closely related, observations suggest that papaya is the most important primary and secondary source for the spread of PRSV in large plantations and small orchards alike. Hereafter, we will refer to PRSV-p as PRSV.

Much progress has been made in the molecular characterization of PRSV. The positive strand genomic RNA consists of 10 326 nucleotides

and has the typical array of genes found in potyviruses (Shukla *et al.*, 1994). The genome is monocistronic and is expressed via a large polypeptide that is subsequently cleaved to yield all functional proteins. There are two possible cleavage sites, 20 amino acids apart, for the amino terminus of the coat protein (CP) (Quemada *et al.*, 1990; Yeh *et al.*, 1992). These two sites may be functional; the upstream site for producing a functional nuclear inclusion b (NIb) protein (the viral replicase), and the other, to produce the CP present in aphid-transmissible virions. It is impossible to segregate PRSV-p and PRSV-w types by their CP sequences. Within the p-types, however, the CP nucleic acid sequences can diverge by as much as 14%.

1.5 Nontransgenic Efforts to Control Papaya Ringspot Virus

Numerous efforts to identify PRSV resistance in *C. papaya* have failed, and thus control of PRSV through conventional means has been through cross-protection and the development of tolerant varieties. Resistance to PRSV has been identified in the genus *Vasconcellea* (Gonsalves *et al.*, 2006) but unfortunately crosses between *C. papaya* and *Vasconcellea* species are not fertile. However, much progress has been made in utilizing the resistance in *Vasconcellea* through initial crossing with *C. papaya* followed by embryo rescue, and subsequent backcrossing. These efforts have yet not resulted in any commercial varieties. In this section, we briefly describe the use of cross-protection and tolerant varieties that have been deployed commercially. Much information described in the sections on cross-protection and tolerant varieties were taken from reviews by one or more of the authors (Gonsalves, 1998; Gonsalves *et al.*, 2006).

1.5.1 Cross-protection

Cross-protection is the phenomenon whereby plants that are systemically infected with a mild strain of a virus are protected against the effects of infection by a more virulent-related strain (Yeh and Gonsalves, 1994). This practice has long been known and has been used to control citrus tristeza virus for many years (Costa and Muller, 1980). The

key component in cross-protection programs is the availability of a mild strain that effectively protects against the target virus. Two mild strains (Yeh and Gonsalves, 1984), designated PRSV HA 5-1 and PRSV HA 6-1, were selected following nitrous acid treatment of leaf extracts of squash infected with PRSV HA, a severe strain from Hawaii that had been recently characterized (Gonsalves and Ishii, 1980). Greenhouse experiments showed that both strains were mild on papaya and afforded protection against PRSV HA.

The mild strains were first tried in large-scale field plots in Taiwan (Wang *et al.*, 1987; Yeh *et al.*, 1988; Yeh and Gonsalves, 1994). In preliminary results, plants preinoculated with the mild strains did not show complete protection against the severe strain in the field, but did show a delay in the severe effects of the challenge virus. Although this degree of protection was insufficient for plants under severe disease pressure, economic returns could be obtained by isolating orchards as much as possible and by roguing out severely affected trees until the flowering period. About 100 to 200 hectares of papaya were protected by PRSV HA 5-1 yearly from 1985 to 1991 (Yeh and Gonsalves, 1994). The mild strains are no longer used in Taiwan because it does not provide consistent economic returns to the farmers.

The mild strains performed much better in Hawaii, presumably because the strains had been derived from a Hawaiian PRSV isolate. A series of field experiments was conducted with the Hawaiian Solo cultivars line 8, Kamiya, and Sunrise on the island of Oahu (Mau *et al.*, 1989; Ferreira *et al.*, 1992; Pitz *et al.*, 1994). These studies showed that mild strain PRSV HA 5-1 gave good protection against the local strains, although PRSV HA 5-1 produced noticeable symptoms on leaves and fruit, with the degree of symptom severity markedly dependent on the cultivar. Cultivars line 8 and Kamiya were the least affected and could be grown economically, but Sunrise was too severely affected in fruit appearance. Protection of line 8 has been the most successful (Mau *et al.*, 1989). However, cross-protection has not been widely adopted on Oahu, for several reasons: (a) the adverse affects of the mild strain on Sunrise and to a lesser extent on Kamiya, (b) cross-protection requires extra cultural management and care, and (c) the reluctance of farmers to infect their trees with a virus.

1.5.2 Tolerant varieties

Although most papayas are highly susceptible to PRSV, some selections have shown tolerance. Tolerance to PRSV is inherited in a quantitative manner (Conover and Litz, 1978) and has been incorporated into some newer cultivars by recurrent selection programs using papaya lines with moderate to high levels of tolerance crossed with local cultivars, followed by screening for resistant seedling progeny by virus inoculation. In this way, “Cariflora”, tolerant to virus found in South Florida and the Caribbean, was developed (Conover *et al.*, 1986). This selection is dioecious and produces round fruit of yellow-orange flesh. This Florida tolerant papaya does become infected by PRSV but the leaves are symptomless or show only mild mosaic phenotype and are not distorted. The fruit does not show any virus-induced distortion although fruit develop ringspots. Florida tolerant papaya produces acceptable amounts of fruit despite being infected with PRSV. However, the small round fruit and yellow flesh along with the dioeciousness are not ideal characteristics for papaya grown in tropical areas. Nevertheless, it has been used as parental genetic material for a number of breeding programs.

In Thailand, a series of papaya lines developed by crossing the Florida tolerant and local variety “Khakdum” followed by recurrent selection is the result of an ongoing breeding program since 1987 (Prasartsee *et al.*, 1998). “Khakdum” is a popular Thai cultivar with desirable fruit characteristics, but it is very susceptible to PRSV (Nopakunwong *et al.*, 1993). Plants of “Khakdum” are hermaphrodite or pistillate with the hermaphrodite producing oblong fruit of orange-red flesh. Previous trials at the Khon Kaen Horticultural Experiment Station (KKHS) (Prasartsee *et al.*, 1998) showed that Florida-tolerant papaya produced acceptable amounts of fruit despite being infected with PRSV.

Since PRSV tolerance is inherited quantitatively, a recurrent selection scheme was used to maintain tolerance and incorporate desired horticultural characteristics. Thus, only one cross was made initially between “Florida tolerant” and “Khakdum”. Subsequent crosses were made from within the progeny population. The first priority was to maintain a high level of PRSV tolerance

followed by selection of desirable horticultural characteristics. All plants were screened with a severe PRSV isolate from Thapra in Northeast Thailand. Papaya seedlings were exposed to PRSV by mechanical inoculation in the greenhouse or by aphid inoculations under field conditions where incidence of PRSV was high. Greenhouse grown and inoculated plants with mild or no symptoms were transplanted to the field for further evaluation of PRSV tolerance and horticultural characteristics.

The plants derived from seeds of papaya fruit (lines) resulting from reciprocal crosses were evaluated under field conditions. Five lines resulting from those crosses showing good PRSV tolerance were selected in the first cycle. Progeny from these promising lines were evaluated by recurrent selections through the fifth cycle resulting in the successful development of three papaya lines named “Thapra 1”, “Thapra 2”, and “Thapra 3” showing good PRSV-tolerance and horticultural characteristics.

In local field trials, the papaya cultivars “Thapra 1”, “Thapra 2”, and “Thapra 3” that showed good tolerance under heavy PRSV disease pressure at KKHS, Thapra, and Khon Kaen were subsequently tested in other areas in north-eastern, central, and southern Thailand. Data collected over 18 months from the three regional PRSV infection sites showed that “Thapra 1” and “Thapra 2” had better tolerance to virus disease under severe virus pressure compared to “Thapra 3”. “Khakdum” papaya showed the most severe symptoms under severe virus pressure. According to the results of local field trials, “Thapra 2” generally showed the most promise. Trees produced fruit as early as 3 months after transplantation into the field and the first fruit ripened at 6–7 months. The flesh of ripe fruit is light to deep yellow with a mild aroma. It is medium to large in size with long, cylindrical-shaped fruit. The average fruit weight is 1.5 kg with a Brix level of 11.2. “Thapra 2” is good for fresh consumption and desirable for canning as well. In 1997, the Department of Agriculture (DOA) released “Thapra 2” as a recommended cultivar. Its name was changed from “Thapra 2” to “Khakdum Thapra”.

Since the tolerance to PRSV found in “Khakdum Thapra” is a quantitative trait, it can be lost if that trait is not continually evaluated and selected

for in subsequent sexual generations. During large-scale production of seed, the seed source plants must have acceptable levels of tolerance and horticultural properties consistent with the line. To maintain the tolerance and qualities of the line, papaya seeds used for establishment of the plants for seed production should always be obtained from specific “Khakdum Thapra” trees identified as tolerant and having good horticultural characteristics.

Other tolerant lines have also been developed. In Taiwan, Lin *et al.* (1989) reported the development of the hybrid “Tainung No. 5”, from the cross of FL 77-5 (from Florida) and “Costa Rica Red”, with good level of tolerance and horticultural characteristics. It has a strong trunk and shows early fruit bearing and ripening. The height of the first fruit from the base of trunk is about 50–60 cm. The use of tolerant papayas has not resolved the virus problem in the long term, and elite commercial cultivars with significant tolerance have not been developed.

2. TRANSGENIC PAPAYA

Our rationale for developing PRSV-resistant transgenic papaya stemmed from the fact that PRSV was a major threat to the Hawaiian papaya industry, efforts to breed resistant cultivars were not successful, and that in the mid-1980s a major scientific breakthrough provided hope that virus-resistant transgenic plants could be developed. The latter involved the work of Beachy and colleagues (Powell-Abel *et al.*, 1986) who showed that transgenic tobacco expressing the *CP* gene of tobacco mosaic virus (TMV) provided resistance against TMV. The concept of parasite-derived resistance was brought forth at about the same time by Sanford and Johnston (1985). The concept stated that a transgenic plant that expressed a transgene of a pathogen would be resistant to that particular pathogen. Since the early reports, numerous studies have shown that “pathogen-derived resistance (PDR)” works for many plant viruses and crops. Research in the last 13 years has shown that PDR for plant viruses is RNA mediated via the mechanism of post-transcriptional gene silencing (PTGS), which is also referred to as RNA interference. It is beyond the scope of this review to delve into the details

of this mechanism, which has been covered in a number of recent reviews (Lindbo and Dougherty, 2005; Waterhouse and Fusaro, 2006).

2.1 Hawaii Papaya Story

2.1.1 Rationale for developing transgenic papaya to control PRSV in Hawaii

PRSV was first reported in Hawaii in the 1940s on Oahu Island, which at that time produced the bulk of Hawaii’s commercial papaya (Gonsalves, 1998). In the 1950s, PRSV began to severely affect papaya orchards on Oahu Island, which forced the industry to relocate. It is not entirely clear whether the original PRSV reported in the 1940s was the same virus to cause the damage, if new strains of the virus had entered Hawaii, or the original strains had mutated. Nevertheless, by the late 1950s PRSV was so severe on Oahu Island that the papaya industry was relocated to Puna on Hawaii Island. Puna is the district where Hawaii’s active volcanoes are located. Puna was a good region for papaya because of the availability of large tracts of rather inexpensive land to lease or to buy, of an abundance of rainfall (over 254 cm per year) and sunshine, of the presence of people who wanted to farm papaya, of the “Kapoho” papaya cultivar that was ideally suited for the Puna area, and importantly because of the absence of PRSV. By the 1980s, Puna was producing 95% of the state of Hawaii’s papaya, and was Hawaii’s second most important fruit crop behind pineapple.

However, the threat of PRSV to Hawaii’s papaya industry was real since the virus was present in Hilo, which was only about 31 km away. Hawaii recognized that threat and the Hawaii Department of Agriculture (HDOA) had an active program consisting of a few people that continually surveyed the Hilo area for PRSV and rogued infected trees (many of which were in people’s backyards), distributed educational material, and put a quarantine on the transport of seedlings from the Hilo area into Puna. Since it was anticipated that PRSV would eventually enter the Puna district, research was started in 1978 to develop strategies to control PRSV on Hawaii Island. Cross-protection efforts described above were investigated. However, the mild mutant, PRSV HA 5-1 was not used widely because its

effectiveness was limited to closely related isolates (Tennant *et al.*, 1994; Yeh and Gonsalves, 1994) and because it induced significant symptoms on fruit of certain papaya cultivars (Ferreira and Gonsalves, unpublished observations).

2.1.2 1985–1992: development of transgenic papaya

As noted above, Beachy's work on transgenic tobacco against TMV prompted our group, which consisted of Richard Manshardt, Jerry Slightom, and Maureen Fitch, to start work on developing transgenic papaya. The goal was to develop transgenic papaya that expressed the *CP* gene of PRSV HA 5-1, a mild nitrous acid mutant developed for cross-protection. The *CP* transgene that was engineered was actually a chimeric gene consisting of the entire *CP* gene of PRSV HA 5-1 with an additional 6 amino acids from cucumber mosaic virus because we wanted to ensure that a protein would be produced. This approach followed the prevalent hypothesis what resistance was due to the expression of the protein. As noted earlier, later evidence would show that resistance is via post-transcription gene silencing that does not require the expression of a protein. By 1987, we were starting efforts to develop transgenic papaya. Subsequent transformation and regeneration of papaya plants from embryogenic calli derived from zygotic embryos of immature papaya fruit (Fitch and Manshardt, 1990) was a major accomplishment led by Maureen Fitch. Transformation of papaya embryos with the gene gun was chosen because of the ready access to the gene gun at Cornell University at Geneva, and the help of John Sanford, a co-inventor of the technology (e.g., Sanford *et al.*, 1992). Transformation experiments resulted in the successful regeneration of nine transgenic papaya lines that were screened for resistance to PRSV HA (Fitch *et al.*, 1992). Six transformants were of the Sunset cultivar and three of the Kapoho cultivar. "Sunset" is a commercial red-fleshed Hawaiian Solo papaya that is widely grown in Brazil, but not so much in Hawaii. "Kapoho" is a yellow-fleshed Hawaiian Solo papaya that was the dominant cultivar being grown in Hawaii. By 1991, our greenhouse experiments had shown that one transgenic "Sunset" line,



Figure 2 Transgenic papaya R₀ line 55-1 (left) following greenhouse inoculation. Infected nontransgenic papaya (right), 1991

designated 55-1, was resistant to PRSV HA (Figure 2) (Fitch *et al.*, 1992).

2.1.3 1992: the merging of a research program and a disease crisis

Rather than going on the normal route of first obtaining seeds from the original, R₀ resistant line and then testing the resulting R₁ plants, we opted to directly clone the resistant R₀ line and test these clones under field conditions in a small field plot at the University of Hawaii Experiment Station on Oahu Island. This approach would turn out to be pivotal and was guided by the philosophy of the research group; basically, our primary focus was to develop a control for PRSV in Hawaii, and delving into the fine details of the mechanism of resistance was secondary.

A field trial was started in April 1992 on Oahu Island (Lius *et al.*, 1997). By December 1992, field results clearly showed that line 55-1 was resistant to PRSV in Hawaii under heavy virus pressure. By the end of 1992, nearly 100% of the nontransgenic papaya in the field trial was infected while none of the R_0 clones of line 55-1 showed symptoms (Lius *et al.*, 1997). We were convinced that line 55-1 was resistant under severe disease pressure and what remained to be seen was its horticultural suitability. Ironically, in May 1992, PRSV was discovered in Puna where 95% of Hawaii's papaya was being grown. The potential for disaster that we had contemplated over a decade before became a reality. Essentially, the challenge at hand would be to stem the spread of PRSV in Puna and commercialize line 55-1 so it could be used to control PRSV. The steps toward eventual commercialization would include testing the line for resistance, developing a suitable cultivar for the industry, on site field trial, deregulation of the transgenic papaya, obtaining licenses from holders of intellectual property that we had used to develop the transgenic papaya, and production and distribution of seeds to the industry. Furthermore, all of this work was being supported by rather small USDA special grants that had been obtained for research to improve agriculture in Hawaii and the Pacific Basin island nations for which the United States have an interest. Would we be able to overcome all of the obstacles in time to stem the impending disaster to papaya production in Hawaii?

Massive efforts to contain the virus and suppress its spread were made by the HDOA immediately after the initial sighting of the virus in Pahoia, a region of Puna. An account of the chronology of events was recently reviewed by one of us (DG) and is recaptured below (Gonsalves, 1998). HDOA immediately launched efforts to control the spread of PRSV. The area was surveyed and infected trees were rogued. By September 1992, 4915 trees had been rogued in Pahoia, and the number of trees being cut each week had decreased to below 85, providing hope that the virus had been contained (Isherwood, 1992).

However, the hope of containment was short lived. The incidence of PRSV increased dramatically in the region of Kapoho, which was closest to Pahoia, as the program of voluntary cutting of trees was not strictly followed and as farmers

experiencing high infection rates abandoned their fields, thus creating huge reservoirs of inocula that allowed aphids to quickly acquire and spread the virus (Isherwood, 1994). By late 1994, nearly all papaya of Kapoho was infected with PRSV (Figure 3). In October 1994, the HDOA declared that PRSV was uncontrollable and stopped the practice of marking trees for roguing. In less than 3 years, a third of the Puna papaya area was infected. By 1997, Pohoiki and Kahuwai were completely infected. Kalapana was the last place to become heavily infected. In this area, the furthest from the original infection site in Pahoia, the spread of PRSV was slowed by constant roguing of infected plants by farmers, who were encouraged by a small bounty paid by a packing house. Nevertheless, virus infection increased, and growers of many heavily infected orchards ceased to eliminate trees. Sixty trees had been cut in May 1996, 2905 in July, 4312 in December, and 14 493 in June 1997. By September 1997, roguing was also discontinued in Kalapana (M. Isherwood, personal communication). Five years after the onset of the virus in Pahoia, the entire Puna area was severely affected.

2.1.4 1992–1997: development and testing of “SunUp” and “Rainbow”

Line 55-1 was a red-fleshed transgenic “Sunset”, while Kapoho, the dominant cultivar in Hawaii, is yellow fleshed. We failed to obtain a virus-resistant transgenic Kapoho. Thus, our goal was to develop a papaya cultivar that would be yellow-fleshed and yet have properties similar to “Kapoho”. Richard Manshardt accomplished this in the following manner. Since line 55-1 was a hemizygous red-fleshed female, he first crossed 55-1 with a nontransgenic hermaphrodite “Sunset” to obtain F_1 seeds, and then continued a selfing program for two more generations until he had obtained plants of line 55-1 that were homozygous for resistance; i.e., all progeny from the trees were resistant to PRSV isolates from Hawaii. This homozygous 55-1 was subsequently named “UH SunUp” and is now normally referred to as “SunUp”. In its own right, this was indeed a nearly isogenic “Sunset” except that it had the transgene insert that provided resistance. To create a yellow-fleshed papaya with sufficient properties of “Kapoho” such that it



Figure 3 Severely infected papaya orchards in Puna, 1994

might be acceptable to farmers and consumers, homozygous line 55-1 (SunUp) was crossed with nontransgenic “Kapoho” and the F_1 hybrid was named “UH Rainbow” and now is commonly referred to as “Rainbow” (Manshardt, 1998). In retrospect, the decision of starting the April 1992 field trial in Waimanalo, Oahu with clones of the R_0 line 55-1 soon after greenhouse inoculations showed its resistance was very important because it allowed Manshardt to use these and subsequent plants to develop “SunUp” and “Rainbow” in a timely manner.

In October 1995, following permission granted by Animal Plant Health Inspection Service (APHIS), the field testing of Rainbow and SunUp on a farm in Puna that was severely infected with PRSV was begun. Permits were obtained by and the field trial was started under the leadership of Stephen Ferreira of the University of Hawaii at Manoa. The field trial served to validate the virus resistance of line 55-1 in both homozygous (“SunUp”) and hemizygous (“Rainbow”) conditions. It served as a site trial for farmers to observe the performance of the cultivars (which was crucial to farmer acceptance); and as a source of data on the performance of “Rainbow” grown on a solid 1 acre (0.4 hectare) planting and

managed using commercial type practices. Overall, results from the field trial seemed to validate the suitability of the cultivars for growing under Puna conditions, and importantly, it provided hope to the farmers that these cultivars might be the solution to their virus problem. Experimental data were impressive (Ferreira *et al.*, 2002). Resistance of the transgenic papaya was virtually 100% and the average yield of “Rainbow” was about 140 metric tons of marketable fruit per hectare per year, versus about 5.6 metric tons for that of control “Sunrise” papaya, all of which had become infected within 1 year of planting (Figure 4). “SunUp” also showed excellent resistance. The field trial clearly demonstrated that “Rainbow” was a viable replacement for “Kapoho”.

2.1.5 1995–1998: deregulation and commercialization of transgenic papaya

Efforts to deregulate the transgenic papaya were started in late 1995 by the same researchers that had created and tested the papaya. This section on “Deregulation and Commercialization” was taken largely from parts of a review by Gonsalves (1998).



Figure 4 Field trial of transgenic papaya in Puna. Severely infected plants (left) and noninfected transgenic Rainbow papaya (right), 1996

Transgenic plants and their products are regulated by APHIS, the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA), the latter being through a consultative process. APHIS assesses the environmental safety of the transgenic product. EPA regards the CP as a pesticide and thus an exemption had to be obtained from tolerance levels of CP in transgenic plants. The FDA assesses the food safety of the transgenic product.

APHIS was largely concerned with the potential risk of transgenic papaya on the environment. Two main risks were of heteroencapsidation of the incoming virus with CP produced by the transgenic papaya and of recombination of the transgene with incoming viruses. The former might allow non-vectored viruses to become vector transmissible, whereas the latter might result in the creation of novel viruses. A third concern—that escape of the transgenic genes to wild relatives might make the relatives more weedy or even make papaya more weedy because of resistance to PRSV was of no consequence because there are no papaya relatives

in the wild in Hawaii, nor is papaya considered a weed in Hawaii, even in areas where PRSV is not present. In November 1996, transgenic line 55-1 and its derivatives were deregulated by APHIS (Strating, 1996). This action greatly increased the efficiency of the ongoing field trial because fruit no longer had to be buried at the test site, thus allowing us to sample and send fruit to various laboratories and to the packing house for further testing.

According to the EPA, the CP transgenes are a pesticide because they confer resistance to plant viruses. A pesticide is subjected to tolerance levels in the plant. In the permit application, we petitioned for an exemption from tolerance levels of the CP produced by the transgenic plant. We contended that the CP was already present in many fruits consumed by the public, since much of the papaya eaten in the tropics is from PRSV-infected plants. In fact, we had earlier used cross-protection (the deliberate infection of papaya with a mild strain of PRSV) to control PRSV. Fruit from these trees were sold to consumers. Furthermore, there is no evidence to date that the CP of PRSV

or other plant viruses is allergenic or detrimental to human health in any way. Finally, measured amounts of CP in transgenic plants were much lower than those of infected plants. An exemption from tolerance to lines 55-1 and 63-1 was granted in August 1997.

The FDA is concerned with food safety of transgenic products. This agency follows a consultative process whereby the investigators submit an application with data and statements corroborating that the product is not harmful to human health. Several aspects of the transgenic papaya were considered: the concentration range of some important vitamins, including vitamin C; the presence of *uidA* (encoding β -glucuronidase or GUS) and *nptII* (encoding neomycin phosphotransferase) genes; and whether transgenic papaya had abnormally high concentrations of benzyl isothiocyanate. This latter compound has been reported in nontransgenic papaya (Tang, 1971). FDA approval was granted in September 1997.

In the United States, a transgenic product cannot legally be commercialized unless it is fully deregulated and until licenses are obtained for the use of the intellectual property rights for processes or components that are part of the product or that have been used to develop the product. The processes in question were the gene gun and parasite-derived resistance, in particular, CP-mediated protection. The components were translational enhancement leader sequences and genes (*nptII*, *uidA*, and *CP*). This crucial hurdle involved legal and financial considerations beyond our means and expertise. These tasks were taken up by the industry's Papaya Administrative Committee (PAC) and its legal counsel, Michael Goldman.

Several factors favored the PAC efforts to obtain licenses: (a) although the Hawaiian papaya industry is important to the state, its annual worth is relatively small (annual farm gate value of \$17 million), (b) the holders of the intellectual properties were not actively working toward developing virus-resistant transgenic papaya, (c) the transgenic papaya was urgently needed to rescue Hawaii's papaya industry, and (d) licensing the transgenic papaya in a timely manner would demonstrate goodwill in trying to help a distressed industry. Detracting from the possible success of the PAC in obtaining a license was the expense, the relatively small size of the industry, and the extra

keen public scrutiny that focuses on transgenic products. Failure by growers or shippers to follow the license agreements could be a source of embarrassment to all involved. Nevertheless, license agreements were obtained from all parties in April 1998, allowing the commercial cultivation of the papaya or its derivatives in Hawaii only. Fruits can be sold outside Hawaii, provided that the importing country allows the import and sale of transgenic papaya. Fruit derived from the licensed transgenic papaya grown outside of Hawaii cannot be sold commercially.

Even before license agreements had been obtained, the PAC had commissioned the Hawaii Agricultural Research Center to produce "Rainbow" seeds so that these would be available when the transgenic cultivars were commercialized. Free distribution of seeds sufficient for 1000 acres was started in May 1998, and farmers could now start the reclamation of abandoned and infected papaya fields by the planting of the transgenic papaya, almost exactly 6 years after PRSV was discovered in Puna.

2.1.6 1998–present: impact of the transgenic papaya

The following section points out several ways by which the transgenic papaya has impacted the Hawaiian papaya industry. The section below is largely taken from a recent review by one of us (Gonsalves, 2006). In looking at impact, it is worthwhile to note that papaya plants that become infected within several months in their growth stage will not produce saleable fruit. Thus, all the expenses for land preparation and caring for the papaya will be lost. Under these severe disease conditions, the difference between susceptible nontransgenic and resistant transgenic is a matter of fruit production versus no fruit production.

2.1.6.1 Papaya industry in 1998

The impact of the transgenic needs to be measured based on the situation of the papaya industry in 1998. As noted earlier, efforts of HDOA to contain the virus was discontinued in late 1994, people were subsequently abandoning fields and thus creating

more sources of virus, all of the Puna district was infected with only the Kalapana district having less than full infection. In Kapoho, where one third of Puna's papaya had been growing before PRSV invaded in 1992, was completely infected with many abandoned-infected fields, thus making it impossible to economically establish new papaya fields there. Moreover, much of the papaya that was sold were infected and of poor quality. The papaya production figures of Hawaii bear out the effect that PRSV was having on Hawaii's papaya industry (Gonsalves *et al.*, 2004b). Puna's papaya production had decreased from 21 800 metric tons in 1992 to 11 000 metric tons in 1998. Papaya production in Puna had fallen by 50% in a 6-year span. In 1992, Puna produced 95% of Hawaii's papaya, whereas in 1998 its share of the Hawaii's production was 75%. Other regions, primarily in the Hamakua district of Hawaii Island, on parts of Oahu Island, and on Kauai increased production and were able to pick up some of the production void left by Puna. Even with the establishing of new production areas, Hawaii's overall papaya production had slipped from 22 800 metric tons in 1992 to 14 700 metric tons in 1998.

An important impact of PRSV on the papaya industry was also on the quality of the papaya. Much of the papaya that was being harvested in Puna was infected and not of superior quality, which was the big selling point for the Hawaiian papaya. In fact, the standards of not shipping symptomatic fruit to Japan and the mainland was lifted as a measure to keep the exports of papaya to Japan and mainland at a reasonably good volume. Another impact is on the people associated with the papaya industry. In 1992, Hawaii had eight packing houses, by 1998 only three packing houses were in operation and these were not at running at full capacity as they were in 1992. It was difficult to find noninfected papaya in Hawaii.

2.1.6.2 Adoption

To capture the farmer adoption rate of the transgenic cultivars at a very early stage, a detailed survey of farmers was done from the initial seed distribution and up to September 1999 (Gonsalves *et al.*, 2004a). Personal interviews were conducted with 93 of the 171 farmers who had registered to obtain transgenic papaya seeds in 1998. The data

collected also included information on the size and types of farms, farmer attitudes, and demographic information. To the question on why they wanted to plant "Rainbow", 96% said that it was because of the ability of "Rainbow" to resist PRSV. Interestingly, 71% of the farmers had received information of the transgenic papaya through their own farmer organizations or through PAC.

Farmer adoption of the transgenic papaya was very high. Adoption was defined as to whether the farmer had planted the seeds, and not based on whether the farmer had signed up and obtained seeds. By September 1999, 90% of the farmers had obtained transgenic seeds and 76% of them had planted (adopted) the seeds. The survey also showed that farmers were ready to implement the technology; i.e., 80% of the farmers surveyed planted the seeds within 3 months of obtaining them. As noted earlier, Kapoho area of Puna succumbed to PRSV the earliest. By September 1999, 94% of the farmers in Kapoho who were surveyed had obtained transgenic papaya seeds, and 88% had planted them, and 29% had already begun to harvest fruit.

The transgenic "Rainbow" papaya is now widely planted in Puna, with many formerly abandoned fields being replanted with Rainbow. Monitoring of papaya orchards and observations by growers have clearly indicated that "Rainbow" has remained resistant to PRSV and has produced high yields of marketable fruit. In fact, papaya production increased in 1999, the first increase since 1993. Production statistics show that papaya production in Puna had increased from a low of 11 800 metric tons in 1999 to 18 100 metric tons in 2001. The percentage of transgenic papaya, as of 2005 data have varied from 40–70%, and thus it is obvious that nontransgenic papaya is still being grown in Puna. There are several reasons for this, the most important being the Japan papaya market, which will be described later.

2.1.6.3 Reversed the decrease in papaya production caused by PRSV

In 1992, when PRSV entered Puna that area produced 95% (21 800 metric tons) of Hawaii's papaya; but production decreased whereas by 1998 Puna only produced 75% (11 000 metric tons) of Hawaii's production. Papaya production in Puna

had decreased by 50% in 1998, when compared to 1992s production. Clearly, PRSV was having a devastating affect on papaya production in 1998. Production decreased slightly more in 1999, a year after seeds were released. Puna papaya production showed an increase starting in 2000 to 14 000 metric tons, 2001 with 16 000 metric tons, and 14 800 metric tons in 2002. Furthermore, Puna production accounted for 84% of the production in 2002, compared to the low of 65% in 1999. As of 2005, Puna accounted for 88% of Hawaii's papaya.

2.1.6.4 Enable the production of nontransgenic papaya in Puna

One might ask the logical question: Why does not Hawaii produce only transgenic papaya? It is critical that Hawaii continues to produce nontransgenic papaya to supply the Japan market, as will be discussed below. Arguably, one of the major contributions that the transgenic papaya has made to the papaya industry is that of helping in the economic production of nontransgenic papaya (Gonsalves and Ferreira, 2003). This has occurred in several ways. Firstly, the initial large-scale planting of transgenic papaya in established farms along with the elimination of abandoned virus-infected fields drastically reduced virus inocula. The reduction in virus inocula allowed for strategic planting of nontransgenic papaya in areas that did not have infected papaya trees. In fact, HDOA instituted a plan in 1999 to ensure the production of nontransgenic papaya in Kahuwai area of Puna. Kahuwai was isolated from established papaya fields and prevailing winds in Kahuwai came from the ocean, which borders the area (Gonsalves and Ferreira, 2003). Growers were to monitor for infection and rogue infected plants quickly. Growers who followed the recommended practices were able to economically produce "Kapoho" without major losses from PRSV. Secondly, although definitive experiments have not been carried out, it seems that transgenic papayas can provide a buffer zone to protect nontransgenic papayas that are planted within the confines of the buffer. The reasoning is that viruliferous aphids will feed on transgenic plants and thus be purged of virus before traveling to the nontransgenic plantings within the buffer. This approach also has the advantage of allowing growers to produce

transgenic and nontransgenic papaya in relatively close proximity. Timely elimination of infected trees would need to be practiced to delay large-scale infection of the nontransgenic plants.

2.1.6.5 Allow the growing of papaya in a more limited area in Puna

In the mid-1990s when PRSV was on the rampage in Puna, a number of growers moved their production to new land in the Hamakua district of Hawaii Island. This move was not disruptive to the Hamakua area because lots of agricultural land had become available in the area due to the demise of sugarcane production. With the introduction of the transgenic papaya, growers have moved back to Puna. The transgenic papaya has allowed growers to reclaim their original papaya land, and also grow nontransgenic Kapoho on land previously cultivated with papaya, so long as caution is used in establishing the stands where virus is not present. This in turn has cut down on the amount of new papaya land that has to be cleared in order to raise nontransgenic papaya for the Japan market. In other words, it has helped to slow down expansion of the industry into new lands simply to escape the virus. This situation helps environmentally by preserving forest and other lands that might otherwise be cleared for papaya plantings to escape PRSV infection.

2.1.6.6 Increase the number of papaya cultivars available to Hawaii

A common concern about genetically modified organisms (GMOs) is that they will cause a reliance on growing only transgenic crops and not encourage diversification of varieties. In fact, the transgenic papaya has had the opposite effect in Hawaii. Before 1992, nontransgenic Kapoho accounted for 95% of the state of Hawaii's production. Essentially, the papaya industry had relied on only one variety for many years. The introduction of transgenic papaya has caused the availability of new distinct varieties. While SunUp is a new variety, it essentially is a sib of Sunset and Sunrise. However, Rainbow is a new variety since it is an F₁ hybrid of a cross between nontransgenic Kapoho and SunUp. The

new transgenic cultivar “Laie Gold”, which is a hybrid between “Rainbow F₂” (selfed Rainbow) and the nontransgenic “Kamiya”, also serves a niche market on Oahu Island. Since “Rainbow F₂” is not homozygous for the *CP* gene, “Laie Gold” needs to be micropropagated to achieve uniformity of production. Micropropagation of the papaya also has the added benefit of ensuring the production of only hermaphrodite plants that is demanded by the market, of earlier and lower bearing trees with initially higher yields, and of providing selected, superior clones that could result in improved quality and yield.

2.1.6.7 *Resurgence of papaya cultivation on Oahu*

As noted earlier, the papaya industry originally was centered on Oahu, but production on that island was largely eliminated by PRSV in the 1950s. The availability of PRSV-resistant papaya provided options for papaya growers on Oahu Island. Prior to the release of transgenic papaya, Oahu growers farmed only small plots of papaya due to the effect of PRSV on production. Growers on Oahu enjoy a niche market, currently growing “Rainbow” and “Laie Gold” papaya for residents in Honolulu and other urban areas of the island. Whereas Oahu grew 20 hectares of papaya in 1960, it now grows 57 hectares of virus-resistant papaya. This would not have happened without the release and utilization of the virus-resistant transgenic papaya.

2.1.7 **Challenges of transgenic papaya in Hawaii**

Although a major constraint to papaya production in Hawaii was eliminated with the introduction of PRSV-resistant transgenic plants, Hawaii’s papaya industry still faces a number of challenges. Some of these are: gaining market share in Canada and Japan, growing of nontransgenic papaya without severe affects of PRSV, the durability of the resistance in transgenic papaya, concerns by some organic growers that their crops will be contaminated by pollen flow to their orchards, and the general controversy of GMOs. This section

has used information from a recent review by the authors (Gonsalves *et al.*, 2006).

2.1.7.1 *Supplying nontransgenic papaya to Japanese markets*

Japan and Canada are large markets for the Hawaii papaya industry. Currently, Japan accounts for 20% of Hawaii’s export market, while Canada accounts for 11%. Canada approved the import of “SunUp” and “Rainbow” transgenic papaya in January 2003, and transgenic papaya shipments are continuing to Canada. However, the application for sale of transgenic papaya in Japan has not yet been approved. Meanwhile, it is critical that papaya shipments to Japan are not contaminated with transgenic papaya fruit. Several steps are being taken to minimize contamination.

At the request of Japanese importers, HDOA adopted an Identity Preservation Protocol (IPP) that growers and shippers must adhere to in order to receive an IPP certification letter from HDOA that accompanies the papaya shipment. This is a voluntary program. Papaya shipments with this certification can be distributed in Japan without delay during the time Japanese officials are conducting spot testing to detect contaminating transgenic papaya. In contrast, papaya shipments without this certificate must remain in custody at the port of entry until Japanese officials complete their spot checks for transgenic papaya. Completing the tests may take several days or a week, during which time fruit lose quality and marketability.

Some significant features of the IPP are that the nontransgenic papaya must be harvested from papaya orchards that have been approved by HDOA (Camp III, 2003). To get approval, every tree in the proposed field must be tested for the transgenic GUS reporter gene that is linked to the virus resistance gene, and found negative. Trees (nontransgenic) must be separated by at least a 4.5 m papaya-free buffer zone, and new fields to be certified must be planted with papaya seeds that have been produced in approved non-GMO fields. Tests for detecting transgenic papaya trees in the field are monitored by HDOA and conducted by the applicant who must submit detailed records to HDOA. Before final approval of a field, HDOA

will randomly test one fruit from 1% of papaya trees in the field. If approved by HDOA, fruit from these fields can be harvested. Additionally, the applicant must submit the detailed protocols that will be followed to minimize the chance of contamination of non-GMO papaya by GMO papaya. This includes a protocol by the applicant on the random testing of papaya before they are packed for shipment. If the procedures are followed and tests are negative, a letter from HDOA will accompany the shipment stating that the shipment is in compliance with a properly conducted IPP.

The above procedure represents a good faith effort by HDOA and applicants to prevent transgenic papaya contamination in shipments of nontransgenic papaya to Japan. It also illustrates meaningful collaboration between Japan and HDOA resulting in continued shipment of nontransgenic papaya to Japan with a minimum of delay once they arrive in Japan, while adhering to the policy that transgenic papaya will not commercially enter Japan until it is deregulated by the Japanese government. These efforts, along with the effectiveness of the transgenic papaya in boosting production of nontransgenic papaya, have allowed Hawaii to maintain significant shipments of the latter to Japan.

2.1.7.2 Deregulation of transgenic papaya in Japan

Details on the efforts to deregulate the transgenic papaya are given in a recent review by the authors (Suzuki *et al.*, 2007). Obviously, deregulation of transgenic papaya in Japan will circumvent much of the concern of accidental introduction of transgenic papaya into Japan. To this end, efforts to allow the transgenic papaya into Japan were initiated by the PAC soon after the transgenic papaya was commercialized in Hawaii. Again, the researchers took the lead in developing the petition. Approval of the transgenic papaya in Japan requires the approval of the Ministry of Agriculture, Forestry, and Fisheries (MAFF) and the Ministry of Health, Labor, and Welfare (MHLW). The petition to the MAFF was approved in December 2000. The petition process for approval by MHLW is still in progress. An initial petition was submitted to MHLW in April

2003. MHLW requested more information. We are hopeful that full approval will be obtained in the near future. The transgenic papaya will need to be labeled, with the specifics of labeling being determined by the Japanese government.

The implications of receiving approval of the transgenic papaya in Japan would have huge benefits to Hawaii's papaya industry and also advance the case of transgenic products outside of the United States. Although effective, the IPP process for minimizing the introduction of transgenic papaya to Japan adds significant costs to the production of papaya. Approval will at least mean that shipment of transgenic papaya and mixed batches of nontransgenic and transgenic papaya could be shipped to Japan without fear of rejection based on having the presence of transgenic papaya.

On a broader level, because the transgenic papaya is a fresh product, consumers will have a clear, side-by-side choice. These conditions will help to answer the questions of consumer acceptance of fresh GMO products outside of the United States in a "real" and "nonacademic" case scenario. Lastly, since the product was not developed by support from multinational companies, the acceptance arguments hopefully will not be about control by large multinational companies. Instead, it is anticipated that product acceptance will be influenced by factors such as of quality, price, advertising, and philosophy of the consumer. Also the arguments should not be on major trade issues because papaya is a low-valued transgenic crop when compared to the currently approved transgenic products in countries outside of the United States. In other words, the transgenic papaya will provide us a chance to analyze factors that supposedly concern the consumers in absence of national media hype on dominance by multinational companies and so on. In this aspect, the transgenic papaya will be a ground breaking biotechnology product.

2.1.7.3 Coexistence

The conditions for deregulation of the Hawaiian transgenic papaya did not stipulate that the transgenic papaya could be grown in only certain locales or regions of the United States. However, there are concerns, especially with organic farmers

that growing of the transgenic papaya would contaminate their products and thus not make them certified or allowable for sale. The US organic rules do state that if a grower growing a product that is certified to be organic and non-GMO at the time of planting and has taken reasonable precaution against contamination, the product can be sold as organic. Nevertheless, coexistence is a contentious issue, even with the transgenic papaya. The fact that Hawaii commercially produces transgenic and nontransgenic papaya in Puna clearly shows that coexistence of transgenic and nontransgenic papaya in the same location is practical and economical. Japan has zero tolerance for having transgenic papaya being sold in Japan. Transgenic papaya has not been detected in nontransgenic shipments to Japan since the IPP program has been in place.

2.1.7.4 Durability of resistance

As will be discussed in Section 2.2.2, the resistance of Rainbow is largely limited to PRSV isolates from Hawaii, while SunUp has broader resistance due to higher dosage of the *CP* gene. However, the resistance of Rainbow in Hawaii has held up to the present time, very likely because the PRSV isolates in Hawaii are homogeneous in their *CP* sequences. The possibility of new virulent strains developing from recombination between PRSV strains in Puna with the *CP* transgene of “Rainbow” is remote. A more realistic danger is the introduction of PRSV strains from outside of Hawaii. Since SunUp should be resistant to many strains of PRSV that might be introduced into Hawaii, one would think that SunUp should be the choice for growing transgenic papaya in Hawaii. However, growers and customers prefer the yellow-fleshed papaya so it is doubtful that acreage grown with SunUp will increase significantly. Given the fact that Rainbow has narrow resistance, current practices of collecting PRSV and inoculating them to Rainbow and SunUp should continue. Since it took over 10 years to develop and commercialize the transgenic papaya, it is absolutely critical that major efforts be made to maintain the effectiveness of the Rainbow and SunUp papaya. As noted above, preventing strains of PRSV from coming into Hawaii is the most important protection.

2.1.7.5 Guarding against large scale resurgence of PRSV in nontransgenic papaya in Puna

PRSV is still in Puna, despite the fact that the transgenic papaya has served to reduce overall infection level. If virus inoculum builds up in Puna, it will become more difficult to economically produce nontransgenic papaya. Strict attention needs to be paid to planting nontransgenic papaya in as much isolation as possible, to timely elimination of infected trees, and to eliminate nontransgenic plantings that are no longer in production. Although important, these simple factors are often not practiced when there are no obvious signs for resurgence of PRSV. We cannot forget the tremendous damage that PRSV did to Hawaii’s papaya industry during the period 1992–1998.

2.1.8 Vectors and transformation protocols

2.1.8.1 Transgene engineering for PRSV resistance in papaya and methods for transformation

The PRSV-resistant transgenic papaya, Rainbow and SunUp were developed by particle bombardment with plasmid pGA482GG/cpPRV4 (Figure 5) (Ling *et al.*, 1991). The parental plasmid pGA482GG from which it was derived contains the genes *nptII* and *uidA* for the selection and visible screening, respectively of transformants as well as a multiple cloning site (MCS) within which the *CP* sequence of the attenuated derivative of Hawaii isolate HA (HA 5-1) was cloned. The PRSV HA 5-1 *CP* gene sequence was originally deciphered from a library of complementary DNAs (cDNAs) derived from purified virus particle RNA and from a partial peptide sequence of a subfragment of the purified *CP* (Quemada *et al.*, 1990). The three transgenes are flanked by transcription and termination signals recognized by the host plant (Figure 6). The *CP* gene, *nptII* and *uidA* “cassettes” are situated within T-DNA (transfer DNA) border sequences that would comprise the transgene insertion ends if in fact this vector was to be used for *Agrobacterium*-mediated transformation.

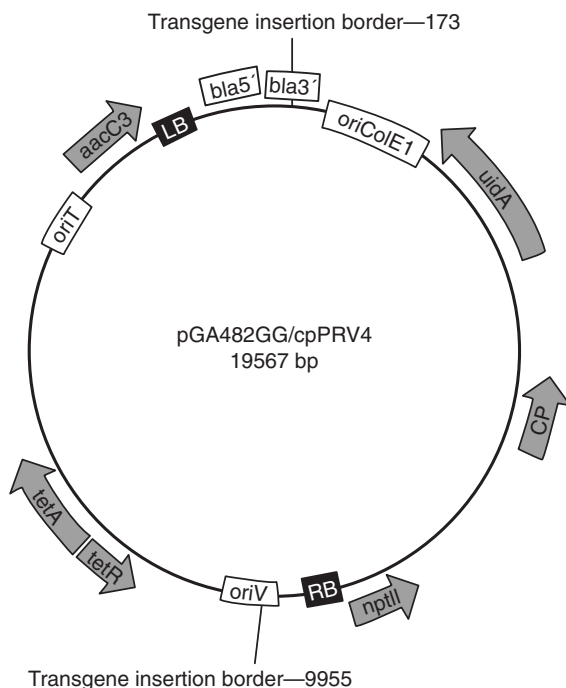


Figure 5 Transformation plasmid pGA482GG/cpPRV4. Transgene insertion borders 173 and 9955 indicate the nucleotide position endpoints of the transgene inserted in the genome of papaya line 55-1 and its derivatives. Target transgenes *nptII*, *CP*, and *uidA* encoding neomycin phosphotransferase, the *CP* gene of PRSV HA 5-1, and β -glucuronidase (GUS), respectively, as well as vector backbone genes *tetA* and *tetR*, and *aacC3* encoding tetracycline resistance and gentamycin resistance, respectively are shown as gray box arrows. Arrows indicate orientation of the respective genes. Light gray boxes represent the nonfunctional 5' and 3' halves of the β -lactamase gene (*bla5'*, *bla3'*, respectively). Replication origins are represented by open boxes and *Agrobacterium* transformation vector T-DNA left border (LB) and right border (RB) segments are represented by black boxes

As mentioned previously, the HA 5-1 *CP* transgene was also engineered to ensure translation by fusion with a 5' untranslated region (UTR) and first 16 amino acids of the cucumber mosaic virus (CMV) *CP* gene as the PRSV *CP* gene sequence is found at the end of the single PRSV-encoded polyprotein and has no translation signals of its own. Expression of the *CP* from this vector was verified by enzyme-linked immunosorbent assay (ELISA) analysis of leaves of transformed papaya and tobacco (Ling *et al.*, 1991; Tennant *et al.*, 2001). Since our original study, research by Dougherty and colleagues with

potyviruses tobacco etch virus and potato virus Y (Smith *et al.*, 1994) showed that resistance was mediated by RNA, via the mechanism of PTGS (Baulcombe, 1999, 2002). We and others have also reported evidence that PDR in transgenic papaya is mediated by RNA-based mechanisms (Tennant *et al.*, 2001; Lines *et al.*, 2002; Bau *et al.*, 2003; Davis and Ying, 2004). Indeed, reports on CP-based resistance via protein expression are largely limited to the case of TMV (Goldbach *et al.*, 2003).

In addition to the genes required for transgene function in the host plant, vector pGA482GG also contains genes *aacC3* (gentamycin-resistance gene), *tetA*, and *tetR* (tetracycline resistance genes), which contain transcription and translation signals for expression in bacteria and function in positive, antibiotic selection for the plasmid in *Escherichia coli* and *Agrobacterium*. DNA elements *oriColE1* and *oriV* are involved in plasmid replication in *E. coli* and *oriT* is involved in replication and conjugation in *E. coli* and *Agrobacterium*.

To facilitate transformation of pGA482GG/cpPRV4 for the production of SunUp and Rainbow, two parameters were considered; transformation method and tissue type to be transformed. In the initial experiments, particle gun mediated or biolistic transformation was utilized. This involved the annealing of purified transformation plasmid to tungsten particles followed by aseptic bombardment into papaya tissue prepared from either embryogenic zygotic embryos, embryogenic calli, or somatic embryos derived from hypocotyls and zygotic embryos (Fitch *et al.*, 1990, 1992; Fitch and Manshardt, 1990). It was established that (2,4-dichlorophenoxyacetic acid; 2,4-D) treated zygotic embryos derived from immature seeds of 90–120-day-old green fruits had the highest transformation capacity following particle bombardment and antibiotic selection compared to embryogenic callus (Fitch *et al.*, 1992). Subsequently, our group reported a modified transformation procedure that included the production of somatic embryos from seed-derived zygotic embryos and limiting the period of antibiotic selection, which greatly increased transformation efficiency (Gonsalves *et al.*, 1998a; Cai *et al.*, 1999). Protocols for efficient production of somatic embryos from hypocotyls and subsequent regeneration were also developed and found to be efficient for papaya transformation (Fitch *et al.*, 1993; Fitch, 1995). This had

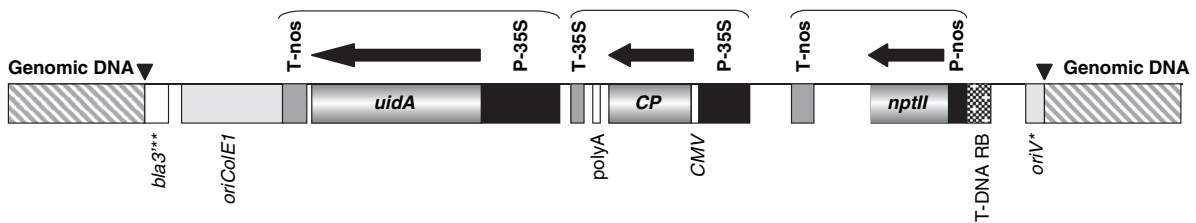


Figure 6 Map of the functional transgene sequences of transformation plasmid pGA482GG/cpPRV4 inserted in papaya line 55-1. The functional transgenes *uidA* (encoding GUS), *CP* (encoding PRSV HA-1 CP), and *nptII* (encoding neomycin phosphotransferase) are shown labeled with black arrows denoting orientation of the genes. Triangles mark the borders of the transformation plasmid sequence insertion and papaya genomic DNA (Genomic DNA). Black boxes denote the transcription signal sequences derived from the *Cauliflower mosaic virus* (CaMV) 35S promoter (P-35S) or the nopaline synthase promoter (P-nos). Dark gray boxes denote transcription terminator sequences derived from CaMV 35S (T-35S) or nopaline synthase (T-nos). The *CP* transgene includes the leader sequence from *Cucumber mosaic virus* (CMV) and a noncoding polyA sequence as marked. Shown also are the plasmid origin of replication sequences *oriColE1* and *oriV*, the T-DNA right border sequence (T-DNA RB) and 3' region sequences of the β -lactamase gene (*bla3'*). Asterisks denote sequence truncation due to the transformation event (*) or truncation of a nonfunctional sequence (**)

the advantage that hypocotyl-derived somatic embryos are easier to procure than the zygotic embryos, which have to be individually excised from seeds of immature fruit of the correct age.

Protocols for the transformation of papaya by *Agrobacterium* have also been established and improved, providing an alternative means for production of transgenic papaya from hypocotyls, zygotic embryos, or petioles (Fitch *et al.*, 1993; Cheng *et al.*, 1996; Yang *et al.*, 1996). One potential advantage of *Agrobacterium*-mediated over biolistic-mediated transformation is that for the most part, at least in other plants, the structure of the integrated transgene DNA is predictable, consisting of the marker gene and gene of interest flanked by the *Agrobacterium* binary vector T-DNA border sequences.

Current surveys on the *CP* gene sequences from different geographic isolates of PRSV indicate that they vary as much as 14% (Jain *et al.*, 2004). This is important to consider since *CP* gene-mediated resistance breakdown has been observed when transgenic papaya was infected with PRSV isolates whose *CP* genes shared only 89.5% nucleic acid identity to the transgene *CP* (Tennant *et al.*, 2001). This phenomenon is thought to be due in part to gene silencing and the role of homology in that process. Based on these empirical observations, papaya have been transformed with a number of different *CP*s in the majority of cases obtained by use of conserved primers to the *CP* gene and polymerase chain reaction (PCR) amplification of PRSV isolates from various geographical

locations for the production of papaya lines resistant to local races of PRSV. Local papaya cultivars have often been chosen as the target of transformation to ensure that the resulting transgenic plants have the desired horticultural properties for production and consumption in the particular region. Various methods and papaya tissue types for transformation have also been adapted or modified. With regards to vector construction, the only major difference compared to the first construct pGA482GG/cpPRV4 among the various research groups is the inclusion of nontranslatable *CP* genes. Use of *CP* transgenes is the most applied approach for conferring virus resistance. However, there is one report of virus resistance conferred by the PRSV replicase (Chen *et al.*, 2001). Transgenic papayas carrying the 3'-truncated and 5'-extended replicase gene of PRSV were resistant to the local PRSV isolate and the mechanism of resistance in this case seems to be RNA mediated, but this remains to be established.

2.1.8.2 Alternative transformation procedures

Alternate transformation procedures have been addressed in a number of the transgenic papaya studies, including new antibiotic selection schemes such as hygromycin resistance, a combination of antibiotic and herbicide phosphinothricin resistance, or the avoidance of antibiotic selection

Table 1 Elements employed for the production of transgenic papaya with novel traits other than PRSV resistance and for the testing of alternative methods for selection and screening of transformants

Gene of interest	Gene product	Gene source	Transgene function	Gene of interest promoter/terminator	Selectable marker gene	Selection/screening	Transformation plasmid	Transformation method	Papaya cultivar	References
<i>bar</i>	Phosphinothricin acetyl transferase	<i>Streptomyces hygroscopicus</i>	Herbicide component phosphinothricin (PPT) inactivation	<i>nos</i> /?	<i>npII</i> / <i>bar</i>	Kanamycin and PPT resistance	pGPTV-Bar	Biolistics	Maradol	Cabrera-Ponce <i>et al.</i> , 1995
CSb	Citrate synthase	<i>P. aeruginosa</i>	Aluminum tolerance	(CaMV) 35S/ <i>nos</i>		Kanamycin resistance	?	Agrobacterium		de la Fuente <i>et al.</i> , 1997
DmAMP1	Defensin	<i>Dahlia merckii</i>	<i>Phytophthora palmivora</i> resistance	(CaMV) 35S/?	<i>npII</i>	Kanamycin/geneticin resistance	<i>DmAMP1</i>	Biolistics	Kapoho	Zhu <i>et al.</i> , 2007
<i>mgfp5*</i>	Green fluorescent protein	<i>Aequoria victoria</i>	Visual marker	(CaMV) 35S/ <i>nos</i>	<i>npII</i>	Geneticin (G418) resistance or GFP fluorescence	pML202	Biolistics	Kapoho	Zhu <i>et al.</i> , 2004b
<i>gusA: mgfp5</i>	β -glucuronidase; green fluorescent protein fusion	<i>E. coli: aequoria victoria</i>	Visual markers	(CaMV) 35S/ <i>nos</i>	<i>hpt</i>	GUS (MUG) activity GFP fluorescence	pCambia 1303	Biolistics	Kapoho	Zhu <i>et al.</i> , 2004b
<i>MSCH</i>	Chitinase	<i>Manduca Sexta</i>	Carmine spider mite tolerance	(CaMV) 35S/ <i>nos</i>	<i>npII</i>	Geneticin (G418) resistance	pBI121/MSCH	Biolistics	Kapoho	McCafferty <i>et al.</i> , 2006
<i>pni</i>	<i>Phosphomannose isomerase</i>	<i>E. coli</i>	Mannose utilization	<i>At Ubg3</i> /?	Same as gene of interest	Mannose utilization	pNOV3610	Biolistics		Zhu <i>et al.</i> , 2005
<i>uidA:npII</i>	β -glucuronidase (GUS); neomycin phosphotransferase fusion protein	<i>E. coli</i> : Tn 5 transposon (<i>Klebsiella</i> , <i>E. coli</i>)	Visual marker/kanamycin resistance	(CaMV) 35S	Same as gene of interest	Kanamycin resistance	pBI426	Biolistics	Maradol	Cabrera-Ponce <i>et al.</i> , 1995
VST1	Stilbene synthase	<i>Vitis vinifera</i> L. (grapevine)	<i>Phytoalexin synthesis</i> , <i>Phytophthora palmivora</i> resistance	<i>Vitis vinifera</i> L., endogenous <i>VST1</i> , pathogen-inducible promoter	Co-transformed with plasmid containing <i>hpt</i>	Hygromycin resistance	?	Co-transformation by biolistics	Kapoho	Zhu <i>et al.</i> , 2004a

altogether with the use of transgene encoded green fluorescent protein (GFP) for noninvasive, visual detection of transformants, or transgene conferred ability to proliferate on nonmetabolizable sugars such as mannose (Table 1).

The study using hygromycin resistance selection provided proof that this was an effective selectable marker system for transformation of papaya (Zhu *et al.*, 2004a). This is valuable information since the availability of different selectable markers can be useful in producing plants with stacked traits derived from different transgene constructs and transformation events. In addition to testing the efficacy of the hygromycin resistance marker selection system in papaya, the novel approach of co-transformation of the marker gene and gene of interest on separate plasmids was tested. Separation of the selectable marker and gene of interest opens the possibility that the marker gene could integrate at a distinct genomic site and subject to independent segregation from the gene of interest. The rate of co-transformation of the unlinked gene of interest was as high as 50% suggesting that this methodology is sufficiently efficient to make it a feasible alternate approach for obtaining transgenic papaya. In the study utilizing phosphinothricin (PPT) in the selection of papaya transformants (Cabrera-Ponce *et al.*, 1995), it is difficult to assess the efficacy of PPT and the herbicide resistance gene system, since it was used in combination with kanamycin selection.

Although antibiotic selection is a predominant method for selecting transformed plants, the antibiotic resistance genes are often the topic of contention among groups opposed to transgene technology. Antibiotic selection can be efficient in selecting transformants, but the efficiency can vary according to a number of factors including the length of the selection period, the concentration of antibiotic used, cultivar differences, and differential responses to related antibiotics such as kanamycin and G418. The development of transgenic individuals can also be slow since the antibiotic negatively selects against nontransformed cells, which is thought to cause them to produce compounds that are in general inhibitory to cell proliferation. The use of GFP has been used previously for visual screening for transformants in other plants and was shown in papaya to allow

vigorous proliferation and development of both transformed and nontransformed cells (Zhu *et al.*, 2004b). This allowed the efficient isolation and identification of papaya transformants within 3–4 weeks instead of the usual 2–3 months required in the case of antibiotic selection. A second example of the use of a nonantibiotic selection or screening system, used the phosphomannose isomerase gene (*pmi*) from *E. coli* (Zhu *et al.*, 2005). In light of concerns and perceptions on the potential negative impact of transgenes in the environment, the *PMI* gene is attractive as a transformation marker since it is widely distributed in nature. The encoded enzyme *pmi* is however lacking in many plants and is required for growth and development of papaya if sucrose that is normally used in plant tissue culture media is replaced by mannose. Mannose was shown to have inhibitory effects on growth of nontransformed papaya cells, even in the presence of sucrose. Consequently, although the selection process for transformants was long (~8 weeks), it yielded very few escapes and was in fact deemed more efficient as measured by relative numbers of transformants obtained compared to either GFP screening or *nptII* gene and G418 selection.

2.1.9 Properties of PRSV-resistant transgenic papaya

The salient properties of the virus-resistant transgenic papaya are discussed in this section. The transgenic papaya will be discussed by country or state in which they were developed.

2.1.9.1 *SunUp and Rainbow*

Line 55-1, the parent of SunUp and Rainbow, has one insert of the *CP* gene (Fitch *et al.*, 1992; Tennant *et al.*, 2001). Since the R₀ plant of line 55-1 was female, we initially obtained seeds by fertilizing the female flowers with pollen from nontransgenic “Sunset” or “Sunrise”, the parent sib from which Sunset was selected. As expected, roughly 50% of the progenies were transgenic and these would have their *CP* gene in a hemizygous state. The first extensive greenhouse tests done in 1994 clearly showed that the R₁ plants were resistant to Hawaiian strains of PRSV but largely susceptible to PRSV strains from outside

of Hawaii (Tennant *et al.*, 1994). In other words, the resistance of the line 55-1 in the hemizygous state was very narrow. For example, all R₁ plants inoculated with isolates from Thailand, Brazil, Guam, Australia, Bangladesh, India, and Taiwan were infected and developed severe symptoms while a percentage of plants that were inoculated with a strain from Mexico became infected and developed relatively milder symptoms.

In contrast, SunUp, homozygous for the *CP* gene, showed broader resistance than Rainbow, apparently due to increased *CP* gene dosage (Tennant *et al.*, 2001). It showed resistance to many but not all of the PRSV isolates from outside of Hawaii. Evidence such as effect of gene dosage, plant age, lower steady state *CP* RNA levels in SunUp than in Rainbow, and nuclear run on experiments clearly pointed to RNA-mediated protection via the mechanism of PTGS (Tennant *et al.*, 2001). Indeed, transgenic papaya expressing nontranslatable *CP* genes (Gonsalves *et al.*, 1998a) or even segments of *CP* genes showed resistance to PRSV (Gonsalves, unpublished results). Furthermore, the strength of resistance was correlated to the degree of homology of the *CP* transgene to that of the attacking virus. PRSV isolates with lesser homology are able to overcome the resistance of Rainbow and SunUp. The availability of a system to produce infectious viral transcripts of PRSV HA *in vitro* (Chiang and Yeh, 1997) provided a unique opportunity to determine the effect of *CP* homology on resistance of Rainbow and SunUp (Chiang *et al.*, 2001). An infectious PRSV HA hybrid virus containing the entire *CP* gene of PRSV YK overcame resistance of Rainbow while PRSV HA did not. PRSV YK has only 91% homology to the transgene while, as expected, PRSV HA has nearly 100% homology. PRSV HA hybrid viruses with segments of the YK *CP* gene also overcame resistance of Rainbow, but not SunUp.

2.1.9.2 Line 63-1

The original transformation work that resulted in the identification of the line 55-1, the parent of SunUp and Rainbow, also resulted in the subsequent identification of another PRSV-resistant line, designated line 63-1 (Tennant *et al.*, 2005b). In contrast to SunUp, which

was derived by transforming the nontransgenic Sunset, line 63-1 was derived by transforming the nontransgenic Sunrise and unlike line 55-1, R₀ line 63-1 was a hermaphrodite. Both Sunset and Sunrise have excellent flavor and are red flesh and were deregulated by APHIS at the same time. However, commercialization of line 63-1 was not pursued.

Interestingly, line 63-1 has at least two *CP* inserts of PRSV HA 5-1 (Souza Jr. *et al.*, 2005b; Tennant *et al.*, 2005b) that segregate independently and resistance to PRSV isolates show a clear correlation to gene dosage. As noted above, R₁ plants of line 55-1 that are hemizygous for the *CP* gene are resistant to PRSV isolates from Hawaii but susceptible to PRSV isolates from Thailand and Brazil. In contrast, only a percentage (40–60%) of R₁ plants of line 63-1 are resistant to PRSV from Hawaii, but a percentage (26–35%) of the transgenic R₁ plants also show resistance to PRSV from Thailand and Brazil (Tennant *et al.*, 2005b). Further work then showed that R₂ plants from crosses of transgenic R₁ plants were all resistant to PRSV HA, and showed variable but increased percentage of plants that were resistant to PRSV from Brazil and Thailand (Souza Jr. *et al.*, 2005b). And progeny (R₃ plants) from two self-pollinated R₂ PRSV HA-resistant plants showed 100% resistance to PRSV from Hawaii and Brazil, and averaged about 65% resistance to PRSV from Thailand. Sequence analysis showed that PRSV HA has only 89% and 92% homology to PRSV from Thailand and Brazil, respectively (Tennant *et al.*, 2001; Tripathi *et al.*, 2006). Taken together, the results with line 63-1 and also line 55-1 clearly show that gene dosage plays a major role in broadening the resistance of transgenic papaya with the PRSV HA 5-1 *CP* to PRSV isolates outside of Hawaii (Tennant *et al.*, 1994, 2001; Souza Jr. *et al.*, 2005b).

2.1.9.3 Brazil

The *CP* gene of PRSV isolate from the Southeast region of the state of Bahia was used to engineer the PRSV *CP* constructs and transform papaya via biolistic and *Agrobacterium tumefaciens*-mediated transformation. The resulting R₀ plants appeared to be resistant to the homologous virus as well

as to the Hawaiian strain PRSV HA and also an isolate from Thailand (Souza Jr., 1999; Souza Jr. *et al.*, 2005a). Candidate-resistant lines were sent to Brazil in 1999 where they were subsequently analyzed up to the third generation. The research has resulted in selection of 13 R₁ and R₂ populations that have been transferred into the papaya-breeding program at Embrapa Cassava and Tropical Fruits station in Cruz das Almas, Bahia, Brazil (Souza Jr. *et al.*, 2005a). Recently, Brazil has passed legislation to allow further testing of transgenic plants and it is hoped that more intensive work can be resumed to identify and eventually commercialize the PRSV-resistant papaya.

2.1.9.4 Jamaica

Solo type (Sunrise) papaya was successfully transformed with translatable and nontranslatable *CP* gene of PRSV (an isolate from the island of Cayman) from Jamaica by biolistic transformation (Tennant *et al.*, 2002; Fermin *et al.*, 2004b). Transgenic papaya carrying the translatable *CP* gene showed a higher (78%) resistance compared to only 10% for the transgenic plant with nontranslated *CP* gene construct. However, a delayed recovery of symptoms in subsequent new growth was observed even for the transgenic papaya that appeared to be susceptible to virus inoculation.

In 1998, initial field trial was conducted in Jamaica with R₀ plants to test the performance of transgenic papaya under natural field conditions. The field test results were similar to initial virus screening test done in greenhouse and showed 80% resistance in case of transgenic papaya carrying the translatable *CP* gene and 44% for the papaya carrying nontranslated *CP* gene. Further field tests with R₁ transgenic plants showed a similar trend to parental lines with 58% resistance (Tennant *et al.*, 2002), which indicates greater resistance was correlated to translatability of the *CP*. Further field tests have been done to characterize the resistance of the lines (Tennant *et al.*, 2005a). The characteristics (horticultural and nutrition) of transgenic papaya during the entire period of the field trial were within the range documented for nontransgenic papaya. The transgenic papaya fruits were safe for consumption as indicated by rat feeding trials conducted on transgenic

papaya fruits to test the food safety and the result showed no adverse health affects attributed solely to transgenic papaya fruit (Fermin *et al.*, 2004b).

One of the major objectives of the project was to develop the local resistant cultivars by crossing in the resistance transgene into locally favored cultivars such as large-fruited “Santa Cruz Giant” and “Cedro”, which are more preferred by the local markets in Jamaica. The Jamaican transgenic cultivars were targeted for release in 2002 initially but it has been delayed due to the lack of support for a third and final field trial. Thus, the release of transgenic papaya in Jamaica is currently on hold.

A further complication is that Europe is a significant market for Jamaican papaya, and unless the papaya is deregulated by the European Union, transgenic papaya will not be able to be exported to Europe or elsewhere. At this moment, there is still a “GMO controversy” with the deregulation of transgenic products in Europe (Fermin *et al.*, 2004b). Conceivably, this could hold back the commercialization of transgenic papaya in Jamaica for fear that the transgenic papaya from Jamaica may somehow be mistakenly shipped to Europe before it is deregulated there.

2.1.9.5 Thailand

A nontranslatable *CP* gene of a PRSV isolate from Northeast Thailand was used to engineer the two popular Thai papaya cultivars (Khakdum and Khaknun). Initially, transformants (R₀ lines) of “Khakdum” and “Khaknun” were tested for resistance in the greenhouse at the Cornell University and the selected lines were sent to Thailand in 1997 for further testing and multiplication. The performance of R₁ generation transgenic papaya under natural field conditions in 1999 was excellent. An R₃ line of “Khaknun” had been selected based on excellent PRSV resistance and horticultural characteristics in the year 2002. In comparative field trials, the transgenic papaya showed that 97% of the progeny were resistant under intense disease pressure and yielded 63 kg of fruit per tree in the first year, whereas nontransgenic papaya yielded only 0.7 kg per year. Recently, good resistance between the crosses of independent lines of Khakdum has been shown under greenhouse and field conditions

(Sakuanrungsirikul *et al.*, 2005; Gonsalves *et al.*, 2006).

A detailed characterization of properties including molecular, biological, biosafety, and food safety was initiated using material that had been selected for eventual deregulation and commercialization (Sakuanrungsirikul *et al.*, 2005). Nearly all biosafety and food-safety experiments that are mandated by the national committee on biosafety have been completed. However, the process of deregulation of transgenic papaya in Thailand will very likely slow down mainly due to recent controversial events relating to GMOs.

Recently, a group from the Mahidol University has reported the development of transgenic papaya using the CP of PRSV isolated from Ratchaburi province of Thailand (Kertbundit *et al.*, 2007). Biolistics was used to transform local papaya cultivar Khakdum. Eight lines were identified out of 1980 during kanamycin selection. Although all eight selected lines were PCR-positive for CP, only one line showed resistance following greenhouse inoculation with the homologous PRSV isolate. Further analysis of the resistant line showed a high degree of rearrangement of the inserted CP expression cassette and a deletion of 166 bp on the 3' end of the CP transgene was also observed (Kertbundit *et al.*, 2007).

2.1.9.6 Venezuela

To develop PRSV-resistant papaya, a local papaya cultivar Thailandia Roja was transformed via *A. tumefaciens* and the CP gene of a local PRSV was used as the transgene in sense/translatable, sense/untranslatable, and antisense forms (Fermin, 1996). A few putative transformed lines were recovered in 1997 and subsequently crossed. Molecular characterization of transgenic generations R₀, R₁, and R₂ and virus inoculation tests indicated that the resistance is RNA-mediated and showed a promising level of resistance not only to local isolates but also to isolates from Thailand and Hawaii (Fermin *et al.*, 2004a). A small field trial of R₁ papaya was conducted in Merida with a special permit from the Ministry of Health of Venezuela (MHV) and these transgenic papayas performed very well under local virus pressure. Unexpected problems started to emerge caused by GMO activists, during

the period that the PRSV-resistant papaya were ready to flower, and in December 2000 the field trial was set on fire by some of their members (Fermin *et al.*, 2004b). Fortunately, G. Fermin identified two hermaphrodite plants showing high level of resistance from the R₂ generation and has seeds for further multiplication and testing but their use in field trials is still in doubt.

2.1.9.7 Taiwan

PRSV-resistant transgenic papayas were successfully developed by S. D. Yeh's team at the National Chung Hsing University, Taichung (Taiwan) using the *Agrobacterium*-mediated transformation approach (Cheng *et al.*, 1996). The technology for engineering PRSV resistance was similar to that used for Hawaiian transgenic papaya. A severe local PRSV isolate (YK) was chosen instead of a mild one (as was the case for Hawaiian transgenic papaya) as a donor of the transgene (CP gene), which was transformed in local papaya cultivar, Tainung No. 2. The transformants were fully characterized and thoroughly evaluated for virus resistance. Several transformation events yielded 45 putative transgenic lines, which exhibited various levels of PRSV resistance ranging from symptom delay to complete immunity (Bau *et al.*, 2003). The mechanical inoculation studies in greenhouse with homologous PRSV showed that in addition to 16 susceptible lines with delay in symptoms expression, 17 were resistant, 10 were highly resistant, and 2 lines were immune to PRSV YK infection. Interestingly, two immune lines, 18-0-9 and 19-0-1 were not only immune to YK infection but also showed a broad-spectrum, high-level resistance to various strains of PRSV from Hawaii, Thailand, and Mexico (Bau *et al.*, 2003). Molecular characterization of nine selected lines with different levels of virus resistance has shown a negative correlation with degrees of resistance and level of transgene expression, which suggests the mechanism of resistance is RNA-mediated. Segregation studies revealed that the transgene in the immune line 18-0-9 had an inheritance of two dominant loci whereas other four highly resistant lines had single dominant loci.

Transgenic papaya lines carrying the CP gene of PRSV YK were evaluated under natural field conditions for their resistance properties and fruit

production for 4 years (1996–1999) and they performed well in regards to resistance, yield, and fruit quality. Performance of the transgenic lines in the field trials was found to be similar to that of Rainbow and SunUp in Hawaii. During field trials, none of the transgenic lines were severely infected whereas 100% of the nontransgenic plants were showing severe symptoms of PRSV infection 3–5 months after planting. However, 20–30% of transgenic plants showed mild symptoms in the first and second field trials but this did not affect the yield or fruit quality. The transgenic lines were not only protected from PRSV, but also produced 11–56% more marketable fruits compared to nontransgenic papaya (Bau *et al.*, 2004).

The CP-mediated transgenic papaya exhibited high degrees of protection against PRSV in Taiwan during a 4-year repeated field trial (Bau *et al.*, 2004). Unfortunately, 18 months after planting the fourth field trial, unexpected symptoms of severe distortion of leaves, stunting on apex, water soaking on petioles and stem, and yellow ringspot on fruit were noticed on transgenic papaya plants, which were resistant to PRSV infection during previous three field tests. Serological and biological properties of infectious agent were determined (Bau, 2000) and later identified as papaya leaf-distortion mosaic virus (PLDMV), a potyvirus, which was originally discovered in Okinawa, Japan, in 1954 (Maoka *et al.*, 1996). All transgenic papaya lines including the immune one were found susceptible to PLDMV infection when inoculated in greenhouse. Therefore, PLDMV is a potential threat to the transgenic papaya line, which is only resistant to PRSV in Taiwan.

In order to overcome the above-mentioned problem, an untranslatable chimeric construct was designed that contained the truncated PRSV CP and PLDMV CP genes and then transferred to papaya by *Agrobacterium* transformation. Transgenic papaya plants carrying these chimeric transgenes of both viruses have initially shown to have resistance in greenhouse inoculation to both PRSV and PLDMV (Yeh, unpublished results).

Transgenic papaya in Taiwan encountered another challenge at the end of fourth year of field trials, a super virulent PRSV strain 5-19 was able to breakdown the resistance in transgenic papaya lines (Tripathi *et al.*, 2004). Interestingly, the nucleotide sequence homology between the CP transgene and the CP of super strain 5-19

is less divergent than that between the CP transgene and other PRSV geographic strains such as PRSV from Hawaii, Thailand, and Mexico, which were not able to overcome the transgenic resistance (Tripathi *et al.*, 2004). This observation indicates that the sequence divergence between the infecting virus and the transgene is not the major cause of transgenic resistance breakdown. In order to analyze the role of HC-Pro as a gene-silencing suppressor, the infectious recombinant viruses were constructed by replacing an HC-Pro and CP region of PRSV YK with that of 5-19 separately or together and recombinant transcripts were inoculated on transgenic papaya (immune line) for resistance evaluation. Results showed that heterologous HC-Pro region of 5-19 alone provides the ability to breakdown the transgenic resistance even though the sequences of the transgene shares 100% sequence homology with the infecting virus (Tripathi *et al.*, 2003; Yeh *et al.*, 2005). The breakdown of the transgenic resistance by a strong gene-silencing suppressor of a super strain has strong impacts on the application of transgenic papaya for virus control in Taiwan.

2.1.9.8 Australia

The ringspot disease has not been reported to be a major limitation for papaya cultivation in Australia and the virus PRSV was described in 1991. Spread of PRSV has been restricted to only South Queensland (where the virus was first discovered) because of strict quarantine measures. However, researchers in Australia have generated transgenic papaya by biolistic transformation of local cultivars GD3-1-19 and ER6-4 that are resistant to Australian PRSV isolates. It is interesting to note that the CP nucleotide sequence data of domestic PRSV isolates have been shown to vary by only 2%. The transgene that was used for transgenic development was obtained from an isolate of Southeast Queensland and the construct was designed with a premature stop codon in the PRSV CP sequence (Lines *et al.*, 2002), thus it was expected that a functional CP would not be expressed.

The transformation event resulted in two male resistant lines for each cultivar that have multiple inserts. Molecular characterization of the transgenic plants has shown that the best resistance was exhibited by the line with the least detectable

transcript RNA, thus strongly suggesting the involvement of an RNA-silencing mechanism for viral resistance. Number of transgene copies in R_0 plants seems to play a role in the level of resistance, as those with single copies were more susceptible. This observation is consistent with that found for RNA-mediated silencing and PRSV resistance of the original Hawaiian transgenic papayas (Tennant *et al.*, 2001; Lines *et al.*, 2002).

2.1.9.9 Florida (USA)

The transgenic papaya carrying the PRSV *CP* of a Florida isolate (H1K) was developed with the intention of producing cultivars resistant to PRSV of Florida and the Caribbean region as *CP* gene sequences of Florida isolates indicate a closer relationship to PRSV sequences from Puerto Rico and Mexico. Four different types of constructs of the *CP* gene were made. The *CP* constructs (sense, antisense, frame shift, and stop-codon mutation in *CP* sequences) were transformed into immature zygotic embryos of the cultivar F65, which is an ancestor of the PRSV-tolerant cultivar Red Lady, by *Agrobacterium* (Davis and Ying, 2004). All transformants were moderate to highly resistant but none of them were immune to the homologous PRSV even when screening was done at later stages. Interestingly, the transgenic lines derived from sense and antisense constructs were infertile. The remaining lines with stop codon and frame-shift mutation were fertilized with pollen from the local cultivars Red Lady and Experimental No. 15 and cultivars grown at the University of Puerto Rico including "Puerto Rico 6-65", "Tainung No. 5", "Solo 40", and Sunrise. The "Puerto Rico 6-65" and "Tainung No. 5" are PRSV-tolerant varieties whereas "Solo 40" and Sunrise are highly sensitive. Resistance of the R_1 progeny seemed to be influenced to some extent by the particular cross, with progeny from the tolerant "Tainung No. 5" and sensitive Sunrise being more sensitive than that of other combinations.

2.1.9.10 China

The replicase gene, from PRSV was used by researchers in China to develop PRSV-resistant papaya (Chen *et al.*, 2001). For the papaya

replicase construct, the 3' end of the gene was deleted and additional codons were added to the 5' end of the gene. Embryogenic calli of papaya cultivar Tai-nong-2 were transformed by *Agrobacterium*-mediated transformation and the resulting transformants showed varying levels of resistance in response to virus inoculation in the greenhouse (Chen *et al.*, 2001). Field trials with the transgenic papaya showed protection from PRSV infection and an increase in the production of better quality papaya fruits. There was no significant difference found in nutritional quality between transgenic and nontransgenic papaya fruits (Wei *et al.*, 2005).

2.1.10 Properties of transgenic papaya regarding other traits

In addition to research directed at producing papaya resistant to PRSV, a number of groups have successfully produced transgenic papaya with properties that address a number of other causal agents of disease limiting papaya productivity worldwide. These include papaya with tolerance or resistance to fungal or oomycetes pathogens and insects, tolerance to aluminum toxicity, and herbicide tolerance aimed at relieving competition with weeds (Table 1).

Papaya is highly susceptible to root, stem, and fruit rot caused by the oomycete *Phytophthora palmivora* causing severe damage and death to trees especially in poorly drained areas during cool and rainy periods. Two studies investigated the expression of genes for defensin and Stilbene synthase, components of naturally occurring plant pathogen defense mechanisms originating from dahlia and grapevine, respectively and their efficacy against *P. palmivora* in papaya.

The dahlia defensin, DmAMP1 belongs to a group of small, structurally similar, highly basic cysteine rich peptides found ubiquitously in plants. A number of plant defensins including DmAMP1 have been shown to possess antifungal properties and expression of defensins in plants has been shown to confer resistance to certain pathogens. Similarly, ectopic expression of *DmAMP1* from the constitutive cauliflower mosaic virus promoter (CaMV 35S) in papaya was shown to confer resistance to *P. palmivora* as measured by root growth and leaf bioassays (Zhu *et al.*, 2007).

Grapevine stilbene synthase gene (*VST1*) expression in papaya was tested for its role in the production of resveratrol, a compound found in a number of, but not all plants and a member of a class of plant antipathogen compounds known as phytoalexins. It was predicted that resveratrol, could be synthesized in papaya which normally lacks it, since its substrates 4-coumaroyl-CoA and malonyl-CoA are common even in plants that do not synthesize it (Zhu *et al.*, 2004a). Since the substrates are utilized in other metabolic pathways and transgene-mediated overexpression has been shown to cause male sterility, the *VST1* gene's own promoter that induces expression temporally in response to pathogen attack, wounding or ultraviolet (UV) irradiation was utilized in the transgene construct. In papaya, *VST1* messenger-RNA (mRNA) as well resveratrol synthesis was also transiently induced in response to pathogen inoculation indicating that the signal transduction pathway activating gene expression of the grapevine transgene was also functional in the heterologous plant. Papayas expressing the *VST1* transgene were resistant to *P. palmivora* due in part to the slower growth of the pathogen on infected transgenic plants compared to their growth on nontransgenic plants.

Development of transgenic papaya resistant to insect pathogens were also developed (McCafferty *et al.*, 2006). Papayas are attacked by numerous insects that cause premature defoliation, reduced tree vigor, and fruit yield. Among these, the carmine spider mite has the largest host range and is one of the most economically important insects with regard to crop productivity. For construction of papaya resistant to the carmine spider mite, a transgene encoding chitinase from the tobacco hornworm (*Manduca sexta*) was utilized. Expression of chitinase, which degrades the polysaccharide chitin, comprising structural features of organs or cell walls of fungi, nematodes, and insects has shown promise as a biopesticide. Transgenic papayas expressing the *M. sexta* chitinase gene were indeed shown to have elevated levels of resistance to carmine spider mites as compared to wild-type controls.

Environmental stresses are also major factors leading to plant disease and several studies have attempted to find solutions to these problems using transgenic papaya. One of these is toxicity of ionized aluminum that occurs in acid soils,

which comprise 40% of arable land and is thus a major factor limiting productivity worldwide. Current practices to ameliorate this problem are through application of lime that alkalizes the soil. However, this can lead to undesirable effects on the environment due to runoff. To address this, de la Fuente *et al.* (1997) developed papaya that could grow at aluminum concentration levels six times above that which is toxic to wild-type plants via transgene conferred overexpression of citrate, an organic acid that can chelate the toxic form of aluminum. The experimental evidence from that study suggests that aluminum tolerance is conferred by excretion of the overproduced citrate from the roots of transgenic plants into the rhizosphere leading to reduced uptake of aluminum. In the study by Cabrera-Ponce *et al.* (1995) papaya plants resistant to commercially applied levels of PPT, the active component of certain herbicides, were constructed by expression of the PPT inactivating enzyme PPT acetyl transferase encoded by the *bar* gene from the bacterium *Streptomyces hygroscopicus*. Creation of herbicide-resistant lines of papaya affords the option of reducing nutrient competition from weeds through herbicide application. The above-mentioned studies indicate that transgenic papaya can be constructed to resist or be tolerant to pathogens or abiotic stresses in some cases reducing the economic and environmental costs conventionally associated with application of chemicals used to combat these problems.

There has been research initiated in the testing of transgenes to alter other properties unrelated to plant disease such as fruit ripening in papaya. This includes research toward reducing expression of endogenous papaya genes such as those encoding 1-amino cyclopropane-1-carboxylic acid (ACC) synthase (Magdalita *et al.*, 2002) or β -galactosidase (Gonsalves, unpublished data) by antisense or gene-silencing approaches, respectively. The goal of these studies is to retard the ripening process of papaya fruit by reducing production of the fruit-ripening gas ethylene, produced by ACC synthase or to retard softening of papaya fruit by reducing expression of the cell wall degrading enzyme β -galactosidase. If successful, this will demonstrate that transgene technology can be further applied to improving papaya traits involved in fruit quality.

Taken together the numerous examples of improvements in the efficiency of transformation and the varied problems they address suggest that the many tools available through transgene technology can be fully utilized and tailored for practical use in papaya.

3. FUTURE ROAD MAP

3.1 Some Potential Products

The future impact of transgenic papaya for worldwide PRSV control and for contributing in a number of other areas is bright technically for several reasons. The demonstrated resistance to PRSV by transgenic papaya has remained highly effective for nearly a decade. Other countries have developed transgenic papayas that show resistance to strains of PRSV in their countries. A major development is that the genome of SunUp papaya is close to being sequenced (M. Alam, personal communication). This achievement will open up the avenue to a multitude of potentials for various products to come from papaya and also for developing new transgenic products. Some products that come to mind are transgenic papaya with delayed ripening or softening of fruit to help in long distance transport of papaya, developing papaya that are only hermaphrodite by knowing the specific genes that govern the sex of papaya, development of lines of papaya that have increased levels of papain, and development of transgenic papaya with resistance to other important pests and diseases of papaya, such as black spot of fruit and phytophthora blight of papaya. Since transformation of papaya via biolistics (particle bombardment) or *Agrobacterium* is efficient, the development of papaya with the target traits is very doable. The largest challenge will be the regulatory issues, intellectual property issues, and the fact that papaya is not a commodity crop.

3.2 Challenges

Currently, the major obstacles for realizing the potentials of transgenic papaya with various properties rests in the regulatory issues and in whether public institutions will have the interest in pursuing transgenic papaya as a research area

given the fact that it is not a commodity, and is not likely to be developed by private companies. The Hawaiian transgenic papaya story is a testament that a transgenic crop can be developed and commercialized through the efforts of public institutions. However, the transgenic papaya is the only product that has been commercialized by a public institution and this was done nearly a decade ago.

Risks or perception of risks of transgenic crops are not unique to any one crop. Potential barriers to the further development of transgenic crops is more related to the time and money that it takes to do the research and come up with the data that the regulatory agencies need to make a judgment on the safety of the product from the food and environmental standpoint. It is logical to think that private companies will focus on products for which they can expect a reasonable profit and thus it is not surprising that efforts have been focused on commercializing crops such as canola, corn, soybean, and cotton. Papaya is but a tiny fraction of the value of these crops. In summary, practical deployment of the papaya will fall largely to the tasks of public institutions, unless some yet unseen extremely valuable product can be developed from papaya. Nevertheless, the Hawaii papaya story clearly shows that transgenic papaya can be developed by public institutions and be done in a timely manner and have impact. It will be indeed interesting to see if other transgenic papaya will be commercialized in the fairly near future.

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Mango

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Mango, *Mangifera indica* L., cultivation probably originated in India 4000 years ago (DeCandolle, 1884). At that time, mango trees most likely produced small fruit with a thin flesh, and domestication over many centuries resulted in larger fruit with thick flesh. According to Mukherjee (1950) many of these primitive mangoes still occur in Northeastern India; with considerable variation in fruit shape and size. The mango has remained an important religious and cultural symbol for India. Gautama Buddha was presented a mango grove by Amradarika (ca. 500 BC) as a quiet meditation place (Popenoe, 1932). The Indian subcontinent was the birthplace of some of the earliest highly developed civilizations, and India exerted strong cultural, religious, and commercial influence over South and Southeast Asia. Hinduism, Buddhism, and Islam were all introduced into Southeast Asia from India. The word for mango in Malaysia and Java (Indonesia) is “mangga”, which is derived from the Tamil “manga”. Although superior Indian mango selections may have been introduced into Southeast Asia, there is little evidence today and the most important mango selections of Southeast Asia are all of the polyembryonic type, and have traditionally been seed propagated.

It is believed that the Portuguese introduced methods for vegetative propagation into India in

the late 15th century. During the reign of the emperor Akbar (1556–1605), the “Lakh Bagh”, a mango orchard of 100 000 trees, was established in Bihar. Many of the mango cultivars of India originated from selections from the Lakh Bagh, for example, “Alphonso”, “Dashehari”, “Langra”, “Rani Pasand”, “Safdar Pasand”, etc. Mukherjee *et al.* (1983) indicated that remnants of this and other ancient orchards still exist in India, and might provide valuable material for breeding.

Although the center of origin and diversity of the genus *Mangifera* is in Southeast Asia, the origin of *M. indica* is less clear. According to Mukherjee (1951), *M. indica* might have appeared during the Quaternary period. It was once believed that mango must have originated in India and spread from there to Southeast Asia, the New World, and Africa. Northeastern India is at the northern edge of the distribution of *Mangifera* species, and mango might have been first naturalized in India (Hooker, 1876). Taxonomic and molecular evidence indicate that the mango evolved within an area encompassing Northwestern Myanmar, Bangladesh, and Northeastern India.

The spread of mangos beyond South and Southeast Asia probably did not occur before the beginning of the European voyages of the 15th and 16th centuries. Mango seeds are recalcitrant, and mango germplasm in the early days must have been transported as ripe fruit and/or seedlings. Only after the 15th and 16th centuries could grafted plants be transported for long distances. The Portuguese would have

transported the mango from India to their African colonies of Mozambique and Angola; however, there is a possibility that mango was introduced to Africa via Arabia in the 10th century by Omani traders (Purseglove, 1972). From Mozambique and Angola, the mango would have been introduced into Brazil. The Spaniards introduced polyembryonic mango types to their New World colonies through the Pacific trading ports of Acapulco and Panama City. Polyembryonic “Manila” is the most important mango cultivar of Mexico, and is considered to be identical to the Philippine polyembryonic “Carabao”. The mangoes in the West Indies are derived from introductions from Brazil during the mid- to late-18th century. The “No. 11”, a polyembryonic mango seedling from Cuba, was the first introduction into Florida (USA) in 1861. Another polyembryonic selection, “Peach”, was introduced into Florida a few years later (Knight and Schnell, 1993). Except for “Mulgoba” (sic.), the early introductions into Florida were unproductive, and only “Mulgoba” was ever grown commercially.

1.2 Botanical Description

The mango, *M. indica* L., is in the Anacardiaceae family in the order Sapindales. The Anacardiaceae consists primarily of tropical species. In addition to the mango, other important species include the cashew *Anacardium occidentale* L., pistachio *Pistacia vera* L., the mombins *Spondias mombin* L. and *Spondias purpurea* L., and the ambarella *Spondias dulcis* Forst. The family also contains the poison ivies and oaks (*Rhus* spp.) of North America and the rengas (*Gluta* spp.) of Southeast Asia. Some of the *Mangifera* species also produce resinous sap that can induce allergic reactions.

Most *Mangifera* species occur naturally in the Malay Peninsula, the Indonesian archipelago, Thailand, Indochina, and the Philippines (Mukherjee, 1985; Bompard, 1989). Kostermans and Bompard (1993) classified *Mangifera* species based upon floral morphology and identified 69 species in two subgenera *Mangifera* and *Limus* with 11 additional species of uncertain position. Eiadthong *et al.* (1999) used genomic restriction fragment length polymorphism (RFLP) and amplification of chloroplast DNA (cpDNA)

to describe phylogenetic relationships among *Mangifera* species. Molecular biology will probably have a major impact on phylogenetic studies of mango and its relatives.

Mangifera species having a single fertile stamen occur in Northeastern India, Myanmar, Thailand, and the Malay Peninsula. Many *Mangifera* species have small fruits with thin, acidic flesh, large seeds, abundant fiber, and astringent resinous substances close to the skin. Edible fruit is produced by *M. indica* and 26 other species in the genus, primarily those from Southeast Asia (Gruezo, 1992). *Mangifera foetida* fruit are highly astringent, and are used for pickles and as a tamarind substitute (Bompard, 1992b). *Mangifera kemang* and *Mangifera altissima* are consumed as fresh fruit or in salads (Angeles, 1992; Bompard, 1992a). *Mangifera caesia* is cultivated in Java (Bompard, 1992a). *Mangifera pajang* produces large and attractive fruit. *Mangifera odorata* is grown in the humid lowlands of Southeast Asia in areas that are otherwise unsuitable for mango, and can also be utilized as a rootstock for mango (Ochse, 1931; Bompard, 1992c). *Mangifera laurina* and *Mangifera pentandra* are used in salads (Bompard, 1992d). *Mangifera griffithii*, *Mangifera minor*, *Mangifera monandra*, *Mangifera quadrifida*, and *Mangifera similis* are considered to have potential as fresh fruit (Gruezo, 1992). The common mango *M. indica* includes all of the commercial cultivars.

There are two distinct types within *M. indica* based upon their reproduction systems and their centers of diversity: a subtropical group with monoembryonic seed (Indian type) and a tropical group with polyembryonic seed (Southeast Asian). Although a few polyembryonic cultivars do occur along the west coast of India, they probably were introduced from Southeast Asia into Goa by the Portuguese. This is supported by a study of relatedness among polyembryonic and monoembryonic cultivars from the west coast of southern India using genomic and cpDNA RFLP analysis (Kumar *et al.*, 2001), and the conclusion was that polyembryonic mangoes could not have originated in India, and must have been introduced from Southeast Asia.

There have been very few genetic studies involving mango. The chromosome numbers of *M. caesia*, *Mangifera caloneura*, *M. foetida*, *M. indica*, *Mangifera sylvatica*, *M. odorata* and *Mangifera*

zeylanica have all been reported to be $2n = 2x = 40$ and $n = 20$ (Mukherjee, 1950, 1957; Roy and Visweswariya, 1951). The mango is believed to be an allopolyploid (Mukherjee, 1950). Because of the presence of secondary associations that occur during metaphase of meiosis, Mukherjee (1950) suggested that the basic chromosome number was $n = 8$. He also noted that the high number of somatic and nucleolar chromosomes supported this conclusion; however, this has not been confirmed by other researchers. Arumuganathan and Earle (1991) determined that the nuclear DNA content of mango is approximately 0.91 pg (picogram).

Traditional breeding has had relatively little impact on mango cultivar release. This is due to several factors: the long juvenile period of the species, which is approximately 7 years; the long period required for assessing the productivity of seedling trees, which can be 12–13 years; the relatively low success rate of controlled hand pollinations, which varies from 0.23% to 3.85% (Mukherjee *et al.*, 1968; Singh *et al.*, 1980); and consumer resistance to change in the traditional mango-growing cultures. Consequently, virtually all cultivars have been derived from seedlings of open-pollinated trees.

During the 20th century, several new mango cultivars were identified in Florida, USA. A seedling of open-pollinated “Mulgoba” came into production in Florida in 1910. The fruit had a red blush, and the tree was more productive than its parent (Wolfe, 1962). The selection was named “Haden”. Another early selection was “Brooks”, which is a seedling of “Sandersha”. During the 20th century, introductions of mango germplasm into Florida occurred from Southeast Asia, India, and elsewhere. Prior to the use of molecular genetics for genetic diversity analyses and pedigree inferences, it was thought that these introductions constituted a secondary center of mango genetic diversity (Knight and Schnell, 1993; Schnell *et al.*, 1995; Mukherjee, 1997). However, it is now considered that all Florida mango cultivars are derived from as few as four monoembryonic Indian cultivars and a single polyembryonic landrace “Turpentine” (Schnell *et al.*, 2006). The second generation “Haden” progeny include “Cushman”, “Edward”, “Lippens”, “Florigon”, “Palmer”, “Springfels”, and “Van Dyke”; “Keitt” and “Kent” were selected from “Brooks” seedlings.

The next generation of Florida cultivars that were seedlings of “Haden” included “Cogshall”, “Earlygold”, “Glenn”, “Hodson”, “Irwin”, “Ruby”, and “Valencia Pride”; whereas, “Sensation” was selected from a “Brooks” seedling. Florida cultivars are suited for many agroecological areas and bear regularly, although many Indian cultivars have been unproductive outside their center of domestication, and are alternate bearing. Florida cultivars also have a red blush at maturity, firm flesh, a high flesh to seed ratio, and are regular bearing. The attractive red blush is favored by consumers in nontraditional mango-consuming countries (Knight, 1997). “Tommy Atkins”, “Keitt”, etc. are moderately resistant to anthracnose, which is the most important production and postharvest problem in many areas. In the latter half of the 20th century, plantings of Florida cultivars were established in many countries and form the basis of international trade of mango fruit.

1.3 Economic Importance

Mangoes are grown commercially throughout the tropics and also in many subtropical areas. It is the most important fruit crop of Asia, and ranks fifth worldwide in total production (metric tons) among fruit crops, after *Musa* (105 815 354), *Citrus* (105 440 168), grapes (65 584 233), and apples (59 444 377) (FAOSTAT, 2006). According to FAOSTAT (2006), world mango production has increased by 67% between 1990 and 2005, with production of approximately 28 221 510 metric tons in 2005. India produced approximately 51% of the world’s mangoes in 1990, but India’s share had declined to approximately 38% by 2005. The leading producing nations after India are (in metric tons) China (3 450 000), Thailand (1 800 000), Pakistan (1 673 900), Mexico (1 600 000), Indonesia (1 478 204), Brazil (1 000 000), and the Philippines (950 000). Between 1990 and 2005, mango exports increased sixfold from 158 030 to 907 782 metric tons, with a value estimated to be US\$583 763 000 (FAOSTAT, 2006). Major exporting countries are (in metric tons) Mexico (212 505), India (156 222), and Brazil (111 181).

Mangoes represent an important part of the diet in many less developed countries of the subtropics and tropics. Their attractiveness and flavor have

also enhanced the quality of life in these countries. Approximately 1% of mango production is utilized for processing for juice, nectars, preserves (including chutney), fruit leather, frozen pulp, and for flavoring baked goods, ice cream, yoghurt, etc. (Nanjundaswamy, 1997; Raymundo *et al.*, 2008). The seed is the source of the starch “amchur”, and anacardic acid is extracted from the skin. The timber of mango trees is of low quality, and the tree bark is an important source of tannins for curing leather.

1.4 Traditional Breeding: Breeding Objectives, Tools, Strategies, and Achievements

Almost all of the mango cultivars are derived from open-pollinated seedlings (see above). Nonetheless, several horticultural traits involving fruit quality and tree architecture have been targeted for improvement (Iyer and Degani, 1997). (1) Regularity of bearing, particularly in biennial bearing Indian-type mangoes, is a high priority. (2) Shortening the juvenile period to enable early flowering and bearing, i.e., precocity, would bring new orchards into earlier production. (3) A compact or dwarf habit would allow high density planting. (4) Resistance to a number of pests and diseases is very important. Diseases that affect mango production and postharvest include anthracnose, which is caused by *Colletotrichum gloeosporioides* (Penz) Penz & Sacc. in Penz and *Alternaria* rot, which is caused by *Alternaria alternata* (FR.:FR.) Keissel (Dodd *et al.*, 1997; Ploetz and Prakash, 1997). (5) The mango fruit should have skin that has an attractive blush, pleasant taste, and aroma and the pulp should be fibreless. (6) Mango fruit is an important export commodity, and therefore extended shelf life and freedom from physiological disorders that cause precocious fruit ripening, for example, “jelly seed” and “soft nose”, in certain important export cultivars, such as ‘Tommy Atkins’, are important.

Mango cultivars that have emerged from breeding programs and which involved controlled pollinations include the successful “Swarnajehangir”, “Mallika” (“Neelum” × “Dashehari”), and “Amrapalli” (“Dashehari” × “Neelum”) in India (Iyer and Degani, 1998). In Australia, “Calypso” has been rapidly adopted by the mango industry (Iyer and Schnell, 2008).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional breeding has not had much impact on mango cultivar development, and almost all cultivars are selections made among seedlings of open-pollinated trees. It is difficult to make controlled crosses between selected mango trees because of the low frequency of fruit set (<0.01%) (Iyer and Degani, 1997) and the universal occurrence of polyembryony in Southeast Asian mangoes. *M. indica* is extremely heterogeneous due to extensive outcrossing and polyploidy ($2n = 4x = 40$) (Mukherjee, 1950). The long juvenile period, which can be at least 7 years following germination, and the time required for evaluation of the mature tree, i.e., 12–15 years after germination, have also impeded breeding. Cultivars that have been released from contemporary breeding programs for the most part have not been accepted in traditional mango producing countries of South and Southeast Asia, because their fruit quality has generally been considered to be inferior to traditional selections.

Because of the problems associated with conventional breeding of mango, genetic transformation holds promise that existing superior cultivars can be transformed with a gene that mediates an important horticultural trait. Fully ripened mango fruit are highly perishable, largely due to the rapid development of anthracnose. Significant changes occur during fruit ripening that affect texture, and these changes can affect fruit storage. Consequently, mango fruits are usually harvested before maturity, when they cannot ripen normally and their flavor is not fully developed (Medlicott *et al.*, 1988). Postharvest losses in storage and in transit to markets can range from 10% to 80% (Gómez Lim and Litz, 2007). Mango fruit physiology resembles other temperate and subtropical climacteric fruit. The differences with respect to temperate fruit involve major substrates during ripening, the rate of ripening and senescence, the order in which the components of ripening occur and chilling sensitivity (Gómez Lim and Litz, 2007).

Softening of fruit occurs as a result of hydrolysis of different cell wall components, solubilization of pectin (Roe and Bruemmer, 1981; Lazan *et al.*, 1986) and a net loss of arabinose, galactose, and galacturonic acid (Brinson *et al.*, 1988).

Various cell wall hydrolytic enzymes increase in activity during softening (Gómez Lim, 1997a). Mature green mango fruit contains accumulated starch (Subramanyam *et al.*, 1976), which is hydrolyzed during ripening (Morga *et al.*, 1979), resulting in increased total sugars, primarily the monosaccharides glucose and fructose (Selvaraj *et al.*, 1989) and sucrose (Castrillo *et al.*, 1992). The total sugar content varies among cultivars, for example, “Carabao” has one of the highest values (Peacock and Brown, 1984), whereas “Golek” has one of the lowest (Lizada, 1993). The activities of both amylase and sucrose phosphate synthase increase several fold during ripening (Mattoo *et al.*, 1975; Castrillo *et al.*, 1992).

Mango fruits are climacteric with a moderate respiration rate (Kader *et al.*, 1985), since there appears to be a relationship between respiration rate and storage life, reducing respiration should increase postharvest life. With many fruits, the respiration rate can be controlled by temperature management; however, tropical fruits are sensitive to low temperature. Climacteric fruits demonstrate increased ethylene production during ripening, and fruits of different species appear to have different sensitivities to ethylene. The climacteric and the ethylene peak in mango occur simultaneously (Tucker and Grierson, 1987).

Ethylene biosynthesis is mediated by enzymes that catalyze the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC to ethylene, i.e., ACC synthase and ACC oxidase or ethylene forming enzyme (EFE) (Adams and Yang, 1979; Kende, 1993). Burg and Burg (1962) observed a small peak of ethylene during ripening of monoembryonic “Haden” and “Kent”, and this has been confirmed with polyembryonic “Carabao” (Cua and Lizada, 1990) and “Manila” (López-Gómez and Gómez Lim, 1992). Ethylene production increases considerably during the climacteric. “Carabao” mangoes produce ethylene prior to full maturity with the highest rate occurring in the outer mesocarp (Cua and Lizada, 1990). The levels of ACC are similar throughout the mesocarp.

Premature ripening of mango fruit has been a cause of large-scale postharvest spoilage. The current strategies that have been adopted to prevent or reduce this spoilage include harvesting of mature green fruit, exposure of fruit to ethylene adsorbents, including potassium permanganate or activated charcoal/vanadium oxide (Maekawa,

1990) and storage of fruit at optimum temperature. Ripening of mango fruit can be delayed when preclimacteric fruits are treated with 1-methylcyclopropane (Jiang and Joyce, 2000; de Silva *et al.*, 2004; dos Santos *et al.*, 2004). Storage temperature is dependent upon cultivar, preharvest environment (Arpaia, 1993), maturity at harvest (Kader *et al.*, 1985), and storage conditions (Kader, 1994). Storage at temperatures above 8–12 °C causes ripening and senescence, and lower temperatures cause chilling injury.

Mango fruits have poor storage quality, and storage in modified atmospheres causes physiological disorders (Chaplin, 1989), which can affect skin, flesh, and other ripening attributes. Chilling injuries include pitting, darkening, uneven ripening, and susceptibility to decay. Storage life and sensitivity to chilling injury depend on fruit maturity and storage temperature. Fruit harvested at early stages of maturation ripen slowly and are more susceptible to chilling injury (Kader, 1994). Ripe fruits are less susceptible to chilling injury (Medlicott *et al.*, 1990a, b) and can be stored for up to 21 days at <8 °C without deterioration in quality, although fruits may deteriorate rapidly after removal from storage (Johnson *et al.*, 1997).

Storage of mango fruit in modified atmospheres has not been particularly successful, and fruit develop poor color, eating quality, and undesirable flavors. Mango ripening has never been delayed satisfactorily in this way (Chaplin, 1989), although Kader (1994) suggested some modified atmospheres for storage of mango could delay ripening with increased firmness retention.

2. DEVELOPMENT OF TRANSGENIC MANGOES

Premature, ethylene-induced fruit ripening lowers the yield of mango. Because this occurs before shipping or in transit to markets, mango fruits are highly perishable and have a short shelf life. Postharvest problems of many temperate fruits have been addressed by harvesting them at the immature or green stage, and/or by storing them either at low temperatures or in controlled atmospheres. However, mango fruits are susceptible to low temperature, they do not store well if they are harvested at full maturity, and the fruits generally fail to ripen properly if they are immature at the time of harvesting.

Genetic transformation of mango to date has focused on the improvement of fruit quality by controlling ethylene biosynthesis and, therefore, ripening. The strategy that has been utilized has involved the inhibition of expression of genes encoding ethylene biosynthetic enzymes by transformation with antisense genes. Three enzymes mediate the biosynthesis of ethylene: SAM synthase, ACC synthase, and ACC oxidase. ACC synthase and ACC oxidase in the antisense have been used in transformation studies involving tomato, and this strategy has resulted in slowly softening fruits to permit longer vine ripening and good shipping qualities (Oeller *et al.*, 1991; Gray *et al.*, 1992).

Gómez Lim (1997a) isolated ACC synthase and ACC oxidase from a mango cDNA library. He observed that ACC synthase cannot be detected in unripe fruit, and begins to appear in turning fruit and achieves maximum expression in ripe fruit (Gómez Lim, 1997a). Expression of ACC synthase appears earlier in the mesocarp than in the peel. In contrast, the expression of ACC oxidase is similar in both tissues, but is detectable before ACC synthase (Gómez Lim, 1997b). ACC oxidase is probably expressed before ACC synthase and ripening and therefore proceeds from the mesocarp outward toward the peel. ACC oxidase and ACC synthase first appear in the peel of ethylene-treated fruits.

2.1 Donor Gene

The only horticultural trait of mango that has been targeted for improvement by genetic transformation is the control of fruit ripening (Cruz Hernandez *et al.*, 1997). The following genes have been utilized in the antisense: alternative oxidase, ACC oxidase, and ACC synthase. Alternative oxidase cDNA was isolated by *in situ* hybridization of a ripe mango fruit mesocarp cDNA library. ACC oxidase cDNA was cloned from an *Arabidopsis thaliana* cDNA library, and ACC synthase was cloned by polymerase chain reaction (PCR)-amplification from DNA isolated from mango leaves (Gómez Lim, 1993; Cruz Hernandez and Gómez Lim, 1995; Cruz Hernandez *et al.*, 1997).

All of the genes were cloned at the *EcoRI* site in pBLUESCRIPT (Stratagene), and they

were recovered by *SacI-KpnI* double restriction. They were transferred to a pGEM7Zf(+) vector (Promega), flanking the genes with *XbaII* sites and cloned at the *XbaI* site in the pBI121 binary vector and controlled by the Cam 35S promoter.

2.2 Methods of Transformation

The accepted procedure for genetic transformation of mango was developed by Mathews *et al.* (1992, 1993), who reported genetic transformation of mango embryogenic cultures of “Hindi” and “Keitt”, respectively. These studies utilized two different disarmed, engineered strains of *Agrobacterium tumefaciens*, i.e., strain C58C1 containing pGV 3850::1103 with the selectable marker gene neophosphate transferase (*NPTII*) that confers resistance to the antibiotic kanamycin and driven by the cauliflower mosaic virus (CaMV) 35S promoter (Mathews *et al.*, 1993) and strain A208 containing the pTiT37-SE::pMON9749, a cointegrate vector, with genes for *NPTII* and the scorable marker β -glucuronidase (*gus* or *uidA*) both driven by the CaMV 35S promoter (Mathews *et al.*, 1992).

For transforming mango with ACC synthase, ACC oxidase and the EFE genes in antisense, the pBI121I/ACC, pBI121/AOX, and pBI121/EFE vectors were introduced into *A. tumefaciens* strain LBA4404 by electroporation. The *Agrobacterium* was activated with 30 mM acetosyringone for 16 h.

The mango embryogenic pathway has been utilized for transformation; details of this regeneration pathway have been reviewed by Mathews and Litz (1992), Litz *et al.* (1993, 1995, 2008), Litz and Lavi (1997), Litz and Gomez-Lim (2005), and Gómez Lim and Litz (2007). Embryogenic cultures of mango were induced from the nucellus of “Hindi” using standard induction medium consisting of modified B5 major salts (Gamborg *et al.*, 1968), MS (Murashige and Skoog, 1962) minor salts and organics, 400 mg l⁻¹ glutamine, 60 g l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), and 2 g l⁻¹ gellan gum (Litz *et al.*, 1982; DeWald *et al.*, 1989a). Cultures on semi-solid medium were maintained in darkness at 25 °C. Embryogenic cultures were maintained in liquid medium of the same formulation under diffuse light at 25 °C, and were transferred to fresh medium at weekly intervals.

2.3 Selection of Transformed Tissue for Resistance to Kanamycin Sulfate

Mathews and Litz (1990) determined that growth of nontransformed embryogenic suspension cultures was inhibited by $12.5 \mu\text{g ml}^{-1}$ kanamycin sulfate; whereas, growth of larger proembryonic masses was inhibited on semi-solid medium by $200 \mu\text{g ml}^{-1}$ kanamycin sulfate. Embryogenic suspension cultures in their logarithmic phase of growth were passed through sterile filtration fabric ($1000 \mu\text{m}$ pore size), and approximately 300 mg of the large fraction ($>1000 \mu\text{m}$) was abraded with a sterile brush on sterile filter paper. Abraded embryogenic cultures were co-cultured with a log phase culture of acetosyringone-activated *A. tumefaciens* in 50 ml of liquid medium for 3 days. *Agrobacterium* contained vectors pB121I/ACC, pBI121/AOX, and pBI121/EFE.

All genetic transformation reports have followed a two-step selection procedure (Mathews *et al.*, 1992, 1993; Cruz Hernandez *et al.*, 1997). Embryogenic cultures were transferred to semi-solid maintenance medium containing 100 mg l^{-1} cefotaxime and 100 mg l^{-1} kanamycin sulfate. Following the appearance of light colored segments on the co-cultured tissue, putatively transformed embryogenic cultures were transferred to semi-solid maintenance medium containing 200 mg l^{-1} kanamycin sulfate and 100 mg l^{-1} cefotaxime. Cultures in liquid medium were maintained in 125 ml Erlenmeyer flasks under diffuse light in the laboratory at 125 rpm. Cultures on semi-solid medium were maintained in darkness at 25°C .

Putatively transformed embryogenic cultures were transferred to somatic embryo maturation medium containing 200 mg l^{-1} kanamycin and 100 mg l^{-1} cefotaxime. Somatic embryo maturation medium consisted of B5 major salts, MS minor salts and organics, 400 mg l^{-1} glutamine, 60 g l^{-1} sucrose, and 20% (v/v) filtered sterile coconut water from freshly harvested immature nuts (DeWald *et al.*, 1989b). Cultures were maintained in darkness at 25°C .

2.4 Regeneration of Whole Plants

Germinating somatic embryos were moved to light conditions ($60 \mu\text{mol s}^{-1} \text{ m}^{-2}$), and when shoots began to emerge, they were transferred to minimal

medium consisting of B5 major salts, MS minor salts and organics, 30 g l^{-1} sucrose, 0.1% activated charcoal and 2.0 g l^{-1} gellan gum. Of the different procedures that have been attempted to enhance plant recovery, i.e., conversion, micrografting and *ex vitro* grating of somatic embryo shoots on seedling rootstocks has been most effective (Litz, unpublished data).

2.5 Testing

Mango trees are propagated vegetatively. Consequently, many of the outstanding cultivars of traditional mango-producing countries, i.e., India, Pakistan, Myanmar, Thailand, Malaysia, etc., are several hundred years' old. The time required to determine the stability of inheritance of transgenes in superior tree selections would require at least two decades and possibly longer, and is thereby not feasible. Likewise, the long juvenile period of 7 years and the 6 or 7 more years required to evaluate whether productivity has been affected preclude classical approaches to evaluate plants in a breeding program.

Transformation was therefore confirmed by (1) growth and development of putatively transformed embryogenic cultures and somatic embryos in the presence of growth-limiting kanamycin sulfate, (2) the 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-Gluc) reaction for the presence of the *uidA* gene, (3) amplification of the *nptII* gene by PCR, and (4) Southern blot hybridization.

2.6 Specific Regulatory Measures Adopted

Transgenic mangoes have been maintained in a nursery, and have not been transferred to the field.

3. FUTURE ROAD MAP

3.1 Expected Products

It is anticipated that postharvest handling and storage of mango fruit will be greatly improved as a result of this strategy. It has been estimated that postharvest losses of mango fruit in developing countries can exceed 70% of the total production. The losses are due to the development of

anthracnose in rapidly ripening fruit and to the poor infrastructure, i.e., storage facilities, roads, etc. in many mango-producing countries. By lowering the postharvest losses of fruit, the profitability of the industry should be greatly improved, and lower acreage of mango groves would be needed to meet local and international demands.

3.2 Addressing Risks and Concerns

It is not anticipated that there will be any issues related to human health and the environment; however, the introduction of transgenic mangoes into traditional mango-producing areas could have a major impact on patterns of farming. Because of the large postharvest losses of fruit and vegetables in the tropics, there must be greater production to meet demand. This innovation should enable producers to devote less acreage to production of mango, and this would result in much lower production costs, for example, fungicides, fertilizer, etc.

3.3 Expected Technologies

Cruz Hernandez *et al.* (2000) reported the transient genetic transformation of embryogenic “Kensington Pride” and “Carabao” cultures using a biolistic approach and two vectors: (1) pBI426 with *GUS-NPTII* under the control of a double CaMV 35S promoter and (2) pBINGfp-Ser, which contains *NPTII* and the green fluorescent protein gene (*gfp*). Although stable transformants were not recovered, biolistic-mediated transformation might facilitate the transformation procedure with this species.

Ideally, the ACC oxidase, ACC synthase, and *EFE* genes in antisense should be expressed under the influence of a mango fruit ripening-specific promoter. However, there has been no progress in this respect. Other genes that suppress ethylene biosynthesis, including ACC deaminase (Klee *et al.*, 1991) and SAM hydrolase (Good *et al.*, 1994) should also be evaluated for their effects on controlling mango fruit ripening. These genes have both been isolated from microorganisms, and this may cause increased scrutiny with respect to health and safety issues.

3.4 Economic Consequences

This strategy for improving shelf life of mango should have a major impact on the economics of fruit production in developing countries (see Section 3.1). By dramatically reducing postharvest losses, it should be possible to produce more marketable fruits on less acreage. The acceptance of transgenic mango fruits in export markets is an uncertainty, although it is quite likely that they will be accepted quickly in producing countries.

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Avocado

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The avocado is believed to have its center of origin in the humid tropical highlands of central America, embracing Chiapas in southern Mexico, Guatemala, and Honduras. The etymology of avocado is the Nahuatl “ahuacatl”, which was transliterated into Spanish as “aguacate”. According to Smith (1966, 1969), the Mexican race was identified and possibly selected 9000–10 000 years ago. Gama (1994) believed that domestication of avocado probably occurred over a long period of time among meso-American cultures. The avocado is now grown in most of the tropical and subtropical regions.

1.2 Botanical Descriptions

The Lauraceae is an ancient group that consists primarily of tree species, some of which are important, i.e., avocado (*Persea americana*), bay leaf (*Laurus nobilis*), camphor (*Cinnamomum camphora*), and cinnamon (*Cinnamomum zeylanicum*). The genus *Persea* may have originated in Gondwanaland, and radiated to Asia and North America via Europe. The genus spread to South America via Antarctica during the Paleocene, and when the land bridge between North and South America was established during the late Neocene, the genus was reunited. The Southeast Asian *Machilus*, *Nothaphoebe*, and *Alseodaphne* genera

may be congeneric with *Persea* (Kostermans, 1952). Extensive speciation within *Persea* occurred in central America (Scora and Bergh, 1990). Wild *P. americana* trees occur in the forests of these tropical highlands (Kopp, 1966). There are two subgenera within *Persea*, i.e., *Eriodaphne* (South America) and *Persea* (North America) (Kopp, 1966). Scora *et al.* (2002) identified three species within the subgenus *Persea*: *P. schiedeana* Nees, *P. parviflora* Williams, and *P. americana* Mill.

Within *P. americana*, there are eight well-defined subspecies or geographical races that evolved in different climatic environments and in geographical isolation from each other (Scora *et al.*, 2002). The subspecies include *P. americana* var. *nubigena* (Williams) Kopp, var. *steyermarkii* Allen, var. *zentmyerii* Schieber and Bergh, var. *floccosa* Mez., and var. *tolimanensis* Zentmyer and Schieber. Three other subspecies comprise the common avocado (Bergh and Ellstrand, 1986), and include the Mexican *P. americana* var. *drymifolia* (Schlect. and Cham.) Blake, which originated in the highlands of south-central Mexico, the Guatemalan subspecies *P. americana* var. *guatemalensis* Williams, which originated in medium elevations in the tropics, and the Antillean or West Indian subspecies *P. americana* var. *americana* Mill., which originated in the humid lowlands of the central American Pacific coast (Popenoe, 1941; Storey *et al.*, 1986). The ancestry of the Guatemalan race may include var. *nubigena*, var. *steyermarkii*, var. *zentmyerii*, and var. *tolimanensis* (Kopp, 1966; Schieber and Bergh, 1987; Furnier *et al.*, 1990). The Antillean

subspecies may have evolved from the Mexican subspecies according to Williams (1976, 1977).

The avocado tree is evergreen, and is approximately 20 m high at maturity. It is supported by a shallow unsuberized secondary root system, although anchorage roots can penetrate to 3–4 m (Whiley, 1992). Flowers are borne on the branch terminals as panicles of cymes. The fruit is a large, fleshy, pyriform, or globose berry containing a single seed. The chromosome number of the species is $2n = 2x = 24$ (Garcia, 1975), and the haploid genome size is 8.83×10^8 bp (base pair), which is relatively small, only 6× the size of *Arabidopsis thaliana* and similar to that of tomato (Arumuganathan and Earle, 1991).

1.3 Economic Importance

World production of avocados was estimated to be 3 271 962 t in 2005 (FAOSTAT, 2006). The greatest production occurs in Mexico (1 021 515 t), Indonesia (263 575 t), the United States (256 189 t), Colombia (185 811 t), Brazil (182 000 t), Chile (163 000 t), and Dominican Republic (140 000 t). Other important producers are South Africa, China, Venezuela, and Peru. Although avocado is normally consumed as a fresh fruit, it is also a rich source of oil.

The international trade in fresh fruit is very important, with an estimated value of avocado exports in 2004 of US\$606 611 000 (FAOSTAT, 2006). Leading exporting countries include Mexico (US\$211 255 000), Chile (US\$94 624 000), and Spain (US\$86 316 000). Other important exporting countries include Israel, New Zealand, and South Africa. The EU and North America are the biggest importers of avocados, US\$387 012 000 and US\$213 275 000, respectively (FAOSTAT, 2006). “Hass” is the most important export avocado.

1.4 Traditional Breeding

Within its native habitat, the avocado tree is a large spreading canopy species, and like other tropical forest trees, it is genetically heterozygous. Avocado has a long juvenile period of approximately 7 years, and several more years are required to assess productivity of seedling trees. Although the

avocado tree flowers profusely, there is a very high incidence of floral abscission and immature fruit drop. The flowering pattern is dichogamous, protogynous, and synchronous (Whiley, 1992); each flower opens twice during two consecutive days. As a consequence, all open flowers are either functionally male or female. Avocado cultivars are identified on the basis of their flowering pattern, depending on whether the flowers are female in the morning (type A) or in the afternoon (type B), and in this way, self-pollination is prevented.

Conventional breeding programs for avocado improvement have been moderately successful, despite the fact that the leading cultivars are almost all derived from open pollinations. The current status of avocado breeding has been reviewed by Bergh and Lahav (1996) and Lahav and Lavi (2002). Because of the lack of segregating seedling populations, the long juvenile period and the time required for assessing the mature phenotype, very little is known about the inheritance of horticultural traits. According to Lavi *et al.* (1993a, b), most morphological traits are probably coded by several loci with several alleles in each locus. Bergh and Lahav (1996) suggested that the dwarf trait of *P. schiedeana* can be attributed to a single gene.

Many avocado cultivars are hybrids that involve two or more subspecies or races. “Hass” and “Fuerte” are Guatemalan × Mexican hybrids. Early California cultivars originated either as selections made in Mexico and central America, e.g., “Benik”, “Itzamna” and “Nabal”, or from imported seeds, e.g., “Dickinson” and “Fuerte” (Bergh, 1957). “Bacon”, “Hass”, “Pinkerton”, “Reed”, and “Zutano” were selected from open-pollinated seedlings in California, USA, and are grown in Mediterranean and subtropical climates. In South Florida, USA, and other tropical regions, West Indian and Guatemalan × West Indian hybrids are grown.

Conventional avocado breeding has focused on improvement of both rootstocks and scions. The most serious threat to avocado production is the disease *Phytophthora* root rot (PRR), which is caused by the soil-borne pathogenic oomycete *Phytophthora cinnamomi* Rands (Coffey, 1987; Whiley, 1992; Ben-Ya’acov and Michelson, 1995). Within the *Persea* subgenus, there is no resistance to PRR, although resistance to PRR occurs in the subgenus *Eriodaphne*. Species in the latter group

are sexually and graft incompatible with species in the subgenus *Persea* according to Zentmyer (1980), Bergh and Ellstrand (1986), and Lahav and Lavi (2002). The rootstock “Duke” and its progeny, “Duke 7”, “Barr-Duke” and “D9”, “Thomas” and “G6” are tolerant to PRR. Although “Martin Grande” (G755) (*P. schiedeana* × *P. americana*) (Ellstrand *et al.*, 1986) is PRR tolerant, the trees are unproductive. Rootstocks with tolerance to PRR often show no tolerance to the related pathogen, *Phytophthora citricola* (Tsao *et al.*, 1992).

Several major production areas are in the semiarid or arid environments where irrigation water is either scarce or somewhat saline. As a result, rootstocks are being sought that show tolerance to saline conditions. Within the different subspecies/races, West Indian avocados generally have the most resistance to saline conditions, whereas Mexican avocados have the least resistance (Kadman and Ben-Ya’acov, 1976). West Indian avocados also have greater resistance to calcium-induced chlorosis (Ben-Ya’acov, 1972). West Indian avocados do not tolerate heavy and waterlogged soils (Ben-Ya’acov *et al.*, 1974). Production costs could be lowered if the large growth habit of the avocado tree could be controlled with dwarfing rootstocks. The “Colin V-33” selection, used either as an interstock or as a rootstock, has been demonstrated to confer a dwarf habit to the scion according to Sánchez-Colin and Barrientos-Priego (1987).

There are several breeding objectives for scion selections. The market standard for avocado is the black-skinned “Hass”, although the green-skinned “Fuerte” also holds a small, yet significant, market share. Fruit ideally should be approximately 250–350 g (Lahav and Lavi, 2002), with a small seed, either of pyriform or of ovate shape, and an easily removable peel. Many of the cultivars that have emerged from breeding programs have fruits that resemble “Hass”, and indeed, complement “Hass” by bearing in the off-season, e.g., “Gwen”, “Jim”, “Lamb Hass”, and “Reed”. The tropical avocados (West Indian and West Indian × Guatemalan hybrids) are much more heterogeneous with respect to shape and size.

Mexican avocados are quite susceptible to anthracnose, caused by *Colletotrichum gloeosporioides* Penz. (Ruehle, 1963). There are varying levels of resistance to *Cercospora* spot or blotch, caused

by *Pseudocercospora purpurea* Cooke, and avocado scab, caused by *Sphaceloma perseae* Jenkins in certain avocado cultivars.

Avocado fruits of Mexican, and hybrids with Mexican parentage, are strongly climacteric (Adato and Gazit, 1977). There is a fixed climacteric phase followed by a variable lag phase during which low concentrations of ethylene are produced (Eaks, 1980), and this in turn triggers the climacteric as sensitivity to ethylene increases (McMurchie *et al.*, 1972). After ripening has been initiated, it cannot be arrested. Fruits of Guatemalan and Mexican cultivars and their hybrids mature but do not directly ripen, and can be stored “on the tree” for 2–4 months (Whiley, 1992); ripening occurs after the fruits have been harvested. In contrast, West Indian and West Indian × Guatemalan cultivars mature and ripen “on the tree”, and must be picked at maturity. By exploiting this habit and production in different climatic niches, it has been possible for growers in California, USA, to provide “Hass” fruits almost year round (Griswold, 1945). They have been able to market a uniform product; however, year-round production of West Indian and West Indian × Guatemalan fruit in the tropics requires several cultivars, each with a different maturity date (Crane *et al.*, 1996), and there is no market standard for these avocados.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

The avocado tree is a genetically heterogeneous species with a complex breeding system. It has a long juvenile period of approximately 7 years and an equally long period before many mature tree characteristics that impact production can be evaluated. Although there has been some limited success in identifying molecular markers that are associated with phenotypic characteristics, marker-assisted breeding has still not made an impact on avocado improvement. The most important breeding objectives, namely resistance to PRR in rootstocks and improvement of the postharvest storage life of West Indian and West Indian × Guatemalan selections, cannot be achieved by conventional breeding. Particularly for addressing these issues, therefore, a transgenic approach is essential.

2. DEVELOPMENT OF TRANSGENIC AVOCADOS

Control of ripening of avocado fruit is being addressed. Several genes that have been implicated in ripening of avocado fruit have been cloned. A ripening-specific cellulase (*pAV5*) was the first gene to be identified in avocado (Christoffersen *et al.*, 1984). Tucker *et al.* (1987) used *pAV5* to screen a complementary DNA (cDNA) library from ripe "Hass" fruit, and isolated *pAV363*. Cass *et al.* (1990) screened an avocado genomic library with the cellulase cDNA probe and identified *cell*, which is homologous with cellulase cDNA. Only *cell* transcripts have been detected in mesocarp of ripe avocado fruit, and it has also been detected in the fruit abscission zone (Tonutti *et al.*, 1995). Expression of *cell* in the mesocarp during fruit ripening is associated with abscission of mature avocado fruits.

Bozak *et al.* (1990, 1992) and O'Keefe *et al.* (1992) cloned a cytochrome P-450 from the mesocarp of ripe avocado fruit. The gene was designated as *CYP71A1*, and there is accumulation of the *CYP71A1* gene product during ripening, although the function of *CYP71A1* is uncertain. Avocado polygalacturonase (*PG*) has been isolated from a cDNA library by heterologous hybridization with a tomato cDNA probe, and Wakabayashi and Huber (2001) isolated an endo-*PG* from avocado mesocarp. *AVOe3* messenger-RNA has been identified as a ripening-related gene and might be involved in ethylene biosynthesis (McGarvey *et al.*, 1992). Twenty-three cDNA clones have been identified by differential screening of a cDNA library of cold-stored avocado fruit (Dopico *et al.*, 1993). Six of the clones showed increased expression during cold storage and normal ripening, and were homologous with *PG*, endochitinase, a cysteine proteinase inhibitor, and several stress-related proteins.

Although PRR is the most serious production problem of avocado, there have been no reports of the identification of genes that might control this disease. Since there is no resistance within the subgenus *Persea*, candidate genes would have to be cloned in species within subgenus *Eriodaphne*, e.g., *Persea borbonia*, *Persea cinerascens*, *Persea pachypoda*, etc., that are highly resistant to the disease.

2.1 Donor Gene

In order to address postharvest storage of avocado fruit by controlling ethylene biosynthesis, the bacteriophage gene S-adenosyl-L-methionine (SAM) hydrolase (*SAMase*) under the control of a cellulase promoter isolated from avocado fruit has been utilized (Hughes *et al.*, 1987). *SAMase* in the binary vector pAG4092, which used the backbone of the pPZP200 binary vector, was utilized (Agritope, 1999). The pAG4092 has the *NPTII* gene that encodes resistance to the antibiotic kanamycin under the AGT01 promoter located near the left border and the *samK* gene driven by a fruit-specific avocado cellulase promoter located near the right border (Agritope, 1999). The *samK* gene is a modified *samase* and encodes for *SAMase* and catalyzes the conversion of SAM to methylthioadenosine (Good *et al.*, 1994). Since SAM is the metabolic precursor of 1-aminocyclopropane-1-carboxylic acid, the proximal precursor of ethylene, the depleted SAM pool inhibits ethylene biosynthesis (Good *et al.*, 1994; Kramer *et al.*, 1997).

In the absence of candidate genes that might confer resistance to PRR, avocado has been transformed with an antifungal defensin gene *pdf1.2*. The defensin gene was driven by the cauliflower mosaic virus 35S promoter in binary vector pGPTV; the construct also contained the *uidA* reporter gene (GUS— β -glucuronidase) and the *bar* gene for resistance to phosphinothricin, the active ingredient of the herbicide Finale[®]. The *pdf1.2* gene was isolated from *A. thaliana*, and is induced by different phytopathogenic fungi: *Alternaria raphans*, *Alternaria brassicola*, *Fusarium oxysporum* f. sp. *matthiolae*, *F. oxysporum* f. sp. *raphans*, etc. (Epple *et al.*, 1997).

2.2 Transformation Methods

The standard procedure for transforming avocado has involved the use of highly embryogenic cultures (Witjaksono and Litz, 1999a, b; Witjaksono *et al.*, 1999), and has been based upon the initial study of Cruz-Hernandez *et al.* (1998). The complete suppression of embryogenic avocado culture growth occurred on semisolid medium containing 200 mg l⁻¹ kanamycin sulfate. Cruz-Hernandez

et al. (1998) described a two-step selection protocol for genetic transformation of avocado. Embryogenic cultures on semisolid maintenance medium were gently abraded with a soft camel hairbrush, and then incubated with acetosyringone-activated *Agrobacterium tumefaciens* strain 9749 ASE2, which contained a co-integrate vector pMON9749 with a selectable kanamycin sulfate-resistant marker (*nptII*—neomycin phosphotransferase II) and *uid* in liquid maintenance medium for 3 days at 100 rpm. *A. tumefaciens* was eliminated by incubating the cultures in maintenance medium supplemented with 50 mg l⁻¹ kanamycin sulfate and 200 mg l⁻¹ cefotaxime. There was an initial selection for antibiotic resistance in liquid maintenance medium containing 50 mg l⁻¹ kanamycin sulfate for 2–4 months, and a second, more intensive selection with 100 mg l⁻¹ kanamycin sulfate for 2 months. The cultures were finally cultured in maintenance medium containing 200 mg l⁻¹ kanamycin sulfate to eliminate chimaeras. Somatic embryo development occurred on maturation medium without kanamycin sulfate, followed by subculture onto maturation medium containing 200 mg l⁻¹ kanamycin sulfate. Transgenic plants were not regenerated in this early study.

For transforming avocado with *samase* and *pdf1.2*, the pAG4092 and pGPTV-BAR-AFP vectors, respectively, were introduced into *A. tumefaciens* EHA 105 by electroporation. The *Agrobacterium* was activated with 30 mM acetosyringone for 16 h. Embryogenic “Suardia” cultures were genetically transformed with *samase*, and embryogenic “Hass” cultures were transformed with *pdf1.2*. The embryogenic cultures were induced from zygotic embryos extracted from immature avocado fruit. Induction medium consisted of semisolid B5 (Gamborg *et al.*, 1968) major salts, Murashige and Skoog (MS) (Murashige and Skoog, 1962) minor salts, 0.1 mg l⁻¹ thiamine HCl, 100 mg l⁻¹ myo-inositol, 30 g l⁻¹ sucrose, 0.41 μM picloram, and 8.0 g l⁻¹ agar (Witjaksono and Litz, 1999a). Cultures were incubated in darkness at 25 °C.

Embryogenic cultures were maintained either on semisolid medium (MSP), MS basal medium with 0.1 mg l⁻¹ thiamine HCl, 100 mg l⁻¹ myo-inositol, 30 g l⁻¹ sucrose, and 0.41 μM picloram, or as suspension cultures. For suspension cultures, the nitrogen component of MS was modified

accordingly: 12 mg l⁻¹ NH₄NO₃ and 30.3 mg l⁻¹ KNO₃ (MS3:1P). Approximately 200 mg of embryogenic cultures was inoculated into 40 ml of liquid MS1:3P medium in 125 ml Erlenmeyer flasks, which were sealed and maintained on a rotary shaker at 100 rpm and 25 °C under subdued light. Subcultures were at 2-week intervals.

2.3 Selection of Transformed Tissue

2.3.1 *Samase*

The two-step transformation procedure described by Cruz-Hernandez *et al.* (1998) was modified (Efendi, 2003). Embryogenic “Suardia” suspension cultures in their logarithmic (log) phase of growth were passed through sterile filtration fabric (1000 μm pore size), and approximately 300 mg of the large fraction (>1000 μm) was abraded with a sterile brush on sterile filter paper. Abraded embryogenic cultures were co-cultured with log phase acetosyringone-activated *A. tumefaciens* in 50 ml of liquid medium for 3 days. *Agrobacterium* contained vector pAG4092. An embryogenic suspension culture in its log phase of growth was sieved through sterile nylon filtration fabric (mesh size 1.8 mm), and the smaller fraction was used.

The small fraction was subcultured into 80 ml fresh MS 3:1 medium in 250-ml Erlenmeyer flasks supplemented with 50 mg l⁻¹ kanamycin sulfate and 100 mg l⁻¹ spectinomycin to which a 1.0 ml aliquot of 6-h-old acetosyringone-activated *A. tumefaciens* was added. Flasks were maintained at 125 rpm in darkness at 25 °C for 3 days, when the cultures were transferred into fresh MSP 3:1 medium supplemented with 200 mg l⁻¹ cefotaxime and 500 mg l⁻¹ carbenicillin for 8 days with a change of medium after 2 days. Embryogenic cultures were then transferred into fresh MSP 3:1 medium supplemented with 50 mg l⁻¹ kanamycin sulfate and then 4 days later to fresh medium with 100 mg l⁻¹ kanamycin sulfate. Cultures were maintained in MS 3:1 medium supplemented with 100 mg l⁻¹ kanamycin sulfate. Somatic embryo development occurred on semisolid MS medium supplemented with 30 g l⁻¹ sucrose, 20% (v/v) filter-sterile coconut water, and 100–300 mg l⁻¹

kanamycin sulfate (Witjaksono and Litz, 1999b, 2002).

2.3.2 *pdf1.2*

The inhibition of “Hass” embryogenic suspension culture growth by phosphinothricin was reported by Raharjo *et al.* (2008), who reported that 3 mg l^{-1} phosphinothricin inhibited the growth of embryogenic cultures, and was utilized as the basis for selection for cultures transformed with the pGPTV-BAR-AFP construct. The large fraction of embryogenic “Hass” suspension cultures in their log growth phase was collected on sterile filtration fabric ($1000\text{ }\mu\text{m}$ pore size), and approximately 300 mg was abraded with a sterile brush on sterile filter paper. The tissue was then co-cultured with log phase acetosyringone-activated *A. tumefaciens* strain EHA 105 with pGPTV-BAR-AFP in 50 ml of liquid medium for 3 days. The proembryonic masses (PEMs) were then transferred to liquid maintenance medium supplemented with 200 mg l^{-1} cefotaxime and 500 mg l^{-1} carbenicillin, and subcultured 2 weeks later into fresh liquid maintenance medium with 200 mg l^{-1} cefotaxime, 500 mg l^{-1} carbenicillin, and 3.0 g l^{-1} phosphinothricin for 3 months. Embryogenic cultures were selected continuously with phosphinothricin, and stained periodically for *uidA* until they were apparently completely transformed. Transformed embryogenic cultures were transferred to somatic embryo development medium that also contained 3 mg l^{-1} phosphinothricin.

2.4 Regeneration of Whole Plants

The shoots from somatic embryos transformed with *pdf1.2* (“Hass”) and *samase* (“Suardia”) were excised and grafted *in vitro* on decapitated “Peterson” seedling rootstocks (Raharjo and Litz, 2005). Rapidly growing transgenic shoots were excised and grafted on “Peterson” rootstocks in the nursery. In order to assure that *pdf1.2* was expressed in roots as well as shoots of “Hass”, *pdf1.2*-transformed scion shoots were air layered, and self-rooted plants were recovered. “Hass” plants contained the *bar* gene, and were resistant to the herbicide Finale, whose active ingredient is

phosphinothricin. In order to expedite flowering in “Suardia” shoots with *samase*, budwood was grafted on “Hass” and “Lula” interstocks.

2.5 Testing

Avocado is a perennial plant with long juvenile period. It is, therefore, impractical to study the inheritance of transgenes in avocado because of the time involved. Because fruit trees are clones, often of great antiquity, the goal of transformation is to modify existing cultivars for one or more horticultural traits. The transgenic plants have been under assessment in a nursery for $2\frac{1}{2}$ years. We have not observed any phenotypic changes in “Hass” plants containing *pdf1.2*, although alterations in plant architecture and leaf morphology have been observed with “Suardia” containing *samase*.

2.6 Regulatory Measures

Transgenic avocado plants have not been transferred from the nursery to the field; however, “Hass” plants containing *pdf1.2* were shipped to California for greenhouse observation and this was under the APHIS (Animal Plant Health Inspection Service (USA)) and California Department of Plant Industries regulations.

3. FUTURE ROAD MAP

3.1 Expected Products

“Hass” avocado that expresses the *pdf1.2* gene constitutively should demonstrate enhanced resistance to fungal pathogens. This should be significant for control of diseases that affect fruit and foliage, such as anthracnose, caused by *C. gloeosporioides*, and *Cercospora* spot caused by *P. purpurea*, and important diseases of rootstocks caused by *Rosellinia necatrix* and other pathogens. “Suardia” transformed with *samase* under the control of an avocado fruit-specific promoter should demonstrate inhibition of ethylene during fruit development. This should enable prolonged storage of fruit “on the tree” and in postharvest storage. This should have a great effect on the

rationalization of tropical avocado cultivars, and create a market standard for Antillean avocados.

3.2 Addressing Risks and Concerns

There should not be any human health or environmental risk with either of these transgenic avocados, although the bacteriophage origin of *samase* may require extra scrutiny before “Suardia” is cleared for commercial production. Both transgenic avocados may find ready acceptance in tropical producing regions for local consumption. Currently, the EU is the largest market for avocado fruit, and acceptance of transgenic fruit there is still uncertain.

3.3 Expected Technologies

Although *samase* is expressed under the control of an avocado fruit-specific promoter, such organ-specific and development-specific promoters are still rare in most tree species. Root-specific promoters would be very useful for controlling pathogenesis-related and defensin genes that target pathogens that attack the root system of avocado, e.g., *P. cinnamomi*. *Persea* species within the *Eriodaphne* are the only source of genes that confer immunity to PRR, and every possible effort should be undertaken to identify and clone these genes.

3.4 Economic Consequences

Success of the *samase* strategy for blocking the biosynthesis of ethylene in avocado fruit is projected to have a major impact on avocado production, particularly with respect to the tropical or Antillean cultivars. Currently, there is no market standard for Antillean avocados. The fruits ripen “on the tree” and have poor postharvest storage life. As a consequence, Florida, USA, avocado producers must grow more than 20 cultivars in order to supply markets for most of the year (Crane *et al.*, 1996). By blocking ethylene biosynthesis, it is our expectation that transgenic Antillean avocados could be stored “on the tree” and could compete with the Mexican and Mexican × Guatemalan cultivars.

Enhancing disease resistance in roots, fruit, and foliage of avocado using the *afp1.2* strategy can only improve the sustainability of avocado production in the humid tropics and the subtropics.

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Kiwifruit

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The name *Actinidia*, attributed by Nathaniel Wallich to plants found in Nepal, derives from Greek *aktis*, which means irradiation due to the radiate stigma of the flowers. The common name, kiwi or kiwifruit, is accepted worldwide for the resemblance with the bird symbol of New Zealand where it was cultivated since 1904 (Ferguson, 1999). Other names were attributed in other countries: “Chinese gooseberries” in the United States, “Groseille de China” in France, “Mao-yantao” or “Mao-ertao”, and “Yang-tao” or “Yang-taw”, with bristly hair and soft hair, respectively, in China, “Actinidia” the plant and “kiwi” the fruit, in Italy. It has become a common fruit over the last 30 years in all the markets of the world.

Actinidia is widespread in the temperate and subtropical regions of the Asian continent. It is considered native to China, since most of the species are spread in the Yangtze river valley offering a bulk of germplasm resources. These huge resources are of immense value in developing new varieties. Among them the two species with the most important economic value are *Actinidia chinensis* var. *chinensis* growing more in the lowlands in the eastern provinces, and *Actinidia deliciosa* in the western, inland, colder areas (Huang and Ferguson, 2001). Until 20 years ago they were classified as one species; however,

A. chinensis fruit is almost hairless and the flesh ranges from a lime green to bright yellow and fruits are sweeter than those of *A. deliciosa*. The most important cultivars grown in the world belong to these two species (Chen, 2003). Other species have been found in the Korea, Japan, Nepal, India, Vietnam, and Russia (Ferguson, 1990a). In these areas, the fruits were collected from the wild, and several of them were introduced for cultivation few years ago and some of them have been selected for the breeding programs.

1.2 Botanical Description

Cultivated kiwi varieties of *A. deliciosa* are characterized by vigorous growth, flexible trunks, and superficial root system, thus strong support structures are needed, increasing the costs of establishment of the orchards. On the contrary *A. chinensis* has a compact vegetative habit (Testolin *et al.*, 1996). Long flexible and climbing vines with large leaves and long internodes are heavily damaged in windy areas where it is possible to observe lower pollination by bees, breaking flowers, leaves, and branches, physiological dysfunctions resulting in hydric disequilibria, causing leaves to dry up, and subsequently, defoliation. The original microenvironment is characterized by high atmospheric relative humidity, mild temperatures, and a limited light intensity during the summer period. Winter and fall frost (−20 °C) can crack bark while late spring frosts, especially

during blossoms, cause necrosis in flowers and tender shoots. Winter temperatures of 8–10°C ensure the chilling requirement for blossom and the total cold timing should be between 1500 and 2000 h for 8°C and 2000 and 3000 h for 10°C (Sale, 1983). The most widely cultivated kiwifruit cultivar, Hayward, needs only 400–600 h below 7°C. In some areas with low winter chilling, spraying dormancy-breaking agents are necessary to increase bud break and fertility (Costa *et al.*, 1997).

Irrigation is essential for keeping soils fresh and also for maintaining high atmospheric humidity in the kiwi orchard. The soil must be rich in organic matter with a neutral or slightly acid pH. Plants do not tolerate highly concentrated nitrates or soluble chlorides in low absorbent soils; thereby soils must have a good drainage system and be rich in humus (Revilla *et al.*, 1992).

Dioecism in kiwi represents a big problem not only for the presence of 15–20% of unproductive pollinators but also for the difficulties in pollination due to asynchrony of blossom with pollinators (Testolin *et al.*, 1990). Flowers are less attractive to insects; and pollen is unsuitable for insect pollination because it is not sticky enough and unsuitable for wind pollination because it is heavy. Fecundation of all ovules is necessary because large fruit size is correlated with the number of seeds. Regular pollination is assured either by artificial pollination or by grafting staminate twigs on the pistillate plant. Dioecism in kiwi is not well defined, both pistils and stamens develop normally but few weeks before blooming one of the two stops growing (Schmid, 1978). Inconstant male and female plants (plants with hermaphrodite flowers) of *A. deliciosa* (Ferguson, 1984; Hirsch *et al.*, 1990; Messina *et al.*, 1990; McNeilage, 1991a, b; Ossani, 1994) and *A. chinensis* (Tang and Jiang, 1995) have been reported. The use of these inconstant staminate plants in genetic improvement programs has given heterogeneous results. Crossing with pistillate plants resulted in only 1–2% of hermaphroditism (Seal and McNeilage, 1989; Buscaroli and Bulzacca, 1994; Testolin *et al.*, 1995; Ferguson *et al.*, 1996); however, in both species the fruit size of hermaphrodite flowers is too small. Chromosome doubling of these plants does not increase the size of the fruits (E. Rugini, personal communication).

1.2.1 Taxonomy

The first botanical name given to the fruit worldwide known as “kiwifruit” was *A. chinensis* by Planchon in 1847. Then, a description based on the smooth skinned and hairy fruit was given by Burbank in 1911. The first botanical distinction of kiwifruit was made by Chevalier (1941) based on the same character that Burbank described, proposing the name of *A. chinensis* var. *deliciosa* for the hairy variety. Later, Liang and Ferguson (1986) proposed the name of *A. chinensis* for the plants with soft-haired fruit, and *A. deliciosa* for the plants with stiff-haired fruits. Since then, the accepted botanical name is *A. deliciosa* (A. Chev.), C.F. Liang and A.R. Ferguson. In the 1990s, cellular biology and molecular systematics and phylogeny have been extensively studied (Cipriani *et al.*, 1995, 1996; Xiao and Hirsch, 1996). In 1998, it was reported that the chloroplast DNA is paternally inherited in the genus *Actinidia*, thus *A. chinensis* is the paternal progenitor of *A. deliciosa* (Cipriani *et al.*, 1998). This discovery confirmed the hypothesis of Testolin and Ferguson (1997) who affirmed that hexaploid *A. deliciosa* ($2n = 174$) derived by polyploidization of the diploid *A. chinensis* ($2n = 58$). These results have been further confirmed by Hirsch *et al.* (2002) who studied the relationships of the two species through chemical means by analyzing their coumarin content (fraxin and esculin). These substances were not present in other *Actinidia* species belonging to the section of the genus.

Over 110 *taxa* and 60 species, belonging to four sections (*Leiocarpae*, *Maculatae*, *Stringosae*, and *Stellatae*) are considered within the genus *Actinidia* Lindl of the *Actinidiaceae* family, and many of those have edible fruit (Liang, 1984); however, the systematical botanic is still under classification because it is a dioecious and polyploid species and these features make genetic studies difficult (Huang *et al.*, 1999). *A. deliciosa*, *A. chinensis*, *Actinidia setosa* are considered to belong to the same complex due to similar morphological traits, and until recently they have been treated as *A. chinensis* by botanists (Liang, 1984), although they differed in ploidy level, fruit size, shape, and taste. The polyploid races of the complex may be derived from the diploid *A. chinensis* genotypes without there being a contribution from any other *Actinidia*

species (Testolin and Ferguson, 1997). The fruit size is directly correlated to the ploidy level and inversely correlated to the plant frost resistance. Only the female cultivar “Hayward” of *A. deliciosa* is intensively cultivated all over the world followed by the pollinator of the male cultivar Tomuri or Matua. Recently, Chinese breeders have released 15 new cultivars deriving from the germplasm source of *A. chinensis*, and these cultivars are widely used in China (Chen, 2003).

Molecular approaches have been attempted for understanding the taxonomy of the genus and for identifying cultivars, such as, DNA-based restriction fragment length polymorphisms (RFLPs) (Crowhurst *et al.*, 1990), microsatellites, or simple sequence repeats (SSRs) (Crowhurst and Gardner, 1991), polymerase chain reaction (PCR)-amplified chloroplast DNA fragments (Cipriani and Morgante, 1993; Cipriani, 1994; Cipriani *et al.*, 1998), and isoenzymes (Messina *et al.*, 1992; Testolin and Ferguson, 1997).

1.2.2 Sex identification

Identification of male and female genotypes and cultivars within the species is the first step toward the correct classification of kiwifruit germplasm. *A. chinensis*, a diploid species, is the progenitor of *A. deliciosa* var. *deliciosa*, which is a hexaploid, $2n = 6x = 174$, and the chromosomes X and Y are not morphologically distinguishable due to their small size (McNeilage, 1997). It is important to identify the sex of the plants at juvenile stage in order to raise material as male and female plant populations before planting an orchard for breeding purposes. The PCR-based random amplified polymorphic DNA (RAPD) technique can be used to identify the dioecy in several species. The breeding programs aimed at selection for fruit characteristics could gain an advantage from molecular sex markers for identifying male seedlings and discarding them at the early stages of growth.

After the early work of Hirsch and Fortune (1984) who used peroxidase for sex determination in *Actinidia*, research in this area has continued with some modifications (Hirsch *et al.*, 1992, 1997) and is further extended to the isoperoxidase, isopolyphenyl oxidase (Auxtova *et al.*, 1994), and polyamines (Biasi *et al.*, 1999). Since this technique

has certain limitations, more attention is being focused on molecular markers.

Cipriani *et al.* (1996) found that none of the 80 RAPD primers (10 bp) tested produced bands linked to the sex. Fraser *et al.* (1997) constructed cDNA libraries from female and male buds of a sibling family of *A. chinensis* at pollen pre- and postmeiotic stage, and found one clone showing high sequence homology to the *fil 1* protein expressed in anther filaments of snapdragon. Harvey *et al.* (1997a, b) used sex-linked RAPD markers (800 bp) and found that sex-determining genes are located in a pair of chromosomes functioning like XX/XY system with male heterogamety. By using amplified fragment length polymorphism (AFLP)-based bulked segregant analysis and then individual analysis of a sibling family of diploid *A. chinensis*, Zhang *et al.* (1998a) observed one male-exclusive and one female-exclusive band.

Besides sex labeling, RAPDs have also been used in cultivar typing: 20 primers were selected for producing polymorphic fragments in 13 kiwifruit cultivars (Cipriani *et al.*, 1996, 1997), and parentage analysis as well as ploidy level of crosses between *A. deliciosa* and *Actinidia arguta* was studied by using flow cytometry and RAPD markers. The results showed the existence of 5x and 2x progenies from 4x and 6x crosses (Chat and Dumoulin, 1997).

Shirkot *et al.* (2002) identified eight sex-linked markers and from seven cultivars of *A. deliciosa* studied, six were female specific and two were male specific in nature.

1.2.3 Mapping

In *A. chinensis*, Weising *et al.* (1996) identified a set of microsatellite markers from a small insert genomic library of DNA, and designed 18 primer pairs according to the DNA sequence of 35 hybridizing clones. Using most of these primer pairs, they successfully fingerprinted a range of closely related varieties in *A. deliciosa*. Huang *et al.* (1998) isolated and sequenced 263 microsatellites-containing clones from *A. chinensis*, with a high degree of polymorphism. These microsatellites were used to support the hypothesis that original diploid species of *Actinidia* were polyploids with

a basic chromosome number $x = 14/15$. A wide range of genetic similarity was observed by using RAPD markers among the 40 taxa, while for varieties of the same species it was 0.54 and for different species it was 0.28. Although the phylogenetic analysis revealed a clear indication that section *Leiocarpace* was a monophyletic group, the subdivision of the other three traditional sections was poorly supported. The majority of the species clustered into geographic subgroups in accordance with their natural distribution (the Yangtze River, Southeastern China, Southern China, and Southwestern China). The intrageneric subdivisions of *Actinidia* appeared to be difficult, but some subdivisions could be explained by the geographic distribution of the species, particularly for species of Liang's sections of *Maculatae* and *Stellatae* (Huang *et al.*, 2002).

Maps were constructed from enriched genomic libraries together with AFLP markers using interspecific crosses between *A. chinensis* and *A. callosa* (Testolin *et al.*, 2001). Later, Frase *et al.* (2004) used microsatellites from expressed sequence tags (ESTs) to evaluate the frequency of occurrence and the level of polymorphism in genetic map construction. They found that EST-derived microsatellites of *A. chinensis* gave large number of possible markers highly polymorphic between the parents and allowed the construction of a map. These results showed that EST libraries are a valuable resource for the supply of markers for genetic mapping of *Actinidia*.

Crowhurst and Gardner (1991) suggested for the first time that kiwifruit may be an allopolyploid species because they found genome-specific repeat sequences in *A. deliciosa*. In addition, 26 restriction sites from five PCR-amplified chloroplast sequences were mapped and analyzed. Three species out of the 20 examined originated through natural interspecific hybridization. *In situ* hybridization with the species-specific repeat sequence has been carried out by Yan *et al.* (1997b), who observed six hybridization sites in some accessions of *A. chinensis*, and all of *A. deliciosa*. These results indicated that the genomes of polyploid *Actinidia* are similar but not identical. The molecular data clearly showed that some taxa, such as *Actinidia rufa* and *Actinidia kolomikta*, occupy a wrong taxonomical position (Cipriani *et al.*, 1998).

The efficiency of two molecular markers (RAPD and SSR) has been compared for investigating the

clonal stability of micropropagated kiwifruit genotypes with interesting agronomic traits maintained in collection. Both molecular marker techniques were able to amplify all of the genotypes, but only SSR markers could detect genetic variation induced in micropropagated plants of cv. Tomuri. (Palombi and Damiano, 2002).

1.3 Economic Importance

In China, most of the orchards are planted with *A. chinensis* accessions (Huang *et al.*, 1999) whereas the world market is based almost exclusively on the pistillate cultivar Hayward, with two pollinators, Matua and Tomuri, selected from *A. deliciosa* by scientists of New Zealand who imported this species around 1930 from China. In this country, the cultivation became economically important from the mid 1960s, and only in early 1970s this species became widely distributed, with little attention to the attitude toward the environment. The other pistillate cultivars like "Abbott", "Allison", and "Bruno", were cultivated together with "Hayward" and they lost their popularity in favor of "Hayward" for its better shape, size, and long storage ability. However, this cultivar is not free from defects, which include high risk of sudden disease occurrence, pest infection or frost damage for its very narrow genetic base, and the dioecism character typical of the genus.

In the recent years, few accessions from cv. Hayward have been selected among the offspring of *in vivo* crosses and from somaclonal variation derived from micropropagated plants. They included hairless fruit, and early fruit ripening, comprising the accession Summer 4605[®] and Summer 3373[®] having 35 and 50 days, respectively earlier ripening than cv. Hayward (Ossani, 1994; Summerkiwi[®] 20010160 and 20010159 patented by V. Ossani from the Istituto Sperimentale di Frutticoltura, Rome (Del Pane, 2005)). Accessions with hairless fruit are susceptible to fruit fly (*Ceratitis capitata*) and for this reason they have not been included in the cultivation.

Recently, the New Zealand cultivar "Zesprigold" (Hort16A) obtained from *A. chinensis*, presents interesting characteristics such as, yellow pulp, higher vitamin C content, sweet flavor, and contemporary ripening to Hayward. The cultivar also has some negative attributes such

as, early blossom and shooting, its fruits are almost hairless and have a mucro that can damage the other fruits during handling. Suitable pollinators used for this cultivar are “K2” and “K3”.

Only few other species with different ploidy levels but with a base chromosome number of 29, seem to be important from an economical point of view, such as: *A. chinensis* ($2n = 2x = 58$), *A. deliciosa* ($2n = 6x = 174$), *A. deliciosa* var. *chlorocarpa* ($2n = 2x = 174$), var. *coloris* ($2n = 6x = 174$), *A. glaucophylla* ($2n = 2x = 58$), *A. guilinensis* ($2n = 2x = 58$), *A. indochinensis* ($2n = 2x = 58$), *A. setosa* ($2n = 2x = 58$), *A. arguta* ($2n = 4x = 116$), *A. arguta* var. *purpurea* ($2n = 8x = 232$), *A. eriantha* ($2n = ?x = 58$), *A. latifolia* ($2n = 2x = 58$), *A. chrysantha* ($2n = 4x = 116$), *A. melanandra* ($2n = 4x = 116$), *A. kolomikta* ($2n = 2x = 58$), and *A. polygama* ($2n = 2x = 58$) (Den and Sen, 1986; Xiong and Huang, 1988; McNeilage and Considine, 1989; Watanabe *et al.*, 1990; Yan *et al.*, 1997a).

1.4 Production, Nutritional Attributes, and Industrial Uses

Kiwifruit has gained enormous popularity in many countries of the world due to its nutritional and medicinal values. World production has increased from the 875 727 metric tons in 1998 to 1 120 938 metric tons during 2005 covering a higher cultivated area of 68 542 ha compared to 52 972 ha of 1998. This includes only four countries as the main producers: Italy (415 052 metric tons), China (340 000 metric tons), New Zealand (318 000 metric tons), and Chile (150 000 metric tons) (Huang and Ferguson, 2003; FAOSTAT, 2006). Only in China about one-quarter of total cultivated areas produced 11 cultivars of *A. chinensis*, the remainder area with 11 cultivars of *A. deliciosa*.

The beneficial effects of an increased consumption of fruit and vegetables against oxidative DNA damage of human cells have been attributed and demonstrated, under *ex vivo* and *in vitro* conditions, to the various antioxidants present in some foods (Collins *et al.*, 2001). The main antioxidant is the vitamin C or L-ascorbic acid and its partially oxidized form: the L-dehydroascorbic acid (DHAA). Kiwifruit, as well as the genus *Citrus* and the guava (*Psidium guajava*) are excellent

sources of vitamin C. The genus *Actinidia* presents large differences in fruit composition among species and often within cultivars (Beever and Hopkirk, 1990; Ferguson, 1990a, 1991; Ferguson, 1990b; Ferguson and MacRae, 1992). There are a considerable number of cultivars and selections in the genus with a wide diversity in size and shape of fruit, hairiness, color of flesh, actinidin content, flavor and taste (Ferguson, 1991; Boyes *et al.*, 1997), and wide variation in vitamin C content (Ferguson and MacRae, 1991). The cultivar Hort16A of *A. chinensis* contains 50% higher vitamin C than the cultivar Hayward (80–120 mg per 100 g fresh weight), and the cv. Sanuki Gold reach more than threefold that of Hayward, as well as cv. Gassan, Issai, and Mitsuko (Nishiyama *et al.*, 2004b), while within the species *A. deliciosa* the range goes from 30 to 400 mg per 100 g of fresh weight. High concentration of ascorbic acid are also reported in *A. arguta* (40–185 mg), *A. chinensis* (up to 180 mg) (<http://www.naturalhub.com>; Chen and Chen, 1998; Muggleston *et al.*, 1998), and the ratio of L-ascorbic acid to total ascorbic acid tend to be higher than that of other species (Nishiyama *et al.*, 2004a): *A. chrysantha* (around 70 mg), and *A. polygama* with very high levels in *A. eriantha* and *A. latifolia* (over 1000 mg per 100 g of pulp) (Ferguson, 1991), and *A. kolomikta* (Kola and Pavelka, 1988). On the other hand, phenols contents and total antioxidant activity of *A. chinensis* rated the second lowest after plum (Imeh and Khokhar, 2002). Like many other fruits, it has low vitamin B and carotene contents. Minerals are also high, especially potassium and magnesium as well as copper, zinc, and manganese. These cations are important for regulating acid/base ratio in the organism, increasing the alkaline reserve in the blood and increasing the pH of the urine (Panatta, 1990).

A negative aspect is that it contains oxalates, which is considered to be a breakdown product of ascorbic acid (Keates *et al.*, 2000; Kostman *et al.*, 2001), and it has adverse nutritional effects because it binds Ca^{2+} , Mg^{2+} , and Fe^{2+} and lowers their availability in the diet by decreasing their absorption, as well as calcium oxalate crystallization and kidney stone formation (Hodgkinson, 1981; Noonan and Savage, 1999). Furthermore, it was found that the needlelike calcium oxalate crystals found in kiwifruit can irritate the mucous membranes of the mouth

(Perera *et al.*, 1990; Walker and Prescott, 2003). Recently, the ascorbic acid and total oxalate were measured in fruit from six genotypes of *A. chinensis*: the whole fruit mean ascorbic acid ranged from 98 to 163 mg per 100 mg of fresh weight, whereas mean oxalate varied between 18 and 45 mg. Ascorbic acid was highest in the inner and outer pericarp while was absent in the seeds. Oxalate was concentrated in the skin, inner pericarp, and seed, but both substances are not concomitants. It may be possible to select cultivars for high vitamin C content without having high levels of oxalate (Rassam and Laing, 2005).

Kiwifruit is involved in allergic reactions causing local or systemic mucosal or both types of symptoms by an IgE-mediated mechanism (Fine, 1981). This immunoglobuline corresponds at a 30 kD protein, the major allergen and the main protein in kiwifruit, it is called actinidin or Act c 1, a proteolytic enzyme belonging to the class of thiol-proteases, a cysteine protease (Pastorello *et al.*, 1998). Researches have demonstrated that there is a difference in actinidin content within the species and cultivars: in the cvs. Sanuki Gold, Ananasnaya, Shinzan, Hirano, Gassan, and Mitsuko, both actinidin concentration and protease activity were much higher than in cv. Hayward, while cultivars First Emperor, Tear drop, Kosui, Awaji, Nagano, and Kosen were lower than Hayward. The allergen is also present in fruits of the newly released cultivar Zespri™ Gold of *A. chinensis* (Lucas *et al.*, 2005). Regarding allergens content within the species, in fruits of *A. arguta* was much higher than in *A. deliciosa* or *A. chinensis* fruits (Nishiyama *et al.*, 2004a, b; Yamanaka *et al.*, 2004). Subsequently, Gavrovic-Jankulovic *et al.* (2005) found that the quality and quantity of allergen content vary in function of the time of fruit harvesting or fruit ripening of *A. deliciosa*: in November harvest it was found the highest allergenic potential of Act c 1 and Act c 2 in fruit extracts. The potential allergenic effect of *A. arguta*, known as hardy kiwifruit, has also been studied by Chen *et al.* (2006) as raw fruit and processed food product. They found that the heat processing alters allergenic protein structure, reduces *in vitro* IgE binding, and reduces the risk of eliciting an allergenic response in those with allergies to raw kiwifruit.

In the ancient Chinese medicine, kiwifruit has been used as therapy for different kinds of cancer. Motohashi *et al.* (2002) studied the anticancer

activity of kiwi extracts of commercial cultivar Hort 16 (Gold) and found that many extracts switches from pro-oxidant action to antioxidant action depending on its concentration and they showed also tumor selectivity, cytotoxic, and antimicrobial activity.

Market acceptance relates to kiwi fruit color, fruit size, texture of flesh, eating quality, price, availability and continuity of supply. Fruit size is one of the most critical parameters in the selection of kiwi. Fruit weight must be superior to 100 g with shape typical of cv. Hayward, dark brown skin. Kiwi plants often require treatment by bioregulators such as the cytokinin-like compound N-(2-chloro-4-puridil-N-phenylurea) (CPPU) (Lewis *et al.*, 1996) and hydrogen cyanamide to enhance fruit size. The hairless skin seems to be more acceptable to consumers. Another important character is pulp color that ideally should be bright green; other colors are not fully acceptable, probably for the absence of bright colors. Fruit flavor is another important criterion in selecting kiwi cultivars and depends on the balance between acidity and sweetness. Fruits of *A. chinensis* are characterized by higher sugar content and better smell than *A. deliciosa*. Furthermore, this species presents a longer harvesting period, from September to November in Northern China (Ferguson, 1984). The newly released cv. Zespri™ Gold (*A. chinensis*) has only been exported from New Zealand since 1998 and then to the United States and Europe in 2000, and its consumption is increasing in many parts of the world. New kiwifruit cultivars from *A. arguta*, known as “baby kiwi”, are also been produced in the United States, Europe, New Zealand, and South America due to the wonderful flavor, good productivity, fruit size, extended maturity dates, good storage and shelf life (Williams *et al.*, 2003).

Fruits are consumed mainly as fresh within 1–6 months after harvesting. A small amount of the total production is used for food industry, such as jams, juices, and syrups. In New Zealand, the residue after processing is used to produce wine (Monastra, 1991).

1.5 Traditional Breeding: Achievements and Limitations

Although the large variability of traits is found in the vast genetic resources within the genus

Actinidia, many interesting agronomic characters are not present in the commercial cultivars such as drought tolerance, adaptation to cool climates, or disease resistance. Interspecific crosses may be carried out, or interesting genes could be identified and transferred to cultivated kiwi. Unfortunately, the genetic improvement by traditional cross-breeding and the potential of *Actinidia* germplasm remains underexploited due to the dioecism of the genus, the unknown genetic background of staminate plants, the relative long juvenile phase, and the presence of at least one-half of staminate plants among the offspring, which increases the cost and time for genetic selection with traditional breeding. Furthermore, the different levels of ploidy among the *Actinidia* species represent another obstacle for interspecific hybridization; so many attempts to produce plantlets between species failed (Pringle, 1986; McNeilage and Considine, 1989; Mu *et al.*, 1990b; Ke *et al.*, 1991). To overcome these difficulties different biotechnological methods, such as protoplast culture, somatic hybridization, embryo rescue, creation of dihaploids lines have been developed.

1.5.1 Protoplast culture

Since the first report on protoplast isolation in the cv. Hayward by Cossio and Marino (1983) several protocols have been described. In some cases only small colonies were formed (Pedroso *et al.*, 1988; Revilla and Power, 1988), while successful results were reached in shoot regeneration from leaf callus and cotyledon protoplasts of *A. chinensis* (Mii and Ohashi, 1988; Tsai, 1988; Xiao *et al.*, 1992). Plant regeneration from protoplasts obtained from preserved callus, leaf and/or cotyledon have been also carried out in *A. deliciosa* (Oliveira and Pais, 1991, 1992; Cai *et al.*, 1992; Xiao *et al.*, 1993). Somaclonal variation on leaf shape and plant morphology was observed as well as variation in the chromosome number (Cai *et al.*, 1992). Protoplasts from different *Actinidia* species were successfully isolated and cultured in *A. arguta* var. *arguta*, var. *purpurea*, cv. Issai, *A. chinensis*, *A. kolomikta*, *A. polygama*, and *A. eriantha* and many plants were obtained (Derambure and Hirsch, 1995; Zhang *et al.*, 1995; Xiao and Hirsch, 1996, 1997). From the regenerated plants of *A. eriantha*, obtained by Zhang *et al.* (1995), changes in chromosome number and ploidy level were found

as well as the multinucleate phenomenon of the cells in most of the plants under study (Zhang *et al.*, 1997, 1998b).

A purification method to obtain populations of leaf protoplasts of Hayward was also developed and the efficiency of β -glucuronidase (GUS) reporter gene expression was compared by direct DNA transfer and plant regeneration (Raquel and Oliveira, 1996).

The first efforts on protoplast fusion were carried out by Xiao and Han (1997) who worked with protoplast from *A. chinensis* var. *chinensis*, *A. deliciosa* var. *deliciosa*, and *A. kolomikta*. Successful results were obtained from both interspecific crosses and RAPD analysis indicated that five clones had RAPD banding patterns corresponding to the parental bandings, and the ploidy level was also changed.

1.5.2 Somatic hybridization

In order to avoid frost damage before harvesting, new cultivars with earlier ripening or frost resistance are recommended in genetic improvement programs. *A. chinensis* could supply valuable genes for crossing in order to obtain earlier harvesting genotype (Testolin *et al.*, 1996). Interspecific somatic hybrids between *A. chinensis* and *A. kolomikta* have been obtained by protoplast fusion: the chilling tolerance under *in vitro* conditions was similar to *A. kolomikta*. The expected cold resistance might be higher than *A. chinensis* plants (Xiao *et al.*, 2004).

1.5.3 Embryo rescue

The first efforts to produce interspecific hybrid with successful results were obtained by Mu *et al.* (1990a) who worked with *A. chinensis* and *A. melanandra*, and the conditions for successfully rescuing embryo were determined by Mu *et al.* Some plantlets were generated, and most of them grew slowly, lacked apical dominance, and did not survive in the greenhouse. The crosses were repeated later and the characteristics of the plants were studied (Harvey *et al.*, 1995). In order to study embryo and endosperm development of the seeds Xi-jin *et al.* (1992) produced hybrids between *A. deliciosa*, *A. eriantha*, and *A. arguta*. Some incompatibility was observed but normal seedlings were produced from embryos of normal

appearance. Further efforts to improve the embryo rescue technique and in order to assess the degree of crossability occurring in the genus *Actinidia*, a set of culture media and techniques were developed to ensure the survival of the embryos at globular and heart stages. In several crosses, embryos at torpedo stage were rescued when incubated in appropriate medium. The embryo rescue was the unique tool that led to hybrid plantlets for four crosses between *A. kolomikta* × *A. chinensis*, *A. polygama* × *A. valvata*, *A. arguta* Issa × *A. polygama*, and *A. kolomikta* × *A. deliciosa*, which permit to deduce the wide interspecific crossability between different sections based on traditional taxonomy (Hirsch *et al.*, 2001).

1.5.4 Homozygous lines

In conventional breeding programs, a pure line or haploidization is developed after several generation of self-pollination, which is not possible to carry out in fruit crops because of their long reproductive cycle, high degree of heterozygosity, and self-incompatibility. The interest of using haploids and doubled haploids lies in the possibility of shortening the time needed to produce homozygous lines compared to conventional breeding. Haplo-diploidization through gametic embryogenesis allows single-step development of complete homozygous lines from heterozygous parents. Embryogenesis and organogenesis were achieved from callus tissue of anthers of *A. chinensis* var. *chinensis* and *A. deliciosa* var. *deliciosa* by Fraser and Harvey (1986) who found that the embryogenic potential was dependent on genotype and sex of plant.

To facilitate the crossing among diploid, tetraploid, and hexaploid and to improve fertility in some hybrids, encouraging results of ploidy manipulation have been obtained by Fraser *et al.* (1991) who tried to double the chromosome number via callus from stems of *A. chinensis* and pollen of *A. deliciosa*. A combination of colchicine treatment and tissue culture regeneration produced tetraploids plants from diploids *A. chinensis* var. *chinensis*. They found also that endosperm culture gave rise mixoploidy plants, but continued culture and regeneration led to the stabilization of chromosome number. The use of oryzaline treatment in one parthenocarpic trihaploid from cv. Hayward was more effective than colchicine in producing

hexaploid and didecaploid plants (Chalak and Legave, 1996). Rugini *et al.* (1996a) observed an increase in ploidy level among regenerated plants from callus in both staminate and pistillate cultivars without the use of chemical treatments. After 14 years of their establishment in the experimental field they have not bloom yet although they present a mature morphological aspect.

1.5.5 Selective pressure and somaclonal variation

Another strategy for inducing genetic variability is manipulating the substrate composition in order to create conditions for a selective pressure. Marino and Battistini (1990) produced somaclones tolerant to high pH under *in vitro* conditions by culturing the *in vitro* explants in media with high pH. Muleo *et al.* (1996) cultured calli in high concentration of glucoselike synthetic compounds: 3-O-Methylglucose and N-Methyl-D-glucamine. Both compounds induced a similar type of genetic variation in regenerated plants, contrary to NaCl at the same water potential. RAPD analysis showed different frequencies and patterns of polymorphic fragments. Although interesting results have been obtained by this technique, it produces some undesirable traits, which do not have agronomic importance. Somaclonal variation for active limestone tolerance has also been obtained by Marino and Bertazza (1996) in *A. deliciosa* from somaclones previously obtained with tolerance to high pH (Marino and Battistini, 1990). Recently, Caboni *et al.* (2003) induced somaclonal variation by *in vitro* organogenesis under selective pressure with high NaCl concentrations, obtaining five tolerant shoot lines of the cv. Tomuri, and RAPD analyses showed polymorphism in two of them. Later, the different responses of the selected clone to salt stress conditions were confirmed from field trial.

The ability to regenerate plants from callus encouraged scientists to induce somaclonal variation for enhancing the ploidy level, increase the fruit size, calcareous, and drought tolerance. Somaclonal variation has been observed in different tissues of *Actinidia* after *in vitro* culture. Hirsch *et al.* (1992) studied the occurrence of variation by culturing different explants such as stem, leaf, root, ovary, anther filament,

mature endosperm, and embryos from *in vitro* regenerated plantlets. Gui *et al.* (1993) reported that endosperm-derived plants had different fruit size and shape, seed number, and chromosome variation (from 58 to 146). Mostly plants were aneuploids with only a few triploids but none of them were parthenocarpic.

1.5.6 Parthenocarpy

Parthenogenesis in *A. deliciosa* was obtained by Padey *et al.* (1990) by using lethally irradiated pollen, and also the parthenogenic response was investigated for Hayward where high rates of trihaploids were detected as well as some hexaploids by spontaneous doubling (Chalak and Legave, 1997). Later, studies on the endosperm development after pollination with irradiated pollen from different male cultivars gave rise that endosperm was autonomous and represent the $2n$ level and 30% of the ovules developed (Musial and Przywara, 1998, 1999).

2. DEVELOPEMENT OF TRANSGENIC KIWIFRUIT

In the last two decades, transgenic plants of *A. deliciosa* have been recovered with some foreign genes of interest: *rolABC*, *rolB*, whole T-DNA, *osmotin*, human epidermal growth factor (*hEGF*). Research on transgenics in *Actinidia* started when Rugini *et al.* (1989) reported genetic transformation and plant regeneration from leaf discs of *A. deliciosa* with the *rol* genes of *Agrobacterium rhizogenes*. Transformation experiments were also tried with *A. rhizogenes* by González *et al.* (1990), and Atkinson *et al.* (1990) with *Agrobacterium tumefaciens* under glasshouse conditions. Subsequently, both direct and indirect gene transfer methods have successfully being tried in three species of the *Actinidia* genus by using protoplasts and different explants of zygotic and maternal origin.

Rugini and Mariotti (1992) developed a procedure to regenerate transgenic plants for most of the genes of the T-DNA of *A. rhizogenes*. Tissues (leaves and shoots) produced abundant hairy roots in auxin-free medium after the co-cultivation with both *A. rhizogenes* wild type

and *A. tumefaciens* carrying *rolABC*, and *rolB* genes, and the selectable marker gene neomycin phosphotransferase II (*nptII*). With *A. tumefaciens* LBA4404 carrying the *rolB*, alone or associated with *A* and *C*, of *A. rhizogenes*, the roots emerged from the inoculation point or at the base of the shoots. Molecular analysis of these roots indicated that a few of them were transgenic probably due to inductive effect of transgenic cells on the nontransgenic neighboring cells. In addition for *rolABC* or for *rolB*, roots still attached to the shoots grew very fast for several days even at high kanamycin concentrations (500–1000 mg l⁻¹). Selection could be possible with detached roots on hormone-free medium, before placing them on medium for regeneration of transgenic shoots.

2.1 Growth Habit Modifications

Modification of the pattern of growth habit may permit labor saving in kiwifruit vineyard management and reduce the cost of production.

The first efforts to obtain *Actinidia* plants with altered phenotype were reported by Rugini *et al.* (1989, 1990, 1991) on the male late-flowering pollinator called “GTH”, which was transformed with the *rolABC* genes of *A. rhizogenes*, cloned in pBin19 and transferred into *A. tumefaciens* strain LBA4404. Transgenic plants, regenerated from leaf disc callus, showed the typical hairy root phenotype as described in herbaceous species (Tepfer, 1984). Furthermore, leaf discs or 3- to 4-node leafy microcuttings of these transgenic plants showed an increased ability to produce roots in auxin-free medium. Subsequently, following the same transformation technique, transgenic plants for *rolABC* and *rolB* were regenerated from the cv. Hayward (Rugini and Mariotti, 1992). Field evaluation of male and female transgenic cultivar is underway since 1998 (Rugini *et al.*, 1999). Firsov and Dolgov (1997) regenerated transgenic plants of *A. kolomikta* having *rolC* gene; all transgenic plants for *rolC*, singly or associated with other genes, showed the morphological dwarf characteristic typical of plants transformed by *rolC*.

Transformation by using the whole T-DNA of *A. rhizogenes* was performed by Yazawa *et al.* (1995) who obtained transgenic plants with “hairy root” phenotype from roots induced by *A. rhizogenes* NIAES 1724 in hypocotyls

of *A. deliciosa* seedlings. A new *A. rhizogenes*-mediated transformation protocol was developed by Yamakawa *et al.* (1996) who obtained direct bud formation on petioles of leaf explants of cvs. Hayward, Abbott, Matua, and Bruno infected by Japanese wild strains of *A. rhizogenes* (ArM 123, IFO14555, A5, and A13).

The controlling mechanism of plant morphogenesis is one of the most important questions in plant biology. Plant homeobox genes are thought to control plant morphogenesis through the regulation of expression of genes involved in plant development. To elucidate the mechanisms of plant morphogenesis, genetic transformation with a synthetic gene encoding the *hEGF* under the control of cauliflower mosaic virus (CaMV) 35S promoter has been done for the first time to produce human bioactive peptides in plants. Young leaves of the regenerated transgenic plants of *A. chinensis* expressed the highest hEGF peptide content, 65 pg mg⁻¹ soluble protein (Kobayashi *et al.*, 1996). Plant growth was also modified by using the rice gene *OSH1*, which contains a homeobox whose product is thought to have DNA binding activity. Kusaba *et al.* (1999) transformed *A. deliciosa* with the rice homeobox containing *OSH1* gene under the control of three different promoters. Only the transgenic plants containing the *OSH1* cDNA under the control of the 35S promoter showed morphological changes such as dissected leaf margin, dwarfism, and loss of apical dominance. Transgenic kiwifruit plants overexpressing *OSH1* showed a suppression of the gibberellin GA20 oxidase activity, while applications of gibberellin (GA) could not completely recover plant morphology and protein profiles. Furthermore, studies on protein content indicated wide range alterations at protein level and protein accumulation (Kita *et al.*, 2006).

2.2 Fungal and Bacterial Resistance

The *osmotin* gene, normally expressed under the stress conditions (Liu *et al.*, 1994; Zhu *et al.*, 1996), was introduced by *A. tumefaciens* strain LBA4404 with plasmid pKYLX71 carrying tobacco *osmotin* gene and *nptII* gene under 35S promoter into leaf discs and petioles of *in vitro*-grown shoots of the cv. Hayward. Regenerated shoots showed deformed leaf lamina, chimerism,

and also spots lacking chlorophyll under *in vitro* conditions; however, each apparent morphological modification disappeared in potted plants as well in the field (Rugini *et al.*, 1999). Another gene used to induce disease tolerance was tried by Nakamura *et al.* (1999), which transformed leaves, petioles, and stem segments of the cv. Hayward with the soybean β -1,3-endoglucanase cDNA under the control of CaMV promoter with *A. tumefaciens* strain LBA4404 harboring a binary vector pROK1a-EG. Young leaves of regenerated plants showed an increase enzymatic activity in many transformants, which reduced the attack of *Botrytis cinerea*. Whereas Kobayashi *et al.* (2000) did not obtain resistant plants of the cv. Hayward to *B. cinerea* after transformation experiments with the *stilbene synthase* gene from *Vitis* spp., but the fruits contained high amount of piceid, which is beneficial on human health.

Regarding bacterial diseases, Balestra *et al.* (2001) investigated the sensitivity of transgenic tissues to *rolABC* genes; how cytokinins and auxins could modify the susceptibility to *Pseudomonas viridiflava* and *P. syringae* pv. *syringae* whether the susceptibility was expressed in the offspring. The staminate cultivar showed high resistance to both pathogens, while the pistillate cultivar resulted to be highly susceptible. Furthermore, the offspring derived from the cross between transgenic staminate cultivar and the pistillate cultivar resulted in susceptible, probably due to the high nitrogen content in the leaves of *rolABC* plants. High resistance to *P. viridiflava* was obtained in those offspring plants lacking the *rolABC* genes. In fact, leaves resulted were tender and greener than those of the nontransgenic plants, except in one genotype, which resulted to have the same level of resistance than staminate plants.

2.3 *Gus*, *cat*, and *nptII* Reporter Genes

In most of the transformation experiments marker genes have been used to optimize the parameters of co-cultivation, culture media, explants, and *in vitro*-grown conditions for developing efficient transformation protocols in morphogenic tissues and protoplasts.

Chloramphenicol acetyltransferase (*cat*) gene has been used by Oliveira *et al.* (1991) to assess the conditions for polyethylene glycol

(PEG)-mediated transfection of kiwifruit protoplasts. Transient expression of *cat* gene was observed in protoplasts of the cv. Harward by using 30% PEG 4000 and by submitting protoplasts to heat shock (45°C, 5 min) prior to transfection. Further research showed a reduction in transient expression rates of either the *cat* or the *gus* genes when electroporation was tried without reproducing plasmid uptake (Oliveira *et al.*, 1994). Raquel and Oliveira (1996) continued to study the transient expression of the *gus* gene in two protoplast populations obtained from *in vitro*-grown leaf material of the same cultivar. One population consisted of hyaline or light green protoplasts originating from epidermis and leaf veins, and the other consisted of green protoplasts very dense in chloroplasts and originating from mesophyll. Transient expression and regeneration ability was more efficient in those from the former population, particularly when subjected to heat shock prior to transfection.

Uematsu *et al.* (1991) successfully introduced *nptII* and *gus* genes into hypocotyls and stem segments of *A. deliciosa* after co-cultivation with a disarmed succinamopine strain of *A. tumefaciens* EHA 101 (derivative of A281), harboring the binary vector pLAN411 or pLAN421 under the control of CaMV 35S or the *nos* promoter. Janssen and Gardner (1993) developed an *Agrobacterium*-mediated gene transfer system with transient GUS expression by comparing several *Agrobacterium* strains, binary vectors, and health of the explant of the same cultivar. Stable kanamycin resistant transgenic plants were obtained at high frequency from the optimized systems. Oliveira *et al.* (1994) also compared the transformation efficiency of explants of stem, petiole, and root, from *in vitro*-grown plantlets of the cv. Hayward. Explants were co-cultivated with *A. tumefaciens* strain LBA4404 carrying p35SGUSINT for 48 h. Some green calli could be recovered from stem and petiole explants under low kanamycin concentration and some adventitious shoots expressed *NPTII* activity.

Fraser *et al.* (1995) compared the amenability of transformation and regeneration procedures of *A. chinensis* and *A. deliciosa* by using the *A. tumefaciens* strains A281 and C58, both carrying the binary vector pKIWI105. During regeneration, shoot initiation from *A. chinensis* was more rapid and prolific than *A. deliciosa*, although *A. chinensis* was more sensitive to

little changes in growth regulator concentration. Comparisons between the different genotypes of *A. chinensis* showed only minor differences in growth regulator preferences. In *A. kolomikta*, an efficient regeneration and genetic transformation method has also been developed by Firsov and Dolgov (1997) with leaf explants co-cultivated with several *A. tumefaciens* strains. Selection of transformants was carried out under high concentrations of kanamycin and hygromycin. Good *gus* expression was observed in a high number of transgenic regenerants. Recently, *gus* activity and molecular evidence for incorporation of the gene was observed in leaf, stem, root, petal, and fruit tissues in transgenic *A. eriantha* plants after two years of transformation experiments (Wang *et al.*, 2006).

2.4 Methods of Gene Transfer

Some procedures are commonly used for transferring genes into the plant cells such as microbe-vectored systems (*Agrobacterium* mediated and virus mediated), artificial delivery system (DNA uptake by diffusion, membrane diffusion, electroporation, laser microbeam, microinjection, and microprojectiles), and natural delivery system (pollen tube pathway and floral tillers) (Dandekar, 1992). In *Actinidia*, both *Agrobacterium*-mediation and protoplast up-take system methods have been used for genetic transformation.

2.4.1 *Agrobacterium*-mediated gene transfer

The development of an efficient *Agrobacterium*-mediated transformation system is a prerequisite for functional genomics studies and for delivery of novel genes into established cultivars. Most of the reports on transformation and isolation of transgenic plants in *Actinidia* have involved *Agrobacterium*-mediated gene. This is affected by the transfer of a segment of the Ti or Ri plasmid known as T-DNA into the nuclear genome of the plant (Chilton *et al.*, 1982). According to Janssen and Gardner (1993) four factors affect transformation frequency: healthy and actively growing source tissue; the ability of *Agrobacterium* to efficiently transform cells; the bacterial strains; and the binary vector used strongly influence the

frequency of transformation. They found that A281 *A. tumefaciens* strain was the most efficient for transformation; the presence of a layer of moistened filter paper between the leaf explants and the co-culture media, and; the presence of 20 μ M acetosyringone in both bacterial culture media and the co-culture media.

The most commonly used strains of *A. tumefaciens* are LBA4404, A281, EHA101, C58, GV3101; and for *A. rhizogenes* the most commonly used strains are NCPPB 1855, NIAES 1724 ArM 123, IFO 14555, A5, A13, and G746. It is important to consider other factors that could affect transformation efficiency such as: (a) co-cultivation period variability (from 24 to 36 h); (b) use of scratching agents during the co-cultivation of the explants with *Agrobacterium*, such as carborundum granules (Rugini *et al.*, 1991); and (c) addition of antibiotics such as cefotaxime or carbenicillin, to the regeneration medium for inhibiting bacterial growth. Carbenicillin is commonly used since this antibiotic does not affect the regeneration process in kiwi.

2.4.2 Artificial delivery systems

The most promising delivery systems used in *Actinidia* is DNA uptake by diffusion using PEG-mediated transfection and electroporation (Oliveira *et al.*, 1991; Raquel and Oliveira, 1996). Electroporation involves the formation of transient pores in biological membranes by the discharge of altering electrical current. This is suitable to species not susceptible to *Agrobacterium* infection (Gupta *et al.*, 1988). The use of protoplasts in these systems is necessary, since they have an exposed plasmalemma, thereby highly efficient plant regeneration protocols are necessary.

2.5 Selection of Transformed Tissue

After the introduction and integration of alien genes into plant cells, only the transformed cells should divide and subsequently differentiate into either shoots or embryos. In order to select transformed cells a selectable marker is included together with the gene of interest. The *nptII* gene, encoding resistance to the antibiotic kanamycin, has been widely used in transformation

experiments, both in herbaceous and in woody plants (Dandekar *et al.*, 1993). This gene is able to inactivate also other aminoglycoside antibiotics such as paromomycin, neomycin, and geneticin rather than kanamycin (Yoshikura, 1989). Efficient selection of the transformed cells is usually carried out with kanamycin at concentration from 20 to 100 mg l^{-1} depending on the explant. Suitable concentration of kanamycin and the time to apply the selective pressure must be initially determined for each kind of tissue of the genotype to prevent regeneration from untransformed cells and at the same time to avoid inhibition of cell division and differentiation of transformed cells. In some other transformation experiments, *cat* gene was used, as a reporter gene, to assess the conditions for PEG-mediated transfection in protoplasts (Oliveira *et al.*, 1991).

Several studies have been conducted to determine the optimal conditions for the selection of transgenic cells after co-cultivation with *Agrobacterium*: Rugini *et al.* (1991) found that untransformed callus and apical shoots stopped growing in a medium containing 20 mg l^{-1} of kanamycin. On the contrary, the shoot could regenerate from putative transformed callus, selected in a medium containing 100 mg l^{-1} kanamycin. Uematsu *et al.* (1991) obtained shoots in the medium containing 25 mg l^{-1} kanamycin. Janssen and Gardner (1993) regenerated kanamycin resistant shoots from leaf discs of *A. deliciosa* cv. Hayward in presence of 100 mg l^{-1} kanamycin.

Experiments with protoplasts have demonstrated that 25 mg l^{-1} of kanamycin led to the gradual loss of the organogenic potential; however, the reduction of kanamycin concentration to 18 mg l^{-1} allowed plantlet regeneration (Oliveira *et al.*, 1994).

Different strategies have been tried to select transgenes. Janssen and Gardner (1993) added kanamycin after 2 days of co-cultivation with *Agrobacterium*, while Rugini *et al.* (1991) applied kanamycin (100 mg l^{-1}) after 2 weeks, then shoots were subcultured twice in the proliferation medium containing 50 mg l^{-1} kanamycin before transferring them to rooting medium. In order to avoid escapes, further selection was done by transferring selected shoots in the rooting medium containing kanamycin. In kiwi, the time for applying selection pressure with antibiotic is not a critical factor given its high ability

to regenerate, even in the presence of high antibiotic concentration. However, late kanamycin application, especially when the explant formed a big mass of callus is not so effective because it requires longer selection time, and the risk of somaclonal variation is very high, mainly due to an increase of ploidy level (Rugini *et al.*, 1994).

2.6 Regeneration of Whole Plant

Genetic manipulation of the plant genome by development of recombinant DNA techniques, and efficient *in vitro* culture techniques for plant regeneration from cells and tissues could lead to the development of new cultivars with desirable traits (Dandekar, 1992). Kiwifruit has demonstrated relative amenability to *in vitro* multiplication, not only by axillary bud stimulation, but also via organogenesis and, to a lesser extent via somatic embryogenesis. The recovery of transgenic plants depends on the frequency of gene introduction and the ability of the transformed cells to differentiate by efficient regeneration systems. The most common explants used for transformation experiments via *Agrobacterium* were leaf discs (Rugini *et al.*, 1989, 1991; Janssen and Gardner, 1993; Raquel and Oliveira, 1996), hypocotyls (Uematsu *et al.*, 1991; Yazawa *et al.*, 1995), protoplasts isolated from petioles (Oliveira *et al.*, 1991), and petioles of leaf explants (Yamakawa and Chen, 1996). However, according to our experience, the petioles of *A. deliciosa* give the best results in terms of regeneration and transformation efficiency.

2.6.1 Organogenesis

Actinidia species has demonstrated a remarkable organogenetic ability from different types of tissues (Revilla *et al.*, 1992). The morphogenetic responses of the explants vary with the type, combination, and concentration of growth regulators added to the basal medium. Different explant types and culture media have been used for shoot/embryo induction, regeneration, and efficient rooting for different kiwifruit genotypes (Canhoto and Cruz, 1987; Rey *et al.*, 1992; Cai *et al.*, 1993; Gonzalez *et al.*, 1995). Explants used for organogenesis are root and stem segments,

staminal filaments, immature fruits, apical meristems, and endosperms. This has allowed plants of novel genetic constitution derived from interspecific hybrids, buds, nodal segments, shoot tips, cambial tissue, callus from different organs, anthers, hypocotyls, petiole segments, leaves from axenic shoot cultures, and cell suspensions (Revilla *et al.*, 1992). Furthermore, plant regeneration from protoplasts has also been developed (Raquel and Oliveira, 1996).

2.6.2 Somatic embryogenesis

Somatic embryogenesis is a suitable technique for transformation experiments because stable transgenic plants can be obtained from a little starting material. The critical factors in kiwifruit somatic embryogenesis are genotype and explant competence, preconditioning, and culture conditions. Several attempts have been made to induce somatic embryogenesis from different tissues of *A. chinensis*, *A. arguta*, and *A. deliciosa*. In most cases, only globular stages and embryoids have been obtained and regeneration of somatic seedlings is still restricted to a few genotypes and explants (Oliveira, 1998). *A. chinensis* var. *chinensis* is highly responsive to embryogenesis. So far, explants tested for somatic embryogenesis showed different behaviors. Root, stem (Harada, 1975), and stamen filaments (Brossard-Chriqui and Tripathi, 1984) gave rise only to embryoids; endosperm tissues allowed to regenerate, some plants from embryoids (Gui *et al.*, 1982; Huang *et al.*, 1983), while Mu *et al.* (1990a) did not obtain complete plants with the same tissue of several interspecific crosses of *Actinidia*. Anthers of *A. chinensis* differentiated embryoids, which developed into bipolar heart-shaped structures (Fraser and Harvey, 1986). Precondition treatments to leaves of *A. deliciosa* cv. Hayward were successful in obtaining different developmental stages of somatic embryos (Oliveira and Pais, 1992).

2.7 Testing for Activity and Stability of Inheritance of Genes; and Adverse Effects on Growth, Yield, and Quality

Agronomic performance and inheritance of the 6-year-old transgenic plants with *rolABC* and *rolB*

and *osmotin* genes of the staminate GTH and the pistillate Hayward have been studied (Rugini *et al.*, 1996b, 1997, 1999; D'Angeli *et al.*, 2001). The most important phenotypic modifications are reported in Table 1. Field evaluations of transgenic *rolABC* plants after 15 years of the creation showed that they maintained a hairy root phenotype showing an increased lateral shoot number, smaller leaves with a narrow angle of insertion with probably modified light interception capacity (Figure 1). The transgenics propagated by cuttings maintained the same "hairy root" characteristics, and plants looked greener and better thriving than controls. However, the number of flowers per plant was lesser than the control plants because more than 60% of the flowers were single. This phenomenon is not constant during the years, depending probably to the environmental conditions before the blossom. This reduction of flower number is a negative feature for staminate plants, and for pistillate plants fruit size was smaller and fruit shape changed to rounder than control plants (Figure 2). When transgenic *rolABC* "GTH" was used as rootstock, the scion showed more branches than those grafted on untransformed rootstocks. In addition, these plants showed potentially useful characteristics with reduced leaf size, expanded root system,

and more drought tolerance (Figure 3). The pistillate cv. Hayward pollinated with transgenic staminate GTH produced regular fruits, and 50% of their seeds produced transgenic plants with characteristic "hairy root" phenotype. Normally, transgenic and nontransgenic offspring produced smaller fruit than the mother plant Hayward, except in one case among over 100 plants observed. Furthermore, the transgenics for *rolABC* showed a different internodes length, number of flowers per plant, and differences in transpiration rate (Rugini *et al.*, 1997, 1999).

The plants transformed with *rolB* were morphologically similar to untransformed controls, but their explants were more sensitive to auxin both for *in vitro* explants and *in vivo* cuttings. In fact, the *in vitro* leaf discs produced roots in the presence of very low auxin concentration and could regenerate shoots with very high 6-benzylaminopurine concentration, prohibiting regeneration of untransformed explants. *In vivo rolB* cuttings showed only abnormal basal callus formation with an auxin concentration allowing satisfactory rooting response in control cuttings. Fruit shape and size were similar to control fruits (Figure 2).

Transgenic kiwi plants of Hayward for the *osmotin* gene were obtained by Rugini *et al.* (1999) and the presence of the *osmotin* gene was

Table 1 Comparison between 8-year-old *rolABC* pistillate "GTH" plants and control plants in the experimental field

Characters	Control GTH	<i>rolABC</i> GTH
One year stem length (cm)	146.00a ^(a)	102.00b
One year shoot internode length (cm)	6.60a	3.40b
Trunk circumference (cm)	20.00a	15.00b
Leaf area (cm ²)	74.00a	35.00b
Leaf area × meter of shoot (m ²)	0.12a	0.10b
Petiole length (cm)	4.40a	1.90b
Petiole diameter (mm)	2.05	2.54b
Number of flowers/1 year vine	147.00a	59.00b ^(b)
Single flowers (%)	10.00a	62.00b ^(b)
Rooting leafy cuttings in October (%)	30.00a	75.00b
Number of roots × cutting in October	2.00a	10.00b
Assimilation [mol m ⁻² s ⁻¹ (CO ₂)]	3.15	(1.30–5.00) ^(a)
Transpiration [mmol m ⁻² s ⁻¹ (H ₂ O)]	3.70	(1.65–3.72) ^(a)
Inheritance of <i>rolABC</i> genes (%)	53	
Drought resistance (both self-rooted and grafted)	Higher	
Susceptibility to winter frost	Higher	
Susceptibility to water stress	Lower	
Susceptibility to bacteria	Slightly higher	

^(a) According to the transgenes

^(b) The average changes considerably according to the year



Figure 1 Compact vegetative habits of *rolABC* plant (a) and control kiwi plant (b) grown in the experimental transgenic field at the University of Tuscia, Italy

confirmed by D'Angeli *et al.* (2001). Fruit size and shape were similar to fruit from transgenic plants for *rolABC*, that is, smaller and rounder than controls (Figure 2). Resistance tests to *B. cinerea* in the fruits showed different response varying from moderate to high resistance depending of the clone (Figure 4). Moreover, a test for *Botritis* resistance was carried out in potted plants under greenhouse conditions. The susceptibility was determined by measuring the area of necrosis induced by mycelium or spores applied by discs of potato agar dextrose on the leaf lamina. A group of

plants showed to be more tolerant to the pathogen than both regenerated plants and the mother plant (Rugini *et al.*, 1999).

After artificial infection with *Pseudomonas syringae* and *P. viridiflava*, T₁ plants (susceptible Hayward × tolerant GTH) were found to be more sensitive to these bacteria as compared to the untransformed plants (Rugini *et al.*, 1999). A positive correlation between bacterium susceptibility and high nitrogen content was observed, which characterize the transgenic plants (Balestra *et al.*, 2001).

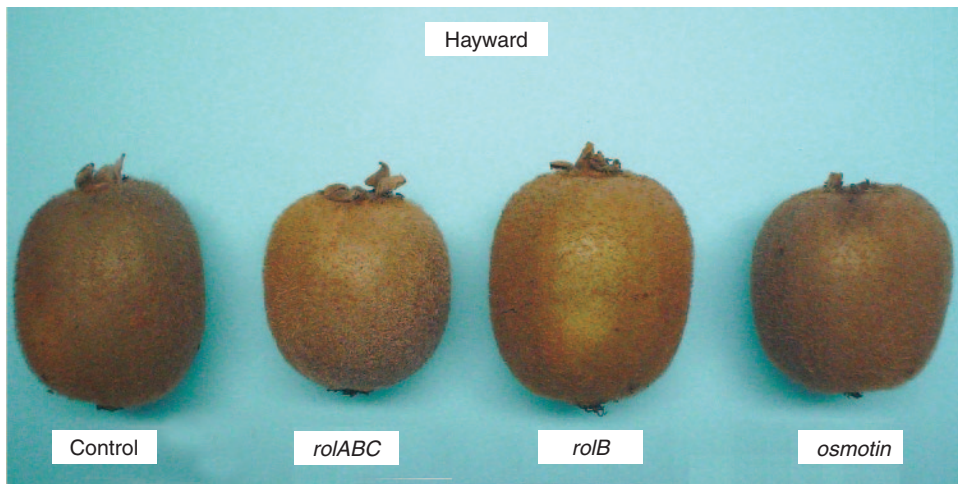


Figure 2 Fruit shape and size of cultivar Hayward and transgenic Hayward for *rolABC*, *rolB*, and *osmotin* genes

Overexpression of genes for modifying both resistance or susceptibility to pathogens and the sensitivity to hormones of the plant is under study (Fladung and Gieffers, 1993; Storti *et al.*, 1994).

Fung *et al.* (1998) studied the inheritance and expression of *nptII* and *gus* genes within progenies of *A. deliciosa* transgenic plants. The detailed

examination of two plants suggested that there were multiple copies of both the *nptII* and the *uidA* genes. None of the actively expressing copies of *nptII* or *uidA* were linked. Expression of GUS was variable in some progeny plants, consistent with the idea that gene silencing phenomena were operating.



Figure 3 Response of *rolABC* plants and control plants grown in the experimental transgenic field at the University of Tuscia, Italy to hydric stress conditions during dry season



Figure 4 Response of kiwifruit from transgenic plant for the *osmotin* gene, and control fruit after inoculation with *Botritis cinerea*

3. FUTURE ROAD MAP

Albeit all the benefits of the consumption of kiwifruit, the world market is small as compared with other fresh fruits. The commercial kiwi cultivars have some deficiencies that limit the marketing because there are still very few types of kiwifruit available for consumption. The potential for development of improved agronomic traits in kiwifruit employing tradition breeding and biotechnology is high. The extensive genetic variation within the genus *Actinidia* provides the opportunity for developing new and different cultivars. For example, in *A. eriantha* the vines are relatively small and nonvigorous, flowers form all over the vine including on lower axillary branches and produce the third largest fruit of the species in the *Actinidia* genus (Liang, 1980) with very high vitamin C content, and some genotypes have peelable skins. Furthermore, it flowers prolifically under greenhouse conditions and has a low requirement for winter chilling than *A. deliciosa* and *A. chinensis* (Seal, 2003; Wang *et al.*, 2006). Breeders need access to a diverse germplasm collection and must have the knowledge of the genetic variation present in them. In traditional breeding, the long generation time is the major constraint as well as the choice of the parents, especially the males, and also the different ploidy level can restrict crossing options.

The main desirable characteristics in *Actinidia* include the production of hermaphrodite plants, improvement in fruit quality, production of parthenocarpic fruits, improvement in abiotic and biotic stress tolerance, alteration of vegetative

habit, and regulation of fruit ripening. Genetic transformation programs are focused on solving the main problems by transferring one or more foreign genes, or by limiting the expression of the endogenous ones. Currently, several genes are available with known origins; others are under characterization and isolation with their own promoters from several woody plants, including kiwi (Owens, 1995).

3.1 Hermaphroditism and Parthenocarpy

Dioecism is the major limiting factor for kiwi productivity. More work is needed on the physiological and biochemical aspects of hermaphroditism. Also, the identification and isolation of genes involved in sex determination may be useful to transform genotypes of high agronomic and commercial value. The introduction of the parthenocarpic trait may help to solve problems related to fructification due to the negative dioecism character. Unfortunately, *Actinidia* species do not show any natural parthenocarpy characteristic as in aubergine (Rotino *et al.*, 1997).

3.2 Regulation on Fruit Ripening and Quality

Fruit ripening is a complex process, which involves both an increase in respiration and ethylene production, which are influenced by the orchard condition, the fruit position in the plant, and

harvest and postharvest handling (Cooper *et al.*, 2005). Due to the rapid fruit softening at maturity, regulation of fruit ripening by manipulating the genes responsible of this process could make harvesting, shipping, storage, and marketing easier. Major efforts of molecular investigations are focused on the study of genes involved in fruit development and ripening under different strategies, and transgenic plants for the antisense genes should be generated.

The polygalacturonase gene encodes poly (1,4- α -D-galacturonide) glycan hydrolase. It is one of the enzymes involved in the process by breaking down pectin, a major component of the middle lamella (Soda *et al.*, 1986; Wegrzyn and MacRae, 1992; Bonghi *et al.*, 1997). Slacking the activity of the polygalacturonase enzyme would be an important strategy for improving fruit storage and marketing. The nucleotide sequence of the polygalacturonase (PG) gene in Hayward has been determined and compared with tomato protein by Atkinson and Gardner (1993) and the antisense construct may soon be available for kiwi transformation. Later, Wang *et al.* (2000) characterized the expression of three polygalacturonase cDNA clones (CkPG A, B, and C) isolated from fruit of *A. chinensis* and they found that CkPGC gene expression was present in softening fruit, 50-fold higher than A and B as fruit passed through the climacteric, but it was also present during fruit development and harvesting prior to the onset of softening.

Demethylation of the carboxyl group of the polygalacturonic acid by the pectin methylesterase enzyme (PME) causes changes in tissue firmness during fruit maturation (Bordenave, 1996; Micheli, 2001). The pectin methylesterase inhibitor (PMEI), its amino acid sequence, basic structure, and its regulation on cell wall degradation and fruit ripening processes by interaction with PME have been reported in kiwifruit (Balestrieri *et al.*, 1990; Giovane *et al.*, 1995; Camardella *et al.*, 2000; Jiang *et al.*, 2002). Fruit ripening controlled by the production of genetically modified kiwi containing *PMEI* genes is an attractive possibility for inactivating endogenous PME activity. The expression of *PMEI* from the cultivar Hayward was studied in transgenic asparagus for two *PMEI* cDNA clones and it was found *in vivo* inhibition effects on PME activity, but the biochemical effect on cell wall metabolism and their physiological role

during plant development are not clear (Irifune *et al.*, 2004).

MacDiarmid and Gardner (1993) tried to understand why Hayward has storage properties superior to other *Actinidia* spp. They sequenced a cDNA clone with high homology to ACC-oxidase tomato gene and related genes from apple. Ikoma *et al.* (1995) cloned two ACC-synthase genes and found that KWACC2 was actively transcribed only in wounded tissue, while KWACC1 was expressed in both ethylene-treated fruit and wounded tissue. Ren *et al.* (1997a) cloned and sequenced ACC-oxidase gene from cv. Qinnei (*A. deliciosa*) and found that their DNA and amino acid sequences are 95.01% and 95.61% homologous, respectively, to those of Hayward. They also cloned and sequenced a Ca-regulated protein gene from Qinnei fruit and found that the DNA sequence is 86.16% homologous to barley (Ren *et al.*, 1997b). In addition, Xu *et al.* (1998) found that ACC synthase and not ACC oxidase is the key enzyme that controls the rate of endogenous ethylene production in fruits of *A. chinensis* (cvs. Kuimi and Hongxin).

Studies on gene expression during fruit development were carried out by Ledger and Gardner (1994), which obtained five cDNAs differentially expressed during fruit development in Hayward. Only one of them had low levels of expression in young fruit and it is induced late in fruit development and during fruit ripening, while the others are highly expressed only in young fruits but had reduced expression in late development.

Other genes involved in fruit ripening in *A. deliciosa* var. *deliciosa* and *A. chinensis* var. *chinensis* have also been identified including a cDNA clone encoding sucrose-phosphate synthase from ripening kiwifruit (Langenkamper *et al.*, 1998). Six cDNA clones (AdXET1-6) with homology to the xyloglucan endotransglycosylase (*XET*) gene were isolated from ripe kiwifruit mRNA of cv. Hayward and appeared to belong to a closely related family of genes (Schroder *et al.*, 1998), while studies on the expression pattern of *XET* gene in *A. chinensis* suggested that *XET* might be a kind of inductase leading to xyloglucan depolymeration in the fruit cell wall and does not play a key role in softening of kiwifruit after ripening (Chen *et al.*, 1999).

Actinidin, a cysteine protease expressed during fruit development and harvest time (up to 60% of the soluble proteins), is the principal

responsible of allergic reactions. Its contents in the fruit vary among species and cultivars. Possible solutions have emerged from the isolation and characterization of cDNAs encoding actinidin from kiwifruit (Praekelt *et al.*, 1988; Podivinsky *et al.*, 1989; Snowden and Gardner, 1990). Its promoter sequence (Keeling *et al.*, 1990) and its expression in transgenic petunia resembled the pattern of actinidin induction in fruit tissues of kiwifruit (Lin *et al.*, 1993). The precise location and the determination of the function of the actinidin propeptide sequences have been studied through transgenic tobacco plants for preproactinidin lacking the C-terminal propeptide, demonstrating that the actinidin CTPP is required for actinidin processing (Paul *et al.*, 1995).

Finally, fruit size and enhancement of sugar accumulation could be improved by overexpressing gene-regulating ADP-glucose-pirophosphorilase, as observed in potato tubers after introduction of this gene (Stark *et al.*, 1992).

3.3 Abiotic Stress Tolerance

Cold resistant plants are recommended due to the wide diffusion of this species in areas where the risk of early spring frost and winter frost are frequent. The *A. arguta* and *A. kolomikta* have demonstrated frost resistance and could be used to identify genes and introduce into the current germplasm. Transgenic plants with gene encoding antifreezing protein have been generated (Hightower *et al.*, 1991) and the overexpression of *Superoxide dismutase* gene has also been demonstrated to repair frost-damaged cells (McKersie *et al.*, 1993; Van Camp *et al.*, 1994). Overexpression of Arabidopsis *CBF1* gene enhances freezing tolerance by inducing genes associated with cold acclimatization could also be useful (Jaglo-Ottosen *et al.*, 1993).

Salt tolerance has been induced in tobacco plants by introduction of genes for glycerol-3-phosphate acyltransferase (Tarczynski *et al.*, 1993). Furthermore, gene identification and isolation for calcareous and drought tolerance from somaclones regenerated under selective pressure media could facilitate to obtain new cultivars by further genetic transformation (Marino and Battistini, 1990; Muleo *et al.*, 1996).

3.4 Alteration of Vegetative and Reproductive Habit

Genetic improvement in canopy architecture to produce dwarf, semi-dwarf plants with shorter shoots, unifloral buds is required for high-density orchards. Moreover, developing plants with a more expanded root system and/or with reduced water consumption could be of advantage during drought seasons or in areas with scarce water availability and/or sandy soils. Some genes of the TL-DNA of *A. rhizogenes* affecting plant growth and development have already been introduced in kiwi and in other woody fruit plants, such as cherry (Gutiérrez-Pesce *et al.*, 1998; Rugini and Gutiérrez-Pesce, 1999) and in peach (Hammerschlag and Smigocki, 1998). In all these species, reduction of plant size and apical dominance is confirmed and currently, field trials will test their agronomic value.

Another possibility offered by biotechnology for modifying growth and reproductive behavior, is the modification of plant receptors to change light perception. Phytochrome genes (*phyA*, and/or *phyB*, sense or antisense) together with other photoreceptors, control plant development, circadian rhythms, apical dominance, blossoms, growth and fruit ripening, photosynthesis products distribution, development of photosynthetic systems, transpiration control, and hormone synthesis (Tucker, 1976; Vince-Prue and Canham, 1983; Baraldi *et al.*, 1992; Muleo and Thomas, 1993, 1997). Transformation with these genes may help to generate plants with high agronomic value. *In vitro*-grown shoots of the cherry rootstock "Colt" overexpressing *phyA* of rice have demonstrated a reduction of apical dominance with red and far-red light treatments (Muleo and Iacona, 1998). This indicates that the excess of red and far-red light, generated in orchard with high-density plantation, could modify the distribution of the photosynthetic assimilate among the vegetative growing organs.

3.5 Disease and Pest Resistance

The promise of using molecular biology is that it may generate broad resistance mechanisms that have been difficult to manipulate with classical plant breeding approaches. Although currently,

pests and diseases are not the only limiting factor in kiwifruit, due to cultivation of a single variety over a wide range of geographical areas, this may cause concern in the future regarding potential pathogens causing devastating damage. For enhancing protection against fungi, besides *osmotin* gene, as already introduced in the cv. Hayward, several other genes may be introduced after having tested the product of the genes against the main fungi of kiwi such as stilbene synthase gene (Hain *et al.*, 1993), ribosome-inactivation protein gene (Logemann *et al.*, 1992), glucose oxidase gene (Wu *et al.*, 1995), and genes encoding hydrolytic enzymes such as chitinase and glucanase, which degrade fungal cell wall components (Broglie *et al.*, 1991; Yoshikawa *et al.*, 1993).

Special attention is given to polygalacturonase-inhibiting proteins (PGIPs), which specifically inhibit the activity of endo-polygalacturonases released by fungi on invasion of the plant cell wall. The complete nucleotide sequence of a potential kiwifruit cv. Hayward *PGIP* has been determined (GeneBank249063). The expression and activity of kiwifruit PGIP is expected to exhibit similar expression patterns to that shown by other *PGIP* genes of other species.

Kiwi genotypes with less susceptibility to *P. syringae* and *P. viridiflava* have been preliminarily identified in the cv. GTH, so it should be interesting to investigate both the staminate GTH and its offspring both of which showed more tolerance (Balestra *et al.*, 2001). Although it is not easy to introduce bacterial resistance in plants because the product of gene should act in the intercellular spaces; several species with enhanced resistance to bacteria have been obtained by introducing genes encoding bactericidal polypeptide as cecropine (Huang *et al.*, 1997) and its synthetic analog MB39 (Mills *et al.*, 1994), thionin (Carmona *et al.*, 1993), and attacin E (Norelli *et al.*, 1994). Particular attention should be given to human lysozyme, which confers resistance to both fungi and bacterium *P. syringae* in tobacco plants (Nakajima *et al.*, 1997).

There are two promising approaches against insect attack. The *Bt* gene from *Bacillus thuringiensis* (Vaeck *et al.*, 1987) has been successfully introduced in other species with encouraging results regarding field resistance. A cytokinin gene under the control of a wound-inducible promoter (Smigocki *et al.*, 1993) could be an alternative

to prevent leaf roller caterpillars attacking the *Actinidia* spp.

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Passionfruit

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1. INTRODUCTION

1.1 History, Origin, Taxonomy, and Distribution

The genus *Passiflora* is one of the 12 genera in the family Passifloraceae of the order Violaceae, class Magnoliopsida. The center of origin is most likely tropical South America, with northern Brazil as the center of diversity (Leitão Filho and Aranha, 1974). More than 580 species are recognized, with approximately 150 originating from Brazil (Hoehne, 1946; Salomão and Andrade, 1987). The name *Passiflora*, derived from “Passion”, was given by Catholic missionaries in South America. The corona threads of the passionflower were seen as a symbol of the crown of thorns, the five stamens for wounds, the five petals and five sepals as the 10 apostles (excluding Judas and Peter), and the three stigmas for the nails on the cross. The Portuguese name, “maracujá”, is derived from the indigenous Tupi language, meaning “food shaped as a bowl”, due to the large size and shape of the fruit. The Spanish name, “granadilla”, meaning “small pomegranate”, is derived from similarity between passionfruit and pomegranate (called “granada” in Spanish due to the high number of seeds, “granas”, in the fruit).

About 50–60 species produce edible fruits that can be used for human consumption (Coppens D’Eeckenbrugge *et al.*, 2001). However, very few species are grown in a commercial scale for fruit

production. Some species are used as ornamentals or as the source of pharmaceuticals. The main cultivated species include *P. edulis* Sims f. *edulis*, *P. edulis* Sims f. *flavicarpa* Deg, *P. ligularis* Juss, *P. mollissima* (HBK) Bailey, *P. quadrangularis* L., and *P. alata* Dryand (Martin and Nakasone, 1970; Oliveira, 1987).

In most countries where passionfruit is grown, *P. edulis* f. *flavicarpa* (yellow passionfruit) is the most widely planted species. It is believed that the yellow passionfruit is the result of a cross between *P. edulis* f. *edulis* (purple passionfruit) and a closely related species, or of a mutation in *P. edulis* f. *edulis* (Oliveira and Ferreira, 1991). *P. edulis* was described in 1818 by Sims and in 1872 by Masters, and forma *flavicarpa* was proposed in 1933 by Degener (*P. edulis* f. *flavicarpa*). Therefore, forma *edulis* (*P. edulis* f. *edulis*) was necessarily established, in accordance with the International Code of Botanical Nomenclature. Both forms are subspecific categories, often cited erroneously (e.g., *P. edulis* var. *flavicarpa*) or incompletely (e.g., *P. edulis*) (Bruckner *et al.*, 2002). *P. edulis* f. *edulis* and *P. edulis* f. *flavicarpa* have differences in fruit size and external color, flavor, and resistance to a number of diseases (Martin and Nakasone, 1970). The external color of the fruit, purple or yellow, is apparently controlled by one pair of co-dominant alleles, and the F₁ progeny of a cross between the two forms produces fruits with an intermediate, reddish purple color (Nakasone *et al.*, 1967). The species *Passiflora edulis* f. *flavicarpa* will be referred

to simply as yellow passionfruit throughout this text.

1.2 Botanical Description

All species in the genus are characterized by possessing flowers with five stamens, five petals, and five sepals, with an upright ginandrophore with free stamens and three stigma (Salomão and Andrade, 1987). Flowers are usually large (5–7 cm in diameter), white, pink, red, or purple, with a pink, red, or white corona of filaments (Figures 1a and b). Most species grow as perennial or semiperennial vines, with alternate, oblong or oval leaves (Figures 1c and d).

Passionfruit is essentially cross-pollinated. The most efficient pollinating agents are carpenter bees (*Xylocopa* spp.), which are large enough to

reach the stigma after removing pollen grains. European honeybees (*Apis mellifera*) visit the flowers and actually remove pollen, but are not large enough to reach the stigma and pollinate the flower. Therefore, the presence of honeybees can actually decrease fruit yield in commercial plantations. Pollination by wind is negligible due to the large size and sticky nature of the pollen grain (Bruckner *et al.*, 2000). Besides floral structure, cross-pollination is favored by self-incompatibility, which was reported for *P. edulis* as far back as 1868 (Munro cited by Nettancourt, 1977). Self-incompatibility in yellow passionfruit is sporophytic, occurring in the stigma and resulting in inhibition of growth of the pollen tube. Early studies indicated that self-incompatibility was governed by a single gene with multiple alleles (Bruckner *et al.*, 1995). However, analysis



Figure 1 (a) Flower of yellow passionfruit (*Passiflora edulis* f. *flavicarpa*); (b) flower of *Passiflora cincinnata*; (c) yellow passionfruit vine and fruit; (d) aspect of a 1-year-old purple passionfruit (*P. edulis* f. *edulis*) field

Table 1 Chromosome length (μm) and arm ratio of yellow passionfruit, *Passiflora amethystina*, and *P. cincinnata*^(a)

Chromosome	1	2	3	4	5	6	7	8	9	
Chromosome length										Total
<i>P. amethystina</i>	3.23	3.23	3.14	3.14	2.91	2.64	2.62	2.42	2.35	25.68
<i>P. edulis</i> f. <i>flavicarpa</i>	3.16	3.00	2.76	2.79	2.60	2.36	2.15	2.02	1.82	22.66
<i>P. cincinnata</i>	2.77	2.70	2.49	2.19	2.20	2.00	1.87	1.64	1.79	19.65
Arm ratio										
<i>P. amethystina</i>	1.81	1.14	1.20	1.55	1.17	1.18	1.61	1.32	1.11	
<i>P. edulis</i> f. <i>flavicarpa</i>	1.84	1.14	1.16	1.33	1.26	1.21	1.30	1.53	1.16	
<i>P. cincinnata</i>	1.58	1.21	1.26	1.81	1.21	1.30	1.43	1.26	1.32	

^(a)Modified from Cuco *et al.* (2005)

of homozygous families for the different alleles identified new phenotypes in these families, a clear evidence that another gene would be acting (Rêgo *et al.*, 1999). Indeed, the presence of a second gametophytic gene was suggested by the analysis of families originated from crosses among plants that presented differences in reciprocal crosses (Suassuna *et al.*, 2003). Compatibility would require that the maternal plant be homozygous for the gametophytic *G* locus and receive pollen grain from a heterozygous plant. Moreover, both plants must have the same genotype at the sporophytic, *S* locus. The reciprocal cross is incompatible (Suassuna *et al.*, 2003). The plant can also be vegetatively propagated by cuttings or grafting.

Passionflowers are tropical plants and therefore are best adapted to grow at warmer temperatures with high rainfall. Yellow passionfruit is cultivated mostly in tropical lowlands, while purple passionfruit is located mostly in moderately higher altitudes (1000–2000 m). The plant is intolerant to frost.

Despite its economical importance and the interest of botanists, information on passionfruit cytogenetics remains scarce. Only a fraction of the known species of *Passiflora* have been studied in terms of genome composition (Snow, 1993). Most species have $2n = 12$ or 18. However, species with $2n = 14$, 20, 24, 27, 36, or 84 are also known, such as *P. suberosa*, *P. foetida*, and *P. pulchella* (Martin and Nakasone, 1970; Lopes, 1994). The horticulturally important species, such as *P. edulis*, have $2n = 18$.

The karyotypes of yellow passionfruit, *P. amethystina* and *P. cincinnata*, previously used to obtain somatic hybrids by protoplast fusion

(Vieira and Dornelas, 1996), were recently described in detail. Their karyotypes were symmetric, i.e., the species have preferentially metacentric chromosomes with subtle differences in the morphology of some chromosomes among species (Table 1). These data were integrated with the physical mapping of the 45S ribosomal DNA (rDNA) (18S–5.8S–26S rRNA genes) and 5S rDNA loci using fluorescent *in situ* hybridization (FISH), and with the pattern of fluorescent staining with chromomycin A3. FISH signals of 45S rDNA were localized on the secondary constriction and satellite of two chromosome pairs, identified as 8 and 9, in all three species. An additional site was observed on the long arm of chromosome 1 in *P. amethystina* (Cuco *et al.*, 2005).

A number of potentially useful species have been characterized in terms of chromosome number as well as agronomical traits. *P. setaceae* was shown to have $2n = 18$ chromosomes and displaying resistance to sudden death and *Fusarium* wilt (common diseases in commercial fields of yellow passionfruit) (Soares-Scott, 1998). *P. quadrangularis*, *P. serrato-digitata*, and an interspecific hybrid between *P. edulis* \times *P. incarnata* have $2n = 36$ chromosomes (Soares-Scott, 1998). The karyotype of *P. nitida*, an Amazonian species with the potential for commercial cultivation as well as a source of resistance genes to sudden death, was characterized. *P. nitida* has $2n = 18$ chromosomes, with secondary constrictions in pairs 3, 4, and 8. The constriction in chromosome 3 differentiates *P. nitida* from the other species within the genus *Passiflora* (Passos, 1999).

The importance of information on chromosome number is demonstrated by the observation

that interspecific compatibility is high among species with $2n = 18$ chromosomes (Martin and Nakasone, 1970; Soares-Scott, 1998). Interspecific hybrids, either natural or artificial, have been obtained and shown to have high-agronomic potential, especially in terms of disease resistance. However, information on several sections and subgenera remains unavailable.

Genome size variation in some *Passiflora* species has been estimated (Souza *et al.*, 2004). 2C DNA content and genome sizes ranged from 1.83 to 5.36 pg (picogram) and 896–5252 Mbp (mega base pairs), respectively, among the species analyzed, as follows: yellow passionfruit, 3.21 pg and 3146 Mbp; *P. quadrangularis*, 5.36 pg and 5252 Mbp; *P. laurifolia*, 3.88 pg and 3802 Mbp; *P. giberti*, 3.92 pg and 3842 Mbp; *P. edmundoi*, 3.40 pg and 3362 Mbp; *P. mucronata*, 3.43 pg and 3332 Mbp; *P. edulis* f. *edulis*, 3.16 pg and 3096 Mbp; and *P. suberosa*, 1.83 pg and 896 Mbp.

1.3 Economic Importance

Brazil is the world's largest producer and consumer of passionfruit since the mid 1970s. Total production in 2004 reached over 490 000 metric tons in approximately 36 000 hectares, representing 60% of the world's production (IBGE, 2004). Over 95% of the production is comprised of yellow passionfruit, and about 35% of this is destined for processing in the form of concentrated juice. Australia, Colombia, Ecuador, Peru, and South Africa also grow significant acreages of passionfruit, but this acreage is more evenly divided among yellow passionfruit, purple passionfruit, and *P. alata* (sweet passionfruit), and the fruit is mostly destined for fresh market consumption.

For yellow passionfruit, yields can be as high as 75 tons of commercial fruit per hectare with the adoption of a few agronomical practices: use of certified seeds, irrigation, and manual pollination (Meletti and Maia, 1999). Commercial plantations have the potential to remain productive for up to 5 years. However, the incidence of diseases (specially passionfruit woodiness and *Fusarium* wilt) usually restricts commercial production to 1 or 2 years in most regions of Brazil and also in Australia and South Africa.

Besides fruit production for human consumption, several species of passionflower are grown as

ornamentals, due to their spectacular flowers and vigorous evergreen foliage. In addition, medicinal use is quite widespread in Brazil, due to the properties of passiflorine, a natural sedative present at high concentrations in the leaves and fruit of several species (Ruggiero and Nogueira Filho, 1994).

1.4 Traditional Breeding

Passionfruit is a crop of relatively recent commercial cultivation, displaying a high degree of genetic variability for most traits related to plant development and fruit production (Bruckner *et al.*, 2002). For example, a population derived from a single cross between two Brazilian accessions, "IAPAR-06" and "IAPAR-123", was evaluated in two harvest seasons in a triple 10×10 square lattice design and displayed a wide genetic variance for several traits related to yield and fruit quality. The estimate for broad-sense heritability ranged between 53% and 83% (Moraes *et al.*, 2005).

This variability can be easily exploited by mass selection and selected populations can be found regionally in Brazil and other countries. Germplasm characterization started in the late 1970s and is still a major objective of several breeding programs (Bruckner *et al.*, 2002). Most breeding programs employ one (or an association) of three strategies—mass selection, selection with progeny tests, and interspecific hybridization.

The objective of breeding programs emphasizes traits related to fruit production, including those which favor industrial processing: fruit size/weight, firmness, pulp content, shape, color, flavor, and aroma (Vieira and Carneiro, 2004). Acidity and total soluble solids are also important for juice processing.

Most breeding programs are targeted to purple or yellow passionfruit, and the development of cultivars has three main objectives: (i) yield, including a short juvenile period and self-compatibility, (ii) fruit quality, including uniform ripening, and (iii) disease resistance (Bruckner *et al.*, 2002; Vieira and Carneiro, 2004). Of these three, disease resistance has been the least successful and is the one for which biotechnology holds the greatest promises.

Fruit size/weight is an important trait, directly related to yield. Different studies have indicated

large variations for this trait in *Passiflora* germplasm. Most currently available cultivars produce fruits that range from 50 to 130 g. Fruit size/weight is also highly dependent on cultural practices. Manual pollination alone can increase average fruit weight from 62 to 112 g (Ruggiero *et al.*, 1976). Some recently developed interspecific hybrids can reach an average fruit weight of 220 g (Bruckner *et al.*, 2002).

The breeding program at the Instituto Agrônomo de Campinas (IAC, Campinas, São Paulo state) has been the most active and successful in Brazil. Using mass selection with progeny tests in preselected populations of yellow passionfruit, gains of up to 100% in yield, fruit size and weight, and number of seeds per fruit were obtained with four rounds of selection. Gains in total soluble solids and percentage of pulp were significant, although not that high. Some selections had an average fruit weight of 150 g, compared to 90 g for the original genotypes, and average fruit diameter of 9 cm compared to 6 cm (Melleti *et al.*, 1992). Selected populations were then subjected to interspecific hybridization. The descendants producing better fruits were backcrossed with high-yielding ($>30 \text{ t ha}^{-1}$) parents. This work generated eight commercially promising combinations producing large, oval fruit with high soluble solids content. Two additional backcrosses generated additional gains in fruit qualitative traits and yield and resulted in the first three yellow passionfruit cultivars ever released in Brazil, “IAC-273, -275, and -277” (Melleti, 1999). These cultivars produce fruits with an average weight of 170–220 g, 9 cm of longitudinal diameter and 7.3 cm of transversal diameter, 50% pulp, $>15^\circ\text{Brix}$ of total soluble solids, and over 370 seeds per fruit, and reach an average yield of $45\text{--}50 \text{ t ha}^{-1}$ with manual pollination but no irrigation (Melleti, 1999).

The generation of interspecific hybrids is a potential tool for the production of high-yielding, disease-resistant passionfruit cultivars. Traditionally, emphasis has been placed upon the transfer of favorable traits from wild species to *P. edulis*. However, most interspecific hybrids present developmental problems, including male sterility, low viability of pollen, and limited flowering. A *P. nitida* \times *P. edulis* hybrid resulted in a single abnormal descendent, which did not produce a single flower in four years (Soares-

Scott, 1998). A *P. edulis* \times *P. incarnata* hybrid produced viable progeny, although pollen grains had significant variations in size. These variations were shown to be correlated with variations in chromosome number in the pollen grains (Soares-Scott, 1998). A *P. edulis* \times *P. incarnata* hybrid with tolerance to cold and to severe strains of passionfruit woodiness virus (PWV) was obtained in Australia (Nakasone and Paull, 1998) and has been maintained by grafting onto yellow passionfruit root stocks.

Soil-borne diseases and viral diseases are limiting factors for passionfruit production in all areas of the world with significant acreages. The wilt disease caused by *Fusarium* is the most important soil-borne disease of passionfruit, due to its rapid progress that inevitably culminates with plant death and the capacity of the fungus to survive in the soil in the absence of the host for at least 10 years (Oliveira and Ferreira, 1991). In South Africa, the disease is controlled by using *P. caerulea* as root stock (Nakasone and Paull, 1998). Grafting is not a cultural practice in Brazil, but due to the failure of incorporating resistance to *Fusarium* wilt into commercial yellow passionfruit germplasm, the use of tolerant or resistant root stocks, such as *P. alata*, *P. caerulea*, or *P. macrocarpa*, has been investigated and encouraged as a way to control the disease (Bruckner *et al.*, 2002).

Passionfruit woodiness disease (PWD) is the most important viral disease of yellow passionfruit worldwide. The disease is characterized by mosaic and distortion of the leaves and by “woodiness” of the fruit, which become dwarfed and lose much of the pericarp, therefore becoming unmarketable. Until recently, it was attributed to a single potyvirus species, PWV. However, the molecular characterization of viral isolates causing the disease in the African continent and in Brazil identified a second species, cowpea aphid-borne mosaic virus (CABMV), as an etiological agent of the disease (McKern *et al.*, 1994; Sithole-Niang *et al.*, 1996; Nascimento *et al.*, 2006). In fact, CABMV is the predominant, if not the single, etiological agent of PWD in Africa and Brazil. The control of PWD is quite unsatisfactory, due to the noncirculative nature of both PWV and CABMV in their aphid vectors, and the existence of several wild hosts of both viruses, including several leguminous plants (family Fabaceae).

In Australia, the use of tolerant *P. edulis* f. *edulis* and yellow passionfruit hybrids is a common practice to control the disease (Pares and Martin, 1984; Fitzel *et al.*, 1985). However, these hybrids produce fruits with a pinkish color, which is not accepted in some markets. In South Africa, a breeding program was started in 1995 to obtain PWD-resistant cultivars (Breedt, 1997). A total of 27 interspecific hybrids among yellow passionfruit, *P. edulis* f. *edulis*, and *P. quadrangularis* were obtained, which eventually generated three genotypes with tolerance to *Fusarium* wilt. However, resistance to PWD was not obtained. In Brazil, CABMV-tolerant or resistant cultivars are not available, and the prospects for their availability are slim, due to the natural barriers for transfer of sources of resistance from wild *Passiflora* species to yellow passionfruit (Bruckner *et al.*, 2002).

1.5 Molecular Breeding: Linkage Maps and QTL Mapping

The locations on chromosomes of each (or a cluster) of the genes controlling a quantitative trait are called quantitative trait loci (QTL). Nowadays, in addition to the traditional methods for selecting complex characteristics, plant breeders combine phenotypic testing and molecular mapping to dissect the inheritance of quantitative traits. This is a consequence of a huge effort for developing molecular markers in the last 20 years. DNA markers are stable changes in short nucleotide sequences that occur at specific loci. Usually, they follow the same Mendelian rules of inheritance. For this reason, molecular markers have been extensively used for constructing saturated linkage maps and to study genetic diversity.

Linkage maps represent the location and the order of genes and DNA sequences, for example, molecular markers on a chromosome. Depending on the linkage forces, one might expect that molecular marker variants are inherited along with the allele the breeders are interested. Therefore, molecular maps can be used to map loci that control a quantitative trait. A new avenue, called marker-assisted selection has been open in recent years, and it has been proven to be effective especially for traits of low heritability and less costly to evaluate (reviewed by Francia *et al.*, 2005).

The development of genetic maps for auto-incompatible species, such as the yellow passionfruit, is restricted due to the unfeasibility for obtaining traditional mapping populations based on inbred lines, like F_2 , BC_1 , or recombinant inbred lines. Therefore, *Passiflora* linkage maps were constructed using an F_1 population. The two-way pseudotestcross strategy (Grattapaglia and Sederoff, 1994) and dominant markers, for example, random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) were employed (Carneiro *et al.*, 2002; Moraes, 2005; Lopes *et al.*, 2006). These PCR-based markers segregate in the F_1 population in a 1:1 or 3:1 fashion, being recognized as monoparental or biparental loci, respectively. Due to the lack of genetic information from these markers in one of the parents (i.e., homozygous for the null allele), two individual (parental) maps are obtained. On the other hand, if biparental markers are simultaneously analyzed, the homology of the linkage groups should be established. Using the same strategy, parental maps were constructed in other fruit species, such as kiwifruit (Testolin *et al.*, 2001) and apple (Kenis and Keulemans, 2005) as one definitive linkage map for each cultivar is desired.

Based on those linkage maps several genes were mapped in the yellow passionfruit. As mentioned, an F_1 population was evaluated during two harvest seasons, and genetic and phenotypic parameters were estimated for yield and fruit quality traits (Moraes *et al.*, 2005). The population displayed a wide genetic variability, as shown by the high significance of the genotype effects. This has permitted testing for associations between the markers (presence vs. absence) and the expression of the traits in the population. QTL analyses were performed simultaneously on each parental map. The composite interval mapping proposed by Zeng (1993, 1994) was preferred. In order to increase the power of QTL detection, both parental maps were used simultaneously as previously suggested by Hayashi and Awata (2004). A total of 40 QTLs were mapped on the parental linkage groups (Moraes, 2005). Approximately 27% of the QTLs identified had an intermediate phenotypic effect, explaining more than 12% of the phenotypic variance. Among other traits, these loci were detected for fruit yield, total number of fruits, length, and width of fruits (Figure 2).

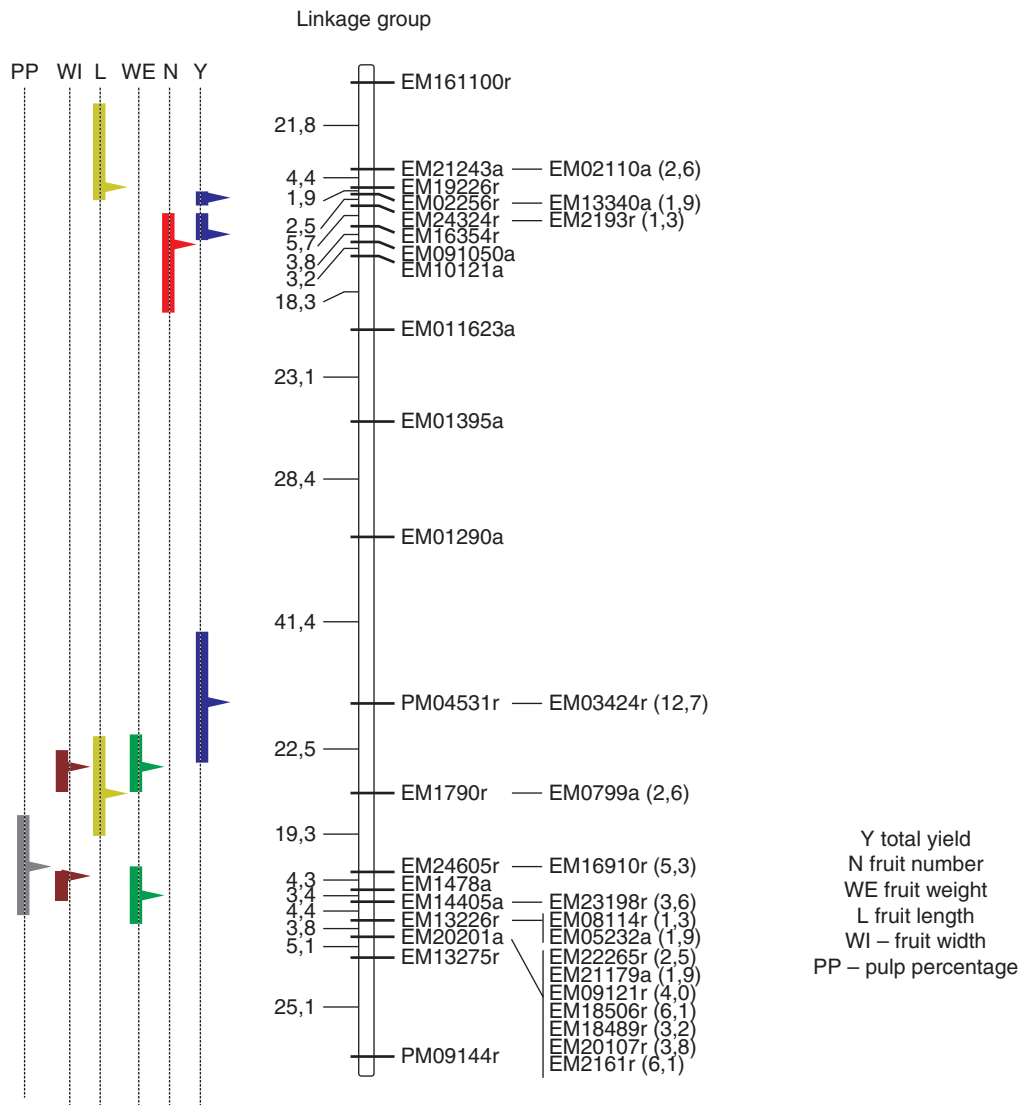


Figure 2 QTL mapping (colored bars, on the left) in one of the yellow passionfruit linkage groups, constructed based on AFLP markers (note that correlated traits, e.g., number, weight, and length of the fruit tend to map on the same regions)

Moreover, important quantitative resistance loci (QRL), related to the response of the same F_1 population to inoculation with isolates of *Xanthomonas axonopodis* pv. *passiflorae* were mapped (Lopes *et al.*, 2006). This pathogen is frequently found in passionflower orchards, being very destructive to the crop. The mechanisms of inheritance of resistance are poorly understood and further breeding progress is difficult to obtain. As reported for other crops (Studer *et al.*, 2006), only a few major QTLs explaining 16.5% of the

total phenotypic variation for diseased leaf area were detected in yellow passionfruit. QRLs for partial resistance tend to map at genomic regions near clustered resistance genes (R) in several plant species. Based on this idea, an investigation to assess the number of analogous R genes in yellow passionfruit was initiated (Consoli *et al.*, 2006). Although many important questions remain to be answered regarding R genes, specific passionflower resistance genes will certainly be cloned in the near future.

1.6 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

As outlined in Section 1.4, conventional breeding is limited, at times quite severely, by natural barriers related to interspecific incompatibility among many species of *Passiflora*, as well as self-incompatibility in most commercially important species, including yellow passionfruit. Also, the semiperennial nature of the plant and its long juvenile period (at least 90 days) necessarily turn conventional breeding into a medium- to long-term endeavor. Therefore, passionfruit is one of the crops in which immediate benefits of transgenic breeding are easier to appreciate. For traits such as virus resistance, in which the resistance gene is derived from the pathogen's own genome and therefore is readily available, resistant plants could, in theory, be generated in a very short period of time. Even for more complex traits, transgenic approaches hold the promise of faster and better results when compared to conventional breeding.

2. TRANSGENIC PASSIONFRUIT

2.1 Tissue Culture-based Techniques Applied to Passionfruit Genetic Transformation

Tissue culture studies in *Passiflora* were initiated as early as 1966 (Nakayama, 1966) with shoot production from stem segments of mature plants of *P. caerulea*. Micropropagation of yellow passionfruit and *P. mollissima* was further reported (Moran Robles, 1978, 1979). Since then, an increasing number of reports on tissue culture-based techniques applied to the genus have been published (reviewed by Vieira and Carneiro, 2004). The development of plant genetic transformation techniques has opened up a new approach to plant breeding of several tropical fruit crops, including passionfruit (Gómez-Lim and Litz, 2004). Genetic transformation is an important strategy for obtaining disease-resistant plants of yellow passionfruit and *P. edulis* f. *edulis*, which is specially relevant since passionfruit woodiness, (caused by PWV or CABMV), *Fusarium* wilt (caused by *Fusarium oxysporum* f. sp. *passiflorae*), and bacterial blight (caused by *Xanthomonas axonopodis* pv. *passiflorae*) are the major limiting

factors for the crop (Vieira and Carneiro, 2004; Alfenas *et al.*, 2005; Trevisan *et al.*, 2006).

2.1.1 Micropropagation

An efficient *in vitro*-based plant regeneration system is a prerequisite for genetic transformation approaches, thereby providing a practical baseline for improved micropropagation systems and/or clonal plant production. Thus, the development of any novel cultural regime that facilitates clonal propagation of commercial *Passiflora* species is of utmost importance (Davey *et al.*, 2003). Although several protocols for *in vitro* propagation and regeneration of *Passiflora* spp. are available (Table 2) and can be used for *Agrobacterium*-mediated transformation, passionfruit micropropagation is far from being routine due to low multiplication rates, even though several studies have been reported. Low regeneration frequencies and inconsistent results continue to offer a challenge to study the effects of explant source on *in vitro* regeneration of *Passiflora* species.

As for other species, the achievement of reliable transformation protocols in the genus *Passiflora* depends on the establishment of an efficient regeneration procedure, high transformation frequency, and optimum selection for regenerating transformed cells. This can be obtained by the interplay of several factors including plant genotype, choice of explant source, concentration and combination of plant growth regulators, nutrient medium composition, and culture environment (light quality and quantity or absence of light; temperature; and gaseous environment) (Phillips, 2004).

De novo regeneration of passionfruit has been achieved via the organogenic pathway from explants derived from seedlings. Organogenesis occurs either from callus (Nakayama, 1966; Moran Robles, 1979; Mourad-Agha and Dexheimer, 1979; Scorza and Janick, 1980; Kantharajah and Dodd, 1990; Vestri *et al.*, 1990; Desai and Mehta, 1985; Monteiro *et al.*, 2000) or directly from the explant (Dornelas and Vieira, 1994; Kawata *et al.*, 1995; Faria and Segura, 1997a; Cancino *et al.*, 1998; Jasrai and Mudgil, 1999; Otahola, 2000). Cytokinins, especially 6-benzyladenine (BA), have been demonstrated to be the most important growth regulator in relation to regeneration

Table 2 Optimal parameters for *Passiflora in vitro* regeneration as determined by different authors^(a)

Species	Source of explant	Hormone type and concentration	Reference
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl segments vertically cultured	3.08 μ M BA	Alexandre, 2006
<i>P. edulis</i> f. <i>flavicarpa</i>	Cotyledon, leaf, and hypocotyls segments	4.4 μ M BAP + 5% coconut water (CW)	Monteiro, 2005
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl segments cultured in different orientations and positions	3.08 μ M BA or 0, 4.54, 9.08, 13.62, and 18.16 μ M TDZ	Felismino, 2005
<i>P. edulis</i> f. <i>flavicarpa</i> , <i>P. cincinnata</i>	Hypocotyl segments	2.2 μ M BA + 10% CW	Reis, 2005
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl segments	4.4. μ M BA	Reis <i>et al.</i> , 2005
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf discs	0, 4.4 and 8.8 μ M BA or 0, 1.1, 2.2, and 3.4 μ M TDZ + AgNO ₃	Trevisan and Mendes, 2005
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl and leaf	4.4 μ M BAP + 5% CW	Fernando, 2005
<i>Passiflora</i> spp.	Endosperm and zygotic embryos (matures seeds)	2,4-D, kinetin, NAA, GA ₃ , BA, IAA, and IBA in several combinations	Guzzo <i>et al.</i> , 2004
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf discs	4.44 μ M BA + 2.32 μ M kinetin	Becerra <i>et al.</i> , 2004
<i>P. edulis</i>	Shoot tips	22.2 μ M BA + 11.6 μ M GA ₃	Isutsa, 2004
<i>P. edulis</i> f. <i>flavicarpa</i> , <i>P. giberti</i> , <i>P. mollissima</i>	Leaf segments	13.2 BA + 9.3 μ M kinetin + Pluronic F-68 (PF) 4.4. μ M BA + 2.32 μ M kinetin + PF	Davey <i>et al.</i> , 2003
<i>P. edulis</i> f. <i>flavicarpa</i>	Shoot tips	No growth regulators + ACC, AVG	Reis <i>et al.</i> , 2003
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl segments vertically oriented	3.08 μ M BA	Couceiro, 2002
<i>P. edulis</i> f. <i>flavicarpa</i>	Cotyledon	8.88 μ M BA	Ribas <i>et al.</i> , 2002
<i>P. actinia</i>	Shoot cultures	4.4. μ M BA + 0.05 μ M IBA	Koch <i>et al.</i> , 2001
<i>P. edulis</i> f. <i>flavicarpa</i>	Shoot cultures	5.02 μ M IAA and precursors or inhibitors of ethylene	Barbosa <i>et al.</i> , 2001
<i>P. edulis</i> f. <i>flavicarpa</i>	Internodal segments	4.4–17.7 μ M BA	Biasi <i>et al.</i> , 2000
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf discs	0.0–5.3 μ M BA	Otahola, 2000
<i>P. edulis</i> \times <i>P. edulis</i> <i>flavicarpa</i>	Cotyledons	10 μ M BA 10% CW	Hall <i>et al.</i> , 2000
<i>P. edulis</i> f. <i>flavicarpa</i> , <i>P. alata</i> <i>P. suberosa</i>	Leaf discs	0.6, 08, 1.0 BA or 0.25–1.0 TDZ 2.2 or 4.4 μ M BA	Monteiro-Hara, 2000
<i>P. suberosa</i>	Leaf discs	0.0, 2.2, or 4.4 μ M BA	Monteiro <i>et al.</i> , 2000
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf discs	1 BA or 1 NAA	Appazzato-da-Glória <i>et al.</i> , 1999
<i>P. caerulea</i>	Leaves	10 μ M BA + 0.1 μ M IAA	Jasrai and Mudgil, 1999
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf	4.4 or 8.8 μ M BA	Barbosa, 1999
<i>P. mollissima</i> , <i>P. edulis</i> f. <i>flavicarpa</i> , <i>P. giberti</i>	Nodal segments	BA and kinetin at various concentrations and combinations	Cancino <i>et al.</i> , 1998
<i>P. edulis</i> f. <i>flavicarpa</i>	Leaf	4.4 μ M BA and transference to 9.9 μ M kinetin + 5 μ M IAA	Silva, 1998
<i>P. edulis</i> f. <i>flavicarpa</i>	Hypocotyl segments, leaf	5 μ M BA + 2 μ M IAA	Faria and Segura, 1997a
<i>P. edulis</i> f. <i>flavicarpa</i>	Shoot apices	2–20 μ M BA + 2 μ M IAA	Faria and Segura, 1997b
<i>P. foetida</i>	Triploid endosperm	8.88 μ M BA	Mohamed <i>et al.</i> , 1996
<i>P. edulis</i> f. <i>flavicarpa</i>	Shoot primordial	1 μ M BA + 1 μ M indole-3-butyric acid (IBA) transference to 10 μ M BA	Kawata <i>et al.</i> , 1995
<i>P. edulis</i> f. <i>edulis</i>	Meristem	BA, IBA, and GA ₃ at various combinations	Biricolt and Chiari, 1994
<i>P. edulis</i> f. <i>flavicarpa</i> , <i>P. mollissima</i> , <i>P. giberti</i> , <i>P. maliformis</i> , <i>P. amethystina</i>	Cotyledons, hypocotyl segments, leaf	8.88 μ M BA + 10% CW	Dornelas and Vieira, 1994
<i>P. edulis</i> \times <i>P. edulis</i> <i>flavicarpa</i>	Adult and juvenile buds	10 μ M kinetin + 5 μ M IAA	Drew, 1991
<i>P. coerulea</i>	Stem and leaves	1 NAA + 13.2 μ M BA	Vestri <i>et al.</i> , 1990
<i>P. edulis</i>	Nodal sections	8.8 μ M BA	Kantharajah and Dodd, 1990
<i>P. suberosa</i>	Shoot apex	0.44 μ M BA	Scorza and Janick, 1980

^(a) Adapted from Vieira and Carneiro (2004)

(Table 2). However, thidiazuron (TDZ) has been used alternatively to induce organogenic responses in passionfruit cultured explants (Table 2).

To better characterize the structural aspects involved in shoot organogenic cultures, anatomical studies have been carried out (Moran Robles, 1979; Scorza and Janick, 1980; Biricolt and Chiari, 1994; Appezzato-da-Glória *et al.*, 1999; Biasi *et al.*, 2000; Reis, 2001; Lombardi, 2003; Dias, 2006; Felismino, 2005). A number of highly comprehensive and descriptive anatomical studies of *in vitro* organogenesis of yellow passionfruit, involving histochemistry, light, and electron (scanning electron microscope and transmission electron microscope) microscopy, have been published (Appezzato-da-Glória *et al.*, 2005; Fernando, 2005). These anatomical characterizations and the better knowledge of the cell and tissue layers involved in the morphogenic events leading to regeneration may contribute positively to increase the efficiency of transformation, as well as laying the basis for the genetic transformation of different *Passiflora* species by means of biolistics- and *Agrobacterium*-mediated gene transfer techniques.

It is well known that light influences the morphology of tissue-cultured plants, and light requirements for morphogenesis are different for each explant type and among different stages of micropropagation. Several authors have discussed the effect of light irradiation on the morphogenic potential of *Passiflora* species (reviewed by Vieira and Carneiro, 2004). However, for yellow passionfruit, a wide range of variation in light irradiation was detected among these reports: dark conditions or $23 \mu\text{M m}^{-2} \text{s}^{-1}$ (Appezzato-da-Glória *et al.*, 1999; Monteiro *et al.*, 2000), $9.75 \mu\text{M m}^{-2} \text{s}^{-1}$ (Ribas *et al.*, 2002), $22\text{--}25 \mu\text{M m}^{-2} \text{s}^{-1}$ (Dornelas and Vieira, 1994; Barbosa *et al.*, 2001; Koch *et al.*, 2001), $36 \mu\text{M m}^{-2} \text{s}^{-1}$ (Reis *et al.*, 2003, 2005), $20\text{--}55 \mu\text{M m}^{-2} \text{s}^{-1}$ (Hall *et al.*, 2000), $56 \mu\text{M m}^{-2} \text{s}^{-1}$ (Moran Robles, 1979), $80 \mu\text{M m}^{-2} \text{s}^{-1}$ (Faria and Segura, 1997a), $90 \mu\text{M m}^{-2} \text{s}^{-1}$ (Faria and Segura, 1997b; Isutsa, 2004), $180 \mu\text{M m}^{-2} \text{s}^{-1}$ (Biricolt and Chiari, 1994), all under 16 h-photoperiod, and $30 \mu\text{M m}^{-2} \text{s}^{-1}$ under continuous light (Kuriyama *et al.*, 1998). However, up to now the only systematic study on the influence of various irradiances on passionfruit *in vitro* regeneration responses was reported by Alexandre (2002, 2006). Several irradiances (12, 25, 50, 100, $150 \mu\text{M m}^{-2} \text{s}^{-1}$) were evaluated,

and hypocotyl segments and stem apices excised from yellow passionfruit plantlets showed a better adaptation to the lowest irradiances (Alexandre, 2006). Markedly, the increase in irradiance incidence on the hypocotyl segments and stem apices affected the ethylene emission rate and CO_2 evolution. In addition to that, there was a relationship between ethylene production and biosynthesis or leaf pigment degradation, and the potential quantum production of photosystem II, whereas the latter is indicated for measuring the plantlet stress levels. In the organogenic process, a direct relationship was observed between sprout formation at the highest irradiances and an increase in peroxidase activity, as well as a decrease in catalase and superoxide dismutase activities.

Regarding the importance of the physiological status of the donor plants of the explants, the age and physiological condition of donor plants was investigated by Becerra *et al.* (2004). The effect of explant source on *in vitro* morphogenesis was studied by comparing the response of explants collected from young plants, *in vitro* plantlets, and reinvigorated adult plants. Highest *de novo* shoot production was observed in explants from 2-months-old plants cultured in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with $4.44 \mu\text{M}$ BA and $2.32 \mu\text{M}$ kinetin. Accordingly, the organogenic response varied with the time of the year when the explants of yellow passionfruit and *P. alata* were collected, something which is apparently related to the environmental conditions in which the seedlings were maintained under greenhouse conditions (Monteiro-Hara, 2000).

The supplementation of the growth media with coconut water (5–10%) improved morphogenic responses of several passionfruit species, including *P. quadrangularis* (Mourad-Agha and Dexheimer, 1979), *P. edulis* f. *edulis* (Kantharajah and Dodd, 1990), yellow passionfruit, *P. giberti*, *P. mollissima* and *P. maliformis* (Dornelas and Vieira, 1994), *P. edulis* \times yellow passionfruit (Hall *et al.*, 2000), and *P. cincinnata* (Lombardi, 2003; Fernando, 2005; Reis, 2005). It appears that for some plant species, including passionfruit, coconut water provides growth-stimulating substances which are essential for optimal regeneration, promoting the rapid development of shoots and increasing shoot number and/or height. The *in vitro* response of yellow passionfruit was also affected by the nature

of the gelling agent added to semisolid or solid media (Reis, 2001; Reis *et al.*, 2005). The gelling agents influenced organogenesis in different ways. Agargel and Phytigel allowed better organogenic responses in relation to agar. A direct relation between agar purity and improved morphogenic responses was not observed. However, the effectiveness of the selective antibiotic kanamycin decreased in media solidified with Phytigel. In view of the results, the appropriate choice of the gelling agent should be taken into account, considering its effects upon *in vitro* morphogenesis and availability of the selective agent.

As a climacteric species (Shiomi *et al.*, 1996; Mita *et al.*, 1998) it is expected that ethylene sensitivity is maintained under *in vitro* culture systems. Therefore, several regeneration protocols have considered the use of ethylene inhibitors as beneficial for enhancing morphogenic responses (Faria and Segura, 1997a; Barbosa, 1999; Barbosa *et al.*, 2001; Reis, 2001, 2005; Reis *et al.*, 2003; Trevisan and Mendes, 2005; Trevisan *et al.*, 2006), suggesting that an improvement on the number of gaseous changes between the internal atmosphere of the culture vessels and the surrounding atmosphere may be beneficial to better manipulate the growth and differentiation of micropropagated passionfruit, as well as lessening the negative effects of ethylene.

2.1.2 Somatic embryogenesis

The first report on the induction of somatic embryogenesis in the genus was for *P. giberti* by Otoni (1995). The author reported the establishment of embryogenic cell suspensions initiated from embryogenic leaf cultures. Cell suspensions, maintained in AA2 medium (Abdullah *et al.*, 1986) were used to establish a regeneration protocol based on isolated and cultured protoplasts (Otoni, 1995; Anthony *et al.*, 1999).

Even though histological analyses suggested that TDZ induces structures that resembled early staged somatic embryos (Monteiro-Hara, 2000; Felismino, 2005), no further confirmation was provided in terms of embryo development and further conversion into plants.

The induction of embryogenic calli lines from hypocotyls, anthers, and zygotic embryo explants of *P. cincinnata* has been recently achieved

(Otoni, unpublished results). Embryogenic lines were maintained in semisolid or liquid media, established from primary or secondary embryos, remaining highly proliferative. Embryo-derived plants have been successfully recovered and acclimatized under greenhouse conditions. Further studies were reported to be directed at synchronizing hystodifferentiation of the embryogenic cultures and to produce synthetic seeds. Furthermore, this morphogenic pathway has been exploited to generate fully acclimatized transgenic plants of *P. cincinnata* expressing β -glucuronidase (*gus*) and green fluorescent protein (*gfp*) genes, by means of sonication-assisted *Agrobacterium* transformation.

2.1.3 Anther culture

P. edulis was assessed for plant regeneration potential using anthers at the tetrad or early-uninucleate microspores stage as explants. However, regeneration was not obtained from calli derived from these anthers, and neither was the callus ploidy level assessed (Tsay *et al.*, 1984). Nevertheless, establishing a protocol for the development of anther culture- or microspore-derived embryogenic haploid lines represents a promising step to generate transgenic plants without hemizygous transformants. Highly proliferative embryogenic calli lines have been established for *P. cincinnata* (Otoni, unpublished results). However, flow cytometry analysis revealed the diploid nature of all regenerated embryogenic-derived plants. Nonetheless, as previously mentioned, these embryogenic lines have been used to generate transgenic plants.

2.1.4 Protoplast-based regeneration systems

Interspecific sexual hybridization has been attempted in several *Passiflora* breeding programs, using wild germplasm to transfer disease resistance and other potentially desirable traits into cultivated species. However, fertile hybrids have been difficult to obtain. Somatic hybridization provides a means of circumventing such sexual incompatibilities and therefore could be used as an alternative method for achieving gene

flow between *Passiflora* species. Indeed, novel fertile intergeneric somatic hybrids have been obtained between cultivated species (specially yellow passionfruit) and several wild *Passiflora* species (Dornelas *et al.*, 1995; Otoni *et al.*, 1995a; Vieira and Dornelas, 1996; Vieira and Carneiro, 2004). Combinations between yellow passionfruit and *P. amethystina* were further grown to maturity and characterized under field conditions (Barbosa and Vieira, 1997; Vieira and Carneiro, 2004).

A prerequisite for the production of somatic hybrids is the development of protoplast-to-plant regeneration systems. To date, plant regeneration from *Passiflora* species has been reported by shoot regeneration via organogenesis from mesophyll cells of cultivated and wild passionfruit species (d'Utra Vaz *et al.*, 1993; Dornelas and Vieira, 1993; Dornelas, 1995; Dornelas *et al.*, 1995; Otoni, 1995; Otoni *et al.*, 1995a, b, 1996).

The relative wide range of *Passiflora* species for which protoplast-to-plant regeneration systems have been established, either from cell suspension cultures (Dornelas, 1995; Dornelas *et al.*, 1995; Otoni, 1995; Anthony *et al.*, 1999) or directly from mesophyll cells, as well as the high protoplast yield, plating, and plant regeneration efficiencies usually observed, should allow the use of *Passiflora* protoplast systems not only for somatic hybridization, but also for physiological studies at the cellular level with the exploitation of possible protoclonal variation and direct gene transfer. Indeed, the later has been attempted by Monteiro (2005). However, due to the easiness of manipulating leaf or hypocotyl explants, as compared to protoplasts, the author chose to incorporate the *attacin A* (*attA*) gene by means of *Agrobacterium tumefaciens*. In conclusion, considering the extensive and reproducible works that have been reported, the protoplast-based regeneration systems already existent for *Passiflora* can be used as a basis for the development of plant transformation protocols or for studies of transient gene expression or viral replication (including virus-induced gene silencing) on freshly isolated and electroporated protoplasts.

2.1.5 *In vitro* conservation

In view of the world tendency to eliminate wild species as a consequence of deforestation, some

Passiflora species are endangered, and genotypes that could have been useful for breeding programs have disappeared. *In vitro* conservation based on shoot cultures (Vieira and Carneiro, 2004) or zygotic embryo culture (Guzzo *et al.*, 2004) not only provides a continuous source of material, but also represents an interesting approach for germplasm collection and preservation. A germplasm collection of *Passiflora* has been established based on shoot culture maintenance of wild species and somatic hybrids, with subcultures at 45–60-day intervals (Vieira, 2000; Vieira and Carneiro, 2004). This technology has also a great application if extended to the conservation of transgenic plants under laboratory conditions, ideally extending this possibility to the ultra-low-temperature conservation via cryopreservation of meristems, internodal or nodal explants, embryogenic cell suspension lines, somatic embryos, zygotic embryos, and also seeds, using vitrification-based procedures (reviewed by Engelmann, 2004).

2.2 Transgenics Developed

A number of reports of genetic transformation of passionfruit have appeared in the literature, starting with Manders *et al.* (1994). All of these reports deal with yellow passionfruit, with the majority concentrating on the validation of genetic transfer and tissue culture regeneration protocols and having no short-term commercial application. The exceptions are the reports by Alfenas *et al.* (2005) and Trevisan *et al.* (2006), which deal with the production of transgenic yellow passionfruit plants resistant to passionfruit woodiness and eventually could lead to large scale deployment.

Manders *et al.* (1994) were the first ones to report the genetic transformation of a species of *Passiflora*. Leaf disks and stem segments of yellow passionfruit were used as explants, which were co-cultivated with *A. tumefaciens* strain LBA4404 harboring the co-integration vector pMON200. Four plants were regenerated from leaf explants after 20 days of co-cultivation on agar-solidified MS medium containing 4.43 μ M 6-benzylaminopurine (BAP) and 86 μ M kanamycin. The four plants were rooted by transfer to MS medium with 14.7 μ M indole-3-acetic acid (IAA), 2.68 μ M α -naphthalene acetic acid (NAA), and 172 μ M kanamycin for 7 days, followed

by MS medium with the same concentration of kanamycin but lacking growth regulators. Rooted plants were transferred to soil and grown to maturity. Three of the plants synthesized nopaline and expressed neomycin phosphotransferase activity. The presence of the *nptII* gene was confirmed by dot blot hybridization and polymerase chain reaction (PCR) analyses in three out of the four plants. These results were expanded by Silva (1998), who demonstrated that foliar explants of yellow passionfruit are tolerant to concentrations as high as 172 μM of kanamycin. Shoot regeneration was actually enhanced at 86 μM of kanamycin. Likewise, root elongation was not inhibited even at 172 μM of kanamycin. Therefore, different selection agents (e.g., hygromycin) should be used to avoid escapes and thus to increase transformation efficiency.

Lin (1998) reported the genetic transformation of purple passionfruit (*P. edulis* f. *edulis*) mediated by *Agrobacterium rhizogenes* strain MAFF03-01724. The bacteria were smeared over a stem segment on MS solid medium. After 4 weeks, adventitious roots derived from tumors were removed and cultured on MS medium with cefotaxime. Shoots were regenerated by transferring roots to MS medium supplemented with BAP and 2,4-dichlorophenoxyacetic acid (2,4-D).

Genetic transformation using biolistics was achieved by Takahashi (2002) and Monteiro (2005), with the objective of obtaining transgenic plants with resistance to *X. axonopodis* pv. *passiflorae* by expression of the *attA* gene, which encoded for a peptide with bactericide properties. It was verified that either 9.47 μM of hygromycin or 86 μM of kanamycin were sufficient to inhibit organogenesis in 60% of the explants. Transient gene expression was effective when plants were bombarded at 1200 psi of helium pressure and 9.5 cm of distance. Co-transformation frequencies of 0.6% were obtained and transformation was verified by PCR.

Reis *et al.* (2007) analyzed the effect of several factors affecting *A. rhizogenes*-mediated genetic transformation of yellow passionfruit and *P. cincinnata*. Suspension cultures of *A. rhizogenes* strain R1601 were grown in Rhizo medium (Tepfer and Casse-Delbart, 1987) supplemented with 172 μM kanamycin and 269.2 μM ampicillin for 16–18 h at 200 rpm and 28 °C. Bacterial cells were centrifuged and resuspended in MS

medium lacking antibiotics for an OD_{600} of 0.25. Rooted hypocotyl segments were used as explants. The distal portion (4–5 mm) of the hypocotyls was punctured with a hypodermic needle 4–5 times to inject the bacterial cells. Explants were transferred in the upright position to test tubes containing half-strength MS semisolid medium (1/2 MS) lacking growth regulators and B5 vitamins, supplemented with 554 μM myo-inositol and 2% sucrose. The medium was solidified with 0.25% Phytigel and the pH was adjusted to 5.8 before autoclaving. After 20–25 days, hairy roots (1.5–2.0 cm in length) induced by *A. rhizogenes* were detached from the shoots and transferred individually to 30 ml semisolid (0.25% Phytigel) MS medium supplemented with 3% sucrose, B5 vitamins, 554 μM myo-inositol, 264 μM kanamycin, and 817 μM timentin. Transformed roots were subcultured every 15 days to selective fresh medium of the same composition, except that the concentration of kanamycin was increased to 334 μM after the fourth subculture. The first responses were observed 20–30 days after inoculation with *A. rhizogenes*. Hairy roots differentiated at the inoculation sites were used to establish individual root clones and to initiate long-term cultures on semisolid medium. The regenerated roots displayed typical features of hairy roots, including plagiotropism, branching, and growth habit. The *nptII* and nopaline synthase (*nos*) genes were detected by PCR in genomic DNA from root clones of both species at the 6th subculture stage, and the *nos* gene was detected in regenerants derived from somatic embryos of *P. cincinnata*. Physiological confirmation of the transformed nature was provided by the auxin autotrophic response and resistance to kanamycin. Spontaneous plant regeneration from roots growing on selective semisolid MS medium devoid of growth regulators was occasionally observed via organogenesis for yellow passionfruit. *P. cincinnata* displayed higher rates of regeneration, and regenerants were recovered via both organogenesis and somatic embryogenesis.

Alfenas *et al.* (2005) reported the production of transgenic plants of yellow passionfruit expressing an untranslatable RNA corresponding to two-thirds of the replicase (NIb, nuclear inclusion b) cistron and one-third of the adjacent coat protein (CP) cistron of a Brazilian isolate of CABMV. The viral fragment was PCR amplified and cloned into

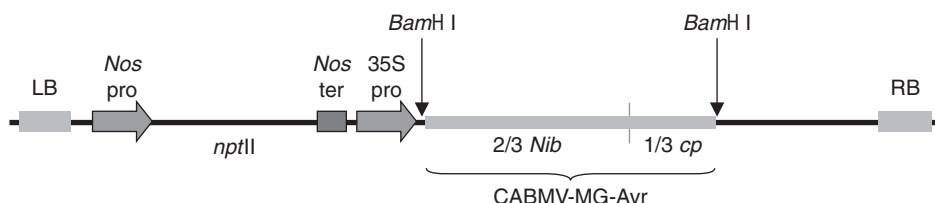


Figure 3 Construct used for genetic transformation of yellow passionfruit by Alfenas *et al.* (2005); LB, left border of the T-DNA in plasmid pBI121; RB, right border of the T-DNA; Nos pro, Nos promoter, which controls the expression of the kanamycin resistance gene *nptII*; Nos ter, transcription termination signal of the Nos gene; 35S pro, cauliflower mosaic virus 35S promoter, which controls transgene expression; the viral-derived fragment corresponds to two-thirds of the Nib protein coding region and one-third of the coat protein coding region of CABMV isolate MG-Avr

the pBI121 binary vector (Figure 3). Recombinant plasmids were transformed into *A. tumefaciens* strain LBA4404. Genetic transformation of yellow passionfruit was accomplished by co-cultivation of *A. tumefaciens* cells and etiolated hypocotyl segments. The explants were immersed in a suspension of *A. tumefaciens* (OD₆₀₀ 0.4) for 20 min, transferred to nonselective MS medium (3% sucrose, 4.4 μ M BAP, B5 vitamins, 277 μ M myo-inositol, 0.26% Phytigel, pH 5.8) and kept in darkness for 48 h. Transgenic plants were regenerated in selective medium containing kanamycin (264 μ M) and cefotaxime (1048 μ M). After elongation, stems were transferred to rooting medium (1/2 MS, 2% sucrose, B5 vitamins, 277 μ M myo-inositol, 1048 μ M cefotaxime, 5 μ M IAA, 0.26% Phytigel, pH 5.8). Rooted plants were then transferred to substrate. Transformation was verified by PCR for 15 out of 16 R₀ plants. The R₀ plants were vegetatively propagated by cuttings and plants developed from the cuttings were sap-inoculated with CABMV isolates MG-Avr and PE-Bnt. Nontransformed plants developed evident mosaic and leaf deformation symptoms upon inoculation with both isolates. Plants obtained from the R₀-transformant TE5-10 developed the same symptoms upon inoculation with isolate PE-Bnt, but did not show any symptoms upon inoculation with isolate MG-Avr. Viral detection by enzyme-linked immunosorbent assay (ELISA) confirmed the visual observations. The transgenic messenger-RNA (mRNA) was detected in a noninoculated plant derived from the susceptible R₀-transformant TE5-4, but was not detected in noninoculated plants derived from the resistant R₀-transformant TE5-10, suggesting that resistance was due to posttranscriptional (RNA) silencing, which was already activated

in TE5-10-derived plants before inoculation with the viral isolates. Resistance in the hemizygous TE5-10 transgenic plant was, therefore, restricted to isolate MG-Avr. This was explained based on the specificity of RNA silencing (Waterhouse *et al.*, 2001), which requires high sequence identity between the target sequence and the sequence used for transformation (Prins, 2003). The nucleotide sequence identity between the CP cistrons from isolates MG-Avr and PE-Bnt is 93% (Nascimento *et al.*, 2006).

In order to verify whether resistance was stably inherited and dependent on gene dosage, the resistant TE5-10 plant was self-pollinated to obtain R₁ progeny homozygous for the transgene (Nascimento, 2006). Self-pollination was accomplished by covering flowers with a paper bag on the day of anthesis and manually pollinating each flower with a cotton swab, twice (the first time at 1:00 PM, right after anthesis, and the second time at 5:00 PM). Out of 300 attempts, three produced viable fruit, from which a total of 231 seeds were obtained. Fifty-one plants were tested for resistance to three viral isolates (MG-Avr, PE-Bnt, and SE-Nps). Most plants were resistant to MG-Avr, but susceptible to the other two isolates.

One plant, labeled TE5-10-15J, was resistant to all the three isolates, displaying no symptoms after inoculation (Figure 4) and testing ELISA negative. Cuttings were obtained from this plant and the resulting plants were inoculated with an additional four isolates (PB-Alh, BA-Itb, ES-Vni, and PA-Iga), showing an equal level of resistance to all of them. Transgenic mRNA was not detected in clonal plants derived from TE5-10-15J, and viral RNA was not detected in these plants after inoculation with isolates MG-Avr, PE-Bnt, and

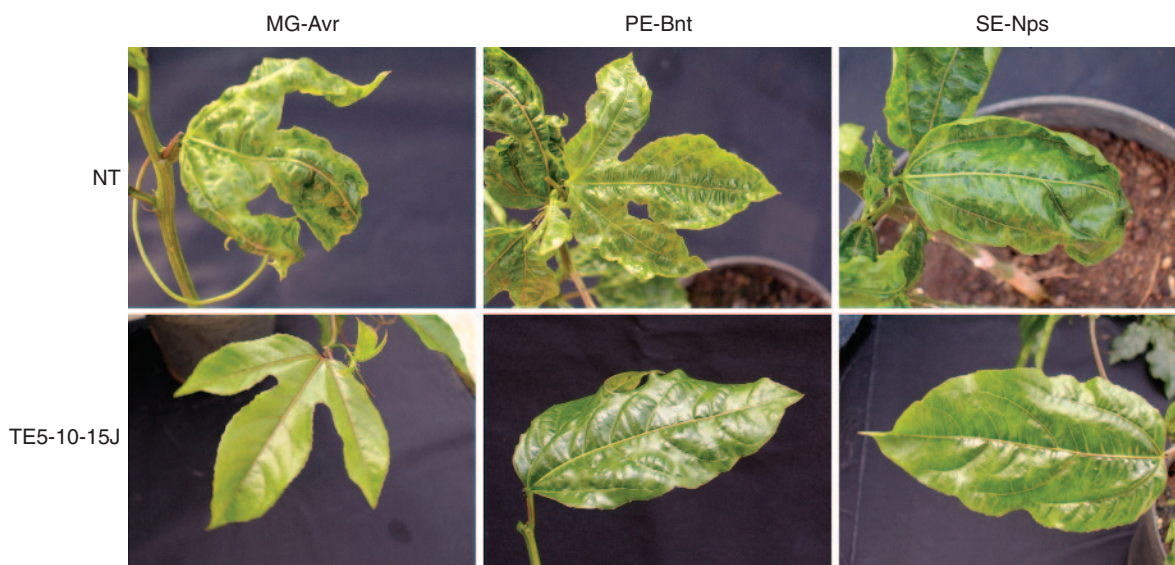


Figure 4 Systemic symptoms observed in R_1 transgenic plants following inoculation with CABMV isolates MG-Avr, PE-Bnt, and SE-Nps; NT, nontransformed plant; TE5-10-15J, plant obtained after self-pollination of the R_0 -transformant TE5-10

SE-Nps. The combined results indicated that the TE5-10-15J plant is resistant to at least seven isolates of CABMV from different geographical regions of Brazil, and that resistance is due to RNA silencing, which is already activated before viral inoculation (Nascimento, 2006).

Trevisan *et al.* (2006) reported similar results. The full-length CP cistron from a severe CABMV isolate (referred to as PWV by the authors) was cloned into the pCAMBIA 2300 binary vector, which was transformed into *A. tumefaciens* strain EHA105. *A. tumefaciens* was cultivated in yeast extract peptone supplemented with kanamycin ($172\ \mu\text{M}$) and rifampicin ($60.7\ \mu\text{M}$) for 48 h. Genetic transformation was achieved by inoculation for 20 min of a bacterial suspension (5×10^8 colony-forming units per milliliter) with leaf disks from the Brazilian cultivars “IAC-275” and “IAC-277”. After inoculation, the explants were transferred to MS medium supplemented with TDZ ($1.14\ \mu\text{M}$), AgNO_3 ($23.5\ \mu\text{M}$), and acetosyringone ($100\ \mu\text{M}$) and incubated for 3 days at 24°C . Explants were then transferred to selection medium consisting of MS supplemented with TDZ, AgNO_3 , kanamycin ($172\ \mu\text{M}$), and cefotaxime ($1048\ \mu\text{M}$). Cultures were incubated in the dark for 6 to 8 weeks at 27°C and then changed to a 16-h photoperiod at $27 \pm 1^\circ\text{C}$. Developed shoots were transferred to

Magenta boxes containing elongation medium (MS supplemented with 10% coconut water and $1048\ \mu\text{M}$ cefotaxime), covered with vented lids and incubated with a 16-h photoperiod for further plant development. After transferring to elongation medium, 119 and 109 plantlets of IAC-275 and IAC-277, respectively, were recovered. Transformation was confirmed in seven of eight plants evaluated by Southern blot analysis, showing different numbers of insertional events. Three transgenic plants (T3, T4, and T7) expressed the expected transcript, but the 32 kDa CP was detected by Western blot in only two plants (T3 and T4). The results of three successive mechanical inoculations against the transgenic plants using three CABMV isolates showed that the primary transformant T2 of “IAC-277” was immune to all isolates. Transgenic plants T1, T5, and T8, which showed higher numbers of transgene insertional events than T2, were all susceptible to infection under high inoculum pressure.

3. FUTURE ROAD MAP

3.1 Expected Products

The most-likely short-term development in terms of passionfruit transgenics are woodiness-resistant

plants. The results obtained by Alfenas *et al.* (2005), Nascimento (2006), and Trevisan *et al.* (2006) are promising and indicate that resistance to passionfruit woodiness can be accomplished by expression of untranslatable (Alfenas *et al.*, 2005; Nascimento, 2006) or translatable (Trevisan *et al.*, 2006) virus-derived genomic fragments. The former approach, which is believed to lead to resistance via RNA silencing, provides a high level of resistance, but this is usually of narrow spectrum. Obtaining homozygous transgenic plants is one way to overcome this problem, as shown by Nascimento *et al.* (2006). Resistance to bacterial or fungal diseases is not realistic in the short term, but could become a possibility once a larger number of genes involved in host-pathogen interactions and plant-defense responses are functionally characterized. Other desirable products would be plants with uniform fruit maturity or self-compatible. However, the biochemical pathways that control fruit setting and maturity, as well as self-incompatibility in yellow passionfruit, are likely to involve a large number of genes and have not yet been dissected and therefore such transgenic plants are not likely to be produced in the near future.

3.2 Risks and Concerns

Transgenic passionfruit plants expressing untranslatable constructs of viral origin do not accumulate transgenic mRNA or proteins and therefore offer no risk for human consumption, even considering that passionfruit is consumed fresh. Concerns due to antibiotic resistance genes, which are part of transgenic constructs have been raised, but any real risk remains to be proven. Even then, it is not unrealistic to assume that transgenic passionfruit could be obtained without using selection markers based on antibiotic resistance. In fact, with the transformation efficiency reported in a number of works, transgenic plants could be regenerated without any selectable marker at all. Therefore, risks for human health are negligible or nonexistent.

The losses caused by passionfruit woodiness are so dramatic and widespread that growers' demand for resistant plants, whether obtained by conventional breeding or engineered resistance, is high. In this context, it is safe to assume that,

once transgenic plants resistant to the disease are available, growers will rapidly adopt them (public opinion issues aside). The possibility exists, therefore, for large-scale planting of one or two yellow passionfruit cultivars, thus severely reducing the genetic basis of the crop. Different alleles to guarantee the compatibility among the orchard plants are also necessary due to the presence of an auto-incompatibility system, as previously mentioned. Such scenario is well known to generate the risk of new epidemics of previously secondary diseases, if the new cultivars are specially susceptible to them. It is important to assure that a number of genetically diverse materials be transformed and made available to growers. Considering that all work carried out to date (at least in Brazil) was done at public institutions, the possibility exists for the free exchange of information and materials among researchers, reducing overhead costs and benefiting the entire passionfruit production chain.

The most difficult issue to be addressed with passionfruit transgenics is certainly gene flow towards wild *Passiflora* species and especially towards commercial fields of nontransgenic yellow passionfruit. As mentioned in Section 1.1, more than 150 species of *Passiflora* are native to Brazil. Very little is known about the actual possibilities of gene flow among different species, so the actual risk is difficult to be assessed. However, it is quite likely that yellow passionfruit pollen can fertilize the flowers of other species, even if at low efficiency. On this regard, further research is necessary to verify the outcrossing rate of different *Passiflora* species and the fertilization efficiency of yellow passionfruit pollen in those species. Gene flow towards nontransgenic yellow passionfruit will be essentially dependent on the insect pollinator, since pollination by wind is negligible. It is generally assumed that carpenter bees have an effective range of 1–3 km, but there is no data on the effective pollination efficiency as a function of distance.

The potential for gene flow in yellow passionfruit was studied by Torres (2003). An experimental plot with 2640 m² was established, consisting of four rows of plants with 210 m each, 3 m apart. Each row consisted of 23 yellow passionfruit plants, planted at 10 m intervals. At one end of each line, four distinct genotypes were planted, and the remaining of each line was planted with clones from the same plant

of a fifth genotype. Due to self-incompatibility, pollination along each line would only occur with pollen from the plants located at the end of each line, carried by the insect pollinator (a study of pollen dispersal from a single plant by wind was also carried out, and the results indicated that gene flow mediated by wind dispersal of pollen does not take place at distances greater than 3 m). Viable fruit were produced in the plants located at the maximum measured distance of 210 m, indicating that gene flow mediated by the pollinator insect could take place at that distance. A decrease in the percentage of pollination along the lines was observed, however, the trend was not statistically significant using either parametric or nonparametric models. It is necessary to test greater distances, preferably in the km range, in order to determine the minimum distance between fields of transgenic and nontransgenic plants to avoid undesired gene flow.

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Persimmon (Kaki)

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The word “persimmon”, used by the Algonquin Indians of Virginia, indicates at least two fruit tree species, with different history, diffusion, and use. One of them, originated in Asia, is Oriental or Japanese persimmon, Kaki or the “apple of the Orient” (botanically named as *Diospyros kaki* L., *Diospyros kaki* L.f., or more precisely *D. kaki* Thunb.). It is by far the most important *Diospyros* for fruit production. The other, known at present as common persimmon, is *Diospyros virginiana* L. of American origin, a relative of *D. kaki*. It is used as a secondary species for fruit production and also as rootstock for Oriental persimmon in some cultivation areas (Yonemori *et al.*, 2000). This chapter is devoted to *D. kaki* and hereinafter its common name “kaki” will be used all along the chapter.

There are no paleobotanical studies on the history of domestication and diffusion in the past of kaki. It is believed to have a Chinese origin, since its wild types abound and a wide range of variability in many traits such as fruit color, shape, sepal duplication, has been observed in temperate areas of China (Wilson, 1929; Grubov, 1967). Kaki, originated in ancient times, has been cultivated for centuries only within its birthplace and in the neighborhood (Sugiura, 1997). Kaki cultivars

are known in China since the Tang dynasty (Li *et al.*, 1996; Wang *et al.*, 1997a, b) and the most ancient records of descriptions of kaki are in Chinese (Kikuchi, 1948). Early in the 14th century Marco Polo recorded that the Chinese used to trade in kaki (Morton, 1987). Hence, China is considered the primary center of diversity (Zheven and Zhukovsky, 1975). Kaki passed from China to the next temperate areas, namely, Japan in the 7th century and Korea in the 14th century, which can be considered secondary centers of diversification. An ancient record regarding the presence of kaki in Japan is reported in the Honzo-Wamei, the oldest encyclopedia of herbs published in 918 and the first Japanese description of a kaki cultivar, “Zenjimarū”, is dated 1241. Korea has old ceremonies that feature the kaki and many local varieties are known in traditional fruit culture (Kang and Ko, 1997). Kaki trees have been grown in Vietnam, Indonesia and the Philippines since a long time (Morton, 1987). Kaki cultivars were introduced in India by the Europeans only in 1929 (Mehta *et al.*, 2005). Now most of the Asian countries including Nepal, Pakistan, and Thailand cultivate kaki.

Records on the presence of kaki in the Mediterranean basin are few. Pliny the Elder (24–79) in the *Historia Naturalis* wrote that “lotus trees in the Piazza del Tempio and by the Temple of Vulcano, well known for the development of

shoots and for the nice shade they produced” were present in ancient Rome. Kaki is called in Italy and Greece also as “loto”, word used also for *Diospyros lotus* L., a relative of kaki of eastern, central and western Asiatic origin. No records are available regarding the presence of kaki in Europe during Medieval and Renaissance periods. Ricci, a Jesuit Monk who traveled to China, mentioned this species in 1613 (Evreinoff, 1948) and kaki was quoted by Trigault in 1615, nevertheless for De Candolle (1778–1841) both *D. kaki* and *D. lotus* were not yet well known species (Occhialini and Tirocco, 1923). In 1860, kaki trees were imported into France (Occhialini and Tirocco, 1923; Morettini, 1949) and 10 years later its cultivation developed in Provence and other Mediterranean French areas, passing to Italy in 1871–1876 and to Algeria in 1984 (Evreinoff, 1948; Morettini, 1949). The appearance of kaki in Spain dates to the end of the 19th century (Climent and Llácer, 2001), even an earlier introduction could have been possible, taking into account the strong relationships between that kingdom and Oriental countries during the colonization period. In Greece and Portugal, kaki may have been present for a relatively long time (Morettini, 1949). The introduction of kaki in Balkan countries seems to have taken place later, middle of the 20th century. *D. kaki* and *D. lotus* were introduced from Russia, where appeared in 1888 (Evreinoff, 1948), to Turkey (Tuzcu and Seker, 1997). In the 20th century the main areas of cultivation in Europe and in the Mediterranean basin were located in Italy, Spain, Israel, Greece, Portugal, Egypt, France, and various countries of the former Yugoslavia, Cyprus, and Morocco.

In North America, kaki was introduced in 1856, when seeds were sent from Japan by Commodore Perry. In 1870 grafted trees were imported by the US Department of Agriculture and distributed to California and the southern states. In the period 1911–1923, other propagation material of kaki were introduced in the United States and plants were sent also to Arizona, Texas, Louisiana, Mississippi, Georgia, Alabama, Southeastern Virginia, and northern Florida (Morton, 1987). Kaki is also cultivated in Mexico. In South America, kaki was introduced in Brazil from France in 1890 as seeds sent by the naturalist Charles Naudin to São Paulo (Picarelli and Mendes, 1989) and then by Japanese immigrants;

the cultivation spread to many states, such as São Paulo, Rio Grande do Sul, Parana, Rio de Janeiro, Minas Gerais, Ceará, Pernambuco, Piauí, Bahia (Morton, 1987). Kaki is also present in Argentina and Chile. Kaki was introduced into Queensland, Australia, around 1885 and then it passed to New South Wales. In 1873, kaki was introduced into New Zealand from Japan.

Hence, kaki has been gaining in popularity worldwide. It is called “kaki” in Japan and most of European countries, “shi” in China, “gam” in Korea, and “persimmon” in Australia and New Zealand. Sharon fruit in the United Kingdom is a trademark name, exclusively reserved for “Triumph” kaki grown in Israel.

1.2 Botanical Description

About 400 species belong to the genus *Diospyros* in the family Ebenaceae, which consists of the two genera, *Euclea* and *Diospyros*, in the order Ebenales. Most of them are of tropical and subtropical origin (Cronquist, 1981; Ng, 1986), but the most common species of temperate climate are *Diospyros kaki* Thunb., often reported as *D. kaki* L. or *D. kaki* L.f., and the relatives *D. lotus* L., *D. oleifera* Cheng, and *D. rhombifolia* Hemsl. All of them have Asian origin, but *D. virginiana* L. is originated from North America. As for fruit production, *D. kaki*, *D. lotus*, *D. oleifera*, and *D. virginiana*, are important, and there are some minor important species, such as *D. digyna*, *D. discolor*, and *D. texana*. The most important species is undoubtedly kaki, *D. kaki*. *D. lotus*, date plum, was known to the ancient Greeks as “the fruit of the Gods”, that is, *Dios pyros*, hence the scientific name of the genus. Date plum is native to Southwest Asia and Southeast Europe, and used as a fruit in Asia and as a rootstock for kaki in China, Israel, Italy, Japan, and the United States. *D. oleifera* is grown in China mainly as a source of tannin and thought to be a parent of *D. kaki* (Ng, 1978). *D. virginiana*, American persimmon, grows wild but has rarely been cultivated. The name “persimmon” comes from “putchamin”, a phonetic rendering of the name used by the Native Americans. *D. rhombifolia* is a bushy plant of Chinese origin, rarely used as a rootstock for kaki, and used as a bonsai, a potted dwarfed tree, in Japan.

Kaki is a single-stemmed (sometimes multi-trunked) deciduous long-living tree, and tends to become a large tree. For example, a huge old tree growing in the Okayama prefecture in Japan is estimated to be 400 years old, has a trunk circumference of 4.5 m, is 17 m in height, and is grafted onto a seedling stock. The trunk is upright and the bark is dark gray, with many irregular cracks. The branches are generally upright, winding and often fragile. One-year shoots are brown or gray, with many pale lenticels, and they are upright or winding. Flowers and then fruits are brought by twigs. The root is pivot and very deep. Kaki leaves are alternate, simple, ovate or obovate. The size varies with the cultivar from 50 cm² to over 200 cm². The edge is winding and the margin is entire. Kaki leaves are often pale green in youth, turning a dark, glossy green as they grow. In autumn, most of them tinge yellow, orange or red before falling.

The flowers are borne in the leaf axils of new shoots originated from a flowering bud of 1-year-old shoots. Kaki has a complex sex expression, since trees can bear separately or together female (pistillate) flowers, male (staminate) flowers and complete (hermaphrodite) flowers, being classified as polygamous dioic. The commercial cultivars exhibit three types of sex expression, those that bear: (1) only pistillate flowers (pistillate type), (2) both pistillate and staminate flowers (monoecious type), and (3) hermaphroditic flowers in addition to pistillate and staminate flowers (polygamomonoecious type) (Yonemori *et al.*, 1993). Female flowers are solitary, larger than male flowers, with yellow-whitish small petals, borne by a green calyx. Male flowers are borne in clusters formed by three small flowers, the central flower being occasionally hermaphrodite. Fruits derived from hermaphrodite flowers are very small with no commercial value. Pollination is carried out by insects, mostly by honey bees, but fruits can be obtained by parthenocarpy. Parthenocarpic fruits are seedless and are preferred by consumers. However, in practice, pollination is desirable to reduce natural fruit drop and improve fruit quality such as size and shape. Hence, commercial orchards in Japan are planted with pistillate-type cultivars and are interplanted with monoecious types as pollinizers. In Italy and Spain, where soft fruits are consumed and the main cultivated varieties (“Kaki Tipo” and “Rojo Brillante”) are

astrigent and very productive by parthenocarpy, seedless fruits are preferred and pollinizers are not desired. Pollinizers are used in Southern Italy in order to obtain hard edible fruits on pollination of variant nonastrigent types (e.g., “Kaki Tipo”).

The fruit is a big berry showing wide variation in size, from 50 g to over 500 g, shape, from acorn to flat to spherical or square, in skin color, from light yellow-orange to dark orange-red or black purple, and in flavor. The 8 ovary lodges, which can bear from none to one seed in each, can be seen in cross-section. The flesh color varies from orange yellow, and sometimes reddish, to reddish brown or bronze depending on the astringency at harvest time and on presence of seed in the fruit.

The most relevant trait by which kaki cultivars are classified is astringency of fruits at harvest time (Yonemori *et al.*, 2000). All cultivars of kaki produce edible fruits when they are jelly soft, i.e., when they are over-ripe; but at harvest time (when fruits are still firm) kaki can be astrigent (not edible) or nonastrigent (edible) depending on the cultivar. Astrigent and nonastrigent types are divided into two subgroups: variant and constant type, depending on the effect of presence of seeds on flesh color. In variant types flesh color changes to dark when pollination of female flower occurs and seeds are present in the fruit, while in constant types flesh color is not affected by presence of seeds (Hume, 1913, 1914). Hence, kaki cultivars are divided into four groups: pollination constant nonastrigent (PCNA); pollination variant nonastrigent (PVNA); pollination variant astrigent (PVA); and pollination constant astrigent (PCA) (Kajiura, 1946). From the group names, it seems that pollination is important, but it is the seeds, not pollination *per se* that influence the fruits. Changes in fresh color and astringency are related to seed formation rather than pollination. At harvest time, PCNA type loses astringency regardless the presence of seeds, hence parthenocarpic seedless hard fruits can be easily handled, transported and marketed; PVNA cultivars bring edible hard and dark fleshed fruits at harvest only when pollination occurs and there is a high number of seeds. PCA and PVA cultivars have astrigent fruits at harvest time, regardless the presence of seeds: PVA fruits with seeds have a dark flesh only next to the seeds. Hence fruits with or without seeds of PCA and PVA cultivars and parthenocarpic seedless fruits of PVNA cultivars, are astrigent at harvest,

and they become edible after artificial removal of astringency or when fruits are overripe. The difference in the cessation of tannin accumulation in fruit, the tannin characteristics (Sugiura *et al.*, 1979; Yonemori *et al.*, 1983; Yonemori and Matsushima, 1987a, b) and the ability of seeds of producing volatile compounds (Sugiura *et al.*, 1979; Sugiura and Tomana, 1983) are under a new classification suggested by Sugiura (1984). He divided kaki cultivars into volatile independent group (VIG) (corresponding to the PCNA type) and the volatile dependent group (VDG), which includes PCA, PVA, and PVNA types.

Astringency is caused by water-soluble tannins, which are found in large special cells, tannin cells, in the fruit flesh. In pollination variant cultivars, seeds exude acetaldehyde, which causes the soluble tannins to be condensed or coagulated and to become insoluble and oxidized: as a result, many brown specks, denaturalized tannin cells, are formed in the flesh, and the flesh color darkens. The seeds of PVNA type exude so much that the fruit becomes nonastringent, but those of PVA exude so little that the fruit is still astringent and those of PCA did not exude it. These fruits can only be eaten firm after the astringency has been removed artificially, for example, by carbon dioxide gas or ethanol treatment. Tannin cells in PCNA type ceases developing at an early stage of fruit development, while tannin cells of other types continue to develop. PCNA vs. non-PCNA is qualitatively inherited. The trait of PCNA of Japanese origin is recessive to that of non-PCNA. Recently, the trait of PCNA of Chinese origin has been suggested to be dominant over that of non-PCNA (Yamada, 2006).

PCNA pomological group, characterized by cultivars with late ripening time, low productivity by parthenocarpy, high susceptibility to physiological disorders (calyx separation and apex breaking), more exigent in terms of mild Autumns, low affinity to grafting on *D. lotus*, presents a narrow genetic variability in respect to PVNA type: in China and Japan the amount of PCNA cultivars is by far inferior to astringent type (Yonemori *et al.*, 2000, 2005).

In Japan the evolution of PCNA is geographically limited to central Japan (Yamada, 1993) and it appears to be quite recent, being "Gosho" the first PCNA to be documented in the 17th century (Kikuchi, 1948), while in 1214

a record of a PVNA cultivar, "Zenjimaru", was found in Japan. Furthermore, 40 PCNA cultivars collected in Japan were grouped into 17 clusters by means of isozyme marker analysis and most of these cultivars were bud mutations of common ancestors, showing very similar morphological traits (Sugiura *et al.*, 1990). The narrow genetic variability of PCNA group in Japan has been confirmed by means of amplified fragment length polymorphism analysis (Kanzaki *et al.*, 2000) and by progeny tests of PCNA \times PCNA crosses, where lethal genes and inbreeding depression have been often observed (Bellini and Giordani, 1998; Yamada, 2005). The evolution of PCNA cultivars in China seems to be precedent, since "Loutian Tianshi" was cultivated for over 900 years; as in Japan, the known PCNA cultivars and strains (about 8 varieties, among which "Loutian Tianshi") developed in a narrow geographical area in Hubei province (Wang *et al.*, 2005; Yonemori *et al.*, 2005). The PCNA cultivars ("Daeon Dangan" and others) presumed to be native to Korea appear to be Japanese cultivars (Yonemori *et al.*, 2000; Park *et al.*, 2005). All varieties of presumed origin in the Mediterranean Sea area belong to astringent type (Giordani, 2002).

Kaki can be considered a species for warm-temperate areas, even if among cultivars there exist many different behaviors in acclimatization. It is moderately resistant to winter frost (tolerating winter temperatures of -15°C), but sensitive to late spring chill and to strong winds mainly during summer and autumn when the weight of fruits can brake the shoots and the fruits can be injured by rubbing. The best soils to grow kaki are those of medium texture, deep and fresh, but this species can grow also on clayey soils. Subacid and subalkaline soils and active limestone soils are good for kaki, while salty soils and those rich in boron should be avoided.

Wild type *D. kaki* is reported to exist in Chinese forests (Wilson, 1929; Grubov, 1967). Phylogenetic studies on chloroplast DNA between kaki (*D. kaki*) and other temperate and tropical *Diospyros* species, indicated that *D. kaki* seems to have a common ancestor with *D. lotus* and *D. virginiana* (Yonemori *et al.*, 1998) and to be closely related to *D. ehretioides* and to *D. oleifera* as was previously indicated by restriction fragment length polymorphism analysis of mitochondrial DNA by Nakamura and Kobayashi (1994), while

D. glandulosa (= *D. roxburghii*) appears more distant despite the observations of Ng (1978) on the basis of morphological similarity and geographical distribution. Recently, Choi *et al.* (2003) investigated genomic similarity among *D. kaki* and nine closely related to distant *Diospyros* species analyzing repetitive DNAs isolated from *D. kaki*, *D. oleifera* and *D. ehretioides*. Repetitive DNA (restricted with *EcoRV*) was present in five closely related species (*D. kaki*, *D. lotus*, *D. glandulosa*, *D. oleifera*, and *D. virginiana*) while *HindIII*-digested repetitive DNA was also found in those species, except for in *D. virginiana*, suggesting that different ancestral species might have been involved in the speciation of *D. virginiana* and *D. kaki*, as was also indicated by Nakatsuka *et al.* (2002) analyzing Ty1-copia group retrotransposons. In the same work, Choi *et al.* (2003) found a very close relationship between *D. kaki* and three diploid *Diospyros* species (*D. glandulosa*, *D. oleifera* and *D. lotus*), confirming the results obtained by Yonemori *et al.* (2008) from studies on the sequences of the internal transcribed spacer and *matK* regions. Such species could have been involved in the speciation of *D. kaki*, which can be an autohexaploid or autoallohexaploid, as suggested also by the observation of segregation pattern of a molecular marker linked to astringency loss (Kanzaki *et al.*, 2001) and four sets of chromosomes carrying 45s ribosomal DNA (Choi *et al.*, 2003).

The somatic chromosome number of *D. kaki* Thunb. is 90 ($2n = 6x = 90$) (Namikawa and Higashi, 1928). But recently, Zhuang *et al.* (1990, 1992) reported the existence of nonaploid cultivars such as “Hiratanenashi”, “Tone Wase”, “Miyazakitanenashi”, and “Watarizawa”, all of them producing generally seedless fruits. The base chromosome number of genus *Diospyros* is presumed to be $x = 15$, and *D. lotus* and *D. oleifera* are diploid ($2n = 30$), *D. rhombifolia* is tetraploid (Zhuang *et al.*, 1990) and Baldwin and Culp (1941) indicated that *D. virginiana* presents two karyotypes ($2n = 60$ and 90). Ploidy variation was observed in kaki progenies of “Fujiwaragoshō” cultivar (Tao *et al.*, 2003); nonaploid plants were obtained by artificial hybridization of hexaploid cultivar with natural unreduced $2n$ pollen (Sugiura *et al.*, 2000); heteroploidy was observed on embryos obtained from poorly filled seeds of the cultivar “Maekawa Jiro” (Hasegawa *et al.*, 1997).

The genome sizes established by flow cytometry for hexaploid and for nonaploid cultivars of kaki were estimated to be 5.00–5.24 pg/2C and 7.51–8.12 pg/2C, respectively (Tamura *et al.*, 1998b); while nuclear DNA contents of *D. virginiana* and of *D. rhombifolia* were 5.12 and 3.76 pg/2C.

1.3 Economic Importance

Kaki has been mainly grown in China, Japan, and Korea. In China, it is one of the most popular fruit crops, and both of the acreage and production ranked first in the world and 9th in China mainland comparing with other fruits (Luo *et al.*, 2005). In Japan, it was the primary fruit crop for a long time, and even today it ranks 4th in production after satsuma mandarin, apple, and pear (Yamada, 2006). In Korea, the cultivation area in 2004 ranked first comparing with other fruits. However, official data on the area coverage and production of kaki all over the world are not easily available. FAO Statistics indicates a world production in 2005 of 2 561 732 t on an area of 730 592 ha, with a positive trend since 1965 (Figure 1). In FAO statistics only 10 countries are listed as kaki producers: China (1 837 000 t), Korea (250 000 t), Japan (230 000 t), Brazil (67 000 t), Italy (50 000 t), Israel (40 000 t), then New Zealand, Iran, Australia, and Mexico with about 1000 t (Figure 2) (FAO, 2006). A main gap regards Spain, where the kaki industry strongly developed in the last 15 years, production for 2005 is estimated in 43 750 t (M.L. Badenes and G. Llacer, personal communication). Chile produced 1300 t from 146 ha in 2005 (ODEPA, 2006). Other countries producing kaki in the Mediterranean basin are Turkey, Greece, Portugal, Cyprus, Slovenia, Montenegro, Albania, Egypt, Macedonia (FYROM), and Czech Republic (for a total estimated production of 30 000–40 000 t). Other countries interested in the cultivation of kaki are Morocco, South Africa, Thailand, Malaysia, and Argentina. The Californian production in 1965 amounted to 2100 t. In 1970, California produced 1600 t, about 92% of the total US crop (Morton, 1987). World production of kaki is increasing. Kaki international trade is quite limited; the main exporters are Israel, Spain, Italy, New Zealand, Australia, and Korea, while the

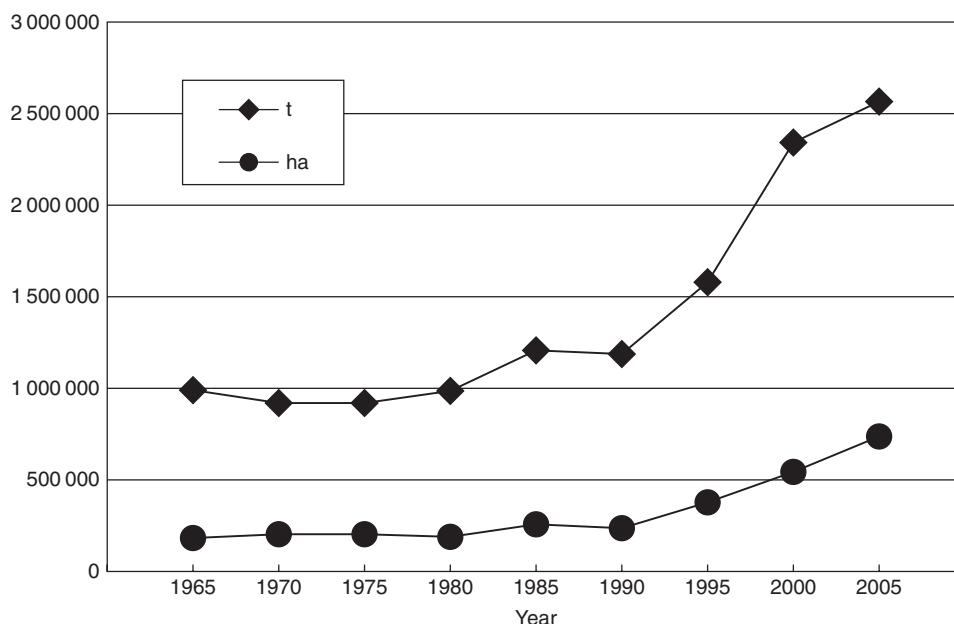


Figure 1 Evolution of kaki production and area coverage (years 1965–2005) (Source: FAO, 2006)

importers are Singapore, Thailand, Hong Kong, Brazil, Germany, Swiss, the United Kingdom, and Belgium; the mean value of exported kaki was US\$1277/t (FAO, 2006).

Kaki is a highly nutritional fruit containing carotenoids, vitamin C, tannins, pectic substances,

and high levels of sugars (Ito, 1971). The sugar content is 14–18 g/100 g fresh weight, fructose and glucose (45% each one on the total) being the most important, while sucrose (generally not present at ripening time), galactose and arabinose are minor components. The total amount of

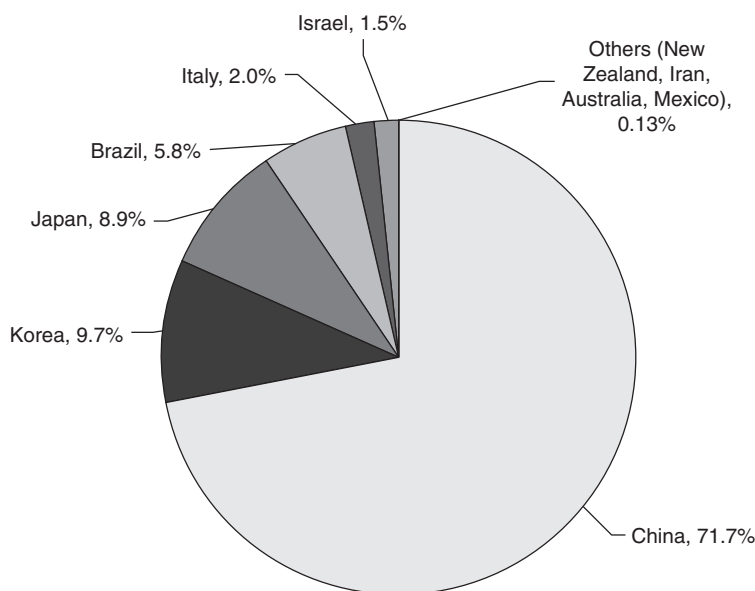


Figure 2 Kaki production (% on the total) by countries in the year 2005 (Source: FAO, 2006)

pectic substances ranges from 0.7% to 1.0% in fresh weight. The amount of soluble tannins ranges from 0% to 4% in fresh weight depending on the cultivar, and of the ripening stage, a leucodelphinidin 3-glucoside named “diospyrin” being the main tannin, while minor components are leucocyanidin, gallic acid, galocatechin, and galocatechin gallate. The total amount of carotenoids ranges between 5 and 6 mg/100 g in fresh weight (10 times higher in the peel): β -cryptoxanthin and lycopene are the major components and β -carotene is also present in small amounts. Kaki is a good source of vitamin C (about 50 mg of ascorbic acid per 100 g of fresh pulp) (Souci *et al.*, 1990). Nineteen amino acids are identified in the flesh, citrulline being the main component. The amino nitrogen is about 15 mg/100 g fresh weight (Testoni, 2002). Kaki is an interesting nutraceutical fruit because of its high amount of carotenoids and polyphenols: β -carotene, lycopene, and β -cryptoxanthin regulate the activity of lipoxygenase in human being, which is responsible for the production of proinflammatory molecules. Tannins have a protective role against free radicals. There have been suggestions that kaki is also beneficial in reducing high blood pressure and has antibacterial effects (Testoni, 2002). Fractioned extracts of kaki peels showed potential antitumor and multidrug resistance reversal activity (Kawase *et al.*, 2003).

Most of kaki production is consumed as fresh fruits (soft or hard) for dessert, but dried fruits are also used mainly in oriental countries and in Europe. Dried fruit is also used for sweetening in various cuisines and confectionary in oriental countries. In Korea, a punch is made from dried kaki. In China and Japan, dried kaki leaves are used for tea. The mushiness and chemical composition (namely, the low content of organic acids) of kaki hinder the industrial preparation of jam. However, fruit can be transformed into juices, mousses, frozen puree for ice cream preparation, alcoholic beverages and its distillates. In Korea, unripe fruit is used for dyeing of garment; in Japan kaki tannin extracted from young fruit has long been used as an antiseptic or water proof for paper used for umbrellas and for fishing fiver nets. Kaki tannin has also been used as a medicine and cosmetic. For traditional Japanese dishes, amber vinegar made from kaki fruit has been used as a flavoring and autumnal red leaves has been used

as a garnish on a dish. Kaki woods are hard and solid enough to be good material for furniture, ornaments, and the head of golf club.

1.4 Traditional Breeding

The perception of quality in kaki for traditional consumers is commonly referred to fruit hardness at consumption, taste, color of skin and of flesh, and presence of seeds, while ripening time can be considered as a marketing requisite. On the other hand, kaki farmers are eager to get a new cultivar with cold, drought, frost, pest, and disease resistances or with higher productivity. In last years, fruit quality has included also nutraceutic characteristics (e.g., vitamin content), healthiness perceived as absence of pesticide (e.g., amount of residues), and environment friendly production (e.g., organic farming, integrated pest management, etc.).

Two types of kaki fruit can be eaten fresh: hard and soft fruits. Hard fruit (i.e., as apple) is produced by PCNA type with or without seeds and by PVNA type with many seeds. Soft and generally seedless fruit, which are consumed overripe, are produced by PCA and PVA with or without seeds and PVNA without seeds. Firm edible fruit can also be obtained by artificial removal of the astringency of non-PCNA types with carbon dioxide or ethanol at harvest, but their eating quality, flesh color and shelf-life are often inferior (Kitagawa and Glucina, 1984; Testoni, 2002) and the work requires much labor, time, and expensive facility for mass production. Hence the evolution of kaki industry and marketing in the last decades, together with handling and transport requirements, and consumer's preference to eat hard fruits, led to consider PCNA cultivars the preferred type (Yonemori *et al.*, 2000; Giordani, 2002).

Hence, the main breeding objective is to obtain PCNA cultivars bringing valuable general characteristics (superior to “Fuyu” and “Jiro”, the leading PCNA cultivars in Japan) such as early ripening time (to broaden market period and to avoid autumn frost injuries during ripening time); high eating quality (high sugar content, juicy and soft melting or crisp flesh); seedless or high and constant productivity by parthenocarp; large fruit with round shape, no physiological disorders

(apex cracking and calyx separation); attractive flesh color (brilliant orange-reddish color); long shelf life and resistance to the most common pests (fruit fly in Mediterranean countries, bark borers, etc.) and diseases; adaptability to different environments (Yonemori *et al.*, 2000; Giordani, 2002; Yamada, 2005). Taking into account kaki propagation, most of the cultivars and rootstocks are recalcitrant to *in vivo* and *in vitro* rooting, hence no self-rooted cultivar or homogeneous rootstock are available. Rootstocks are obtained from seedlings of *D. kaki*, *D. lotus*, and *D. virginiana*, hence their characteristics (such as tree vigor, frost resistance, etc.) are not the same as those of the mother plant. High rooting ability of cultivars and/or rootstocks and cultivar compatibility with *D. lotus*, the main rootstock used in Europe, are also desired characteristics. In Italy and Spain, where “Kaki Tipo” and “Rojo Brillante”, two astringent varieties, are extensively cultivated, a breeding objective is also to obtain high quality, high productive early ripening non-PCNA cultivars (Giordani, 2002). Similar objectives are sought in Korea, where new non-PCNA cultivars with juicy fruits, early ripening, red-ripe soft kaki, suitable for vinegar production and high-quality “Sangjudoongsi” clones are desired (Song *et al.*, 2005). In Japan, a non-PCNA fruit with larger, longer shelf life and better taste is one of the breeding objectives.

The oldest kaki breeding program has been carried on since 1938 in Japan, while other countries involved in kaki breeding were (or still are) China and Korea in Asia, and Brazil, Italy, Spain, Israel, and former Soviet Union (Bellini *et al.*, 1998; Giordani, 2002; Yamada, 2005). The main strategies applied were the selection of superior genotypes within local germplasm (ancestral cultivars and their bud mutations) and intraspecific cross-breeding (recurrent selection and backcross) (Yonemori *et al.*, 2000; Giordani, 2002; Song *et al.*, 2005; Yamada, 2005) and interspecific hybridization (Pasenkov, 1974).

Selection of superior genotypes was carried out mainly in the center of origin (China) and centers of diversification (firstly Japan and Korea, and in more recent times in the United States, Italy, and Spain) (Yonemori *et al.*, 2000). Selection was based on comparative evaluation of morphological characteristics among the collected accessions. In China, 550 genotypes belonging

mostly to the Chinese native germplasm have been collected and evaluated for resistance traits by the National Fruits Germplasm Repository for kaki and 110 accessions, among which Chinese PCNA cultivars are collected in the kaki repository Huazhong Agricultural University (Luo *et al.*, 2005). In Japan, 263 local accessions are listed and widely described by the National Institute of Agrobiological Sciences (NIAS) GenBank (NIAS, 2006) and about 600 genotypes are collected at NIFTS, Akitsu, Hiroshima (Yamada, 2005). In Korea, 233 local cultivars were collected during 1959–1969 at the Experimental Station at Kim-hae (Yonemori *et al.*, 2000); many local varieties from different areas have been morphologically described and a recent survey has been carried out at Jeju Island (Park *et al.*, 2005). By the USDA-ARS National Clonal Repository, few *D. kaki* of Japanese origin are collected together with *D. lotus* and *D. virginiana* accessions (USDA, 2006). Many of the accessions collected in Asia have been analyzed by molecular markers in order to assess distinctness and to study genetic relationships (Yonemori *et al.*, 2000). In Europe, 161 genotypes were collected by national or regional repositories of Italy and Spain, including accessions of presumed local origin, among which “Kaki Tipo” and other PVNA varieties, and “Rojo Brillante”, “Cristalino”, “Picudo”, “Tomatero”, “Xato del Bon Repòs” among others in Spain (Giordani, 2002). Most of the accessions belonging to the European collections have been morphologically described and genetically characterized for distinctness by means of random amplified polymorphic DNA markers (Bellini, 1982; Badenes *et al.*, 2003; Bellini *et al.*, 2003). Other European countries that are developing kaki collections are Romania (11 accessions) and the Czech Republic (28 collected accessions) (Bellini and Giordani, 2005). Turkey holds several collections for a total of 74 accessions (Tuzcu and Seker, 1997).

The problem of cultivar distinctness and identification, far to be solved, did not hinder the activity of selection in collected accessions of kaki taking into account morphological characteristics. The main achievements obtained by preferential selection of superior genotypes within native germplasm and their bud mutation (namely, in the center of origin and in the close diversification areas) are reported in Table 1 together with

Table 1 Superior cultivars obtained by selection from local germplasm and spontaneous bud mutations

Cultivar name	Type	Country	Genetic origin and/or characteristics
Anguyoushi	PCA	China	Big fruit, juicy
Dahongshi	PCA	China	Juicy
Tone Wase, Spur-hiratanenashi, Sugita Wase, Oh-tanenashi, Kohshimaru	PVA	Japan	Bud mutations of “Hiratanenashi”, with different improving characteristics (earlier ripening time, bigger fruits, higher productivity)
Aichi Wase Fuyu, Matsumoto Wase Fuyu, Uenishi Wase, Tanba Wase Fuyu	PCNA	Japan	Bud mutations of “Fuyu”, with different improving characteristics (earlier ripening time, better taste)
Maekawa Jiro, Ichikikei Jiro, Aisyuhou	PCNA	Japan	Bud mutations from “Jiro”, earlier ripening time, better fruit skin color
Kaki Tipo	PVNA	Italy	Uncertain origin, perhaps “Amahyakume” from Japan; big fruit, high eating quality, high productivity, medium late-ripening time
Mercatelli, Moro, Vainiglia	PVNA	Italy	Uncertain origin, perhaps “Zenjimarū” from Japan; male flowers, medium fruit size, good taste; very high parthenocarpic productivity
Rojo Brillante	PVNA PVA (?)	Spain	Mutation of “Cristalino”; big fruits, high flesh quality; attractive peel and shape, very high parthenocarpic productivity

results obtained from presumed local germplasm of European countries, Italy and Spain. Most of the reported varieties are widely cultivated in the countries of origin and abroad (namely, the Japanese PCNA cultivars, “Fuyu” and “Jiro”), nevertheless the variability that they exhibit is not enough to ensure a sound development of kaki industry and to improve many undesirable traits (such as late ripening time, susceptibility to physiological disorders, astringency at harvest time, etc.) borne by these cultivars (Bellini *et al.*, 1998; Yonemori *et al.*, 2000; Giordani, 2002; Yamada, 2005).

Cross-breeding is the main strategy adopted in recent times to obtain high value cultivars. Breeding programs started in 1938 in Japan, and in the second half of 20th century in Korea, Brazil, Italy, Israel and former Soviet Union, while in 2005 a Spanish breeding program started at Valencia (Bellini *et al.*, 1998; Yonemori *et al.*, 2000; Song *et al.*, 2005; Yamada, 2005; M.L. Badenes and G. Llacer, personal communication).

Crossing protocols are quite simple, since pistillate flowers are big and receptive, pollination is easy (often the small staminate flowers are just rubbed on the pistil of female flowers), fruit set ratio is high and up to eight seeds can be obtained per fruit; after stratification, germination rate of seeds is generally high. Taking into account other temperate fruit tree species, showing a long juvenile

unproductive phase, kaki seedlings bear flowers 3–5 years after germination (Bellini *et al.*, 1993). Furthermore, molecular marker-assisted selection has been developed for the trait non-PCNA (Kanzaki *et al.*, 2000, 2001) and it will be used for early selection and grafting of PCNA seedling for promoting early fruiting (Yamada, 2005).

The main results achieved in terms of released cultivars are reported in Table 2. At present, none of the new kaki cultivars obtained by cross-breeding has been widely cultivated. Only few of them represent a small percentage of the whole production, for example “Taishu” in Japan (100 ha in 2003) (Yamada, 2006), and varietal assortment of kaki appears to be rather static in comparison with other fruit tree species (e.g., apple, peach and citrus) where outstanding new released cultivars are immediately grown and they supplant old fashioned cultivars (Yonemori *et al.*, 2000; Giordani, 2002; Yamada, 2005).

Progeny tests carried out in the last 50 years in Japan improved knowledge about inheritance in kaki (Yamada, 2005). Among them, the hereditary behavior of loss of astringency at harvest time is outstanding, given its relevance in cross-breeding.

The studies of Ikeda *et al.* (1985) demonstrated that the trait regulating astringency at harvest is qualitatively inherited, PCNA being recessive to non-PCNA. PCNA F₁ progenies can be obtained only crossing PCNA types, since ancestral

Table 2 Kaki cultivars obtained by cross-breeding release

Cultivar name	Country	Genetic origin	Type	Year of release	Main characteristics
Kaoru	Brazil	—	PVA	—	Big fruit
Suruga	Japan	Hana-gosho × Oku-gosho	PCNA	1959	Big fruit, late ripening, fruit cracking, not juicy
Nishimura Wase	Japan	Fuyu × Akagaki (very improbable)	PVNA	1960	Big fruit, male flowers, early ripening
Izu	Japan	Fuyu × A4 (Oku-gosho × Oku-gosho)	PCNA	1970	Good taste and color; low productivity, weak tree, early ripening
Rossiyanaka	Former Soviet Union	<i>D. kaki</i> × <i>D. virginiana</i>	Astringent	1974	Small fruits, astringent flesh, resistance to frost
Tavrichanka	Former Soviet Union	<i>D. kaki</i> × <i>D. virginiana</i>	Astringent	1974	Small fruits, astringent flesh, resistance to frost
Zvezdochka	Former Soviet Union	<i>D. kaki</i> × <i>D. virginiana</i>	Astringent	1974	Small fruits, astringent flesh, resistance to frost
Fuyu Hana	Brazil	Fuyu × Hana-gosho	PCNA	1984	Large fruits, male flowers, seeded fruits
Pomelo	Brazil	—	PCA	1985	Big fruit, early ripening time
Rubi	Brazil	—	PCA	1985	High eating quality
Shinshuu	Japan	Okitsu-20 (Fukuro-gosho × Hana-gosho) × Okitsu-1 (Oku-gosho × Oku-gosho)	PCNA	1990	Medium-early ripening, high sugar content, susceptible to skin damage
Youhou	Japan	Fuyu × Jiro	PCNA	1990	Intermediate ripening, high parthenocarpic productivity
Taishuu	Japan	Fuyu × Ili G-16 (Jiro × Okitsu-15)	PCNA	1994	Big fruits, high quality
Yubeni	Japan	Matsumoto Wase-Fuyu × F2 (Jiro × Oku-gosho)	PCNA	1997	Attractive skin, late ripening
Tanrei and Kinshuu	Japan	Okitsu-2 (Fuyu × Oku-gosho) × Okitsu-15 (Oku-gosho × Hana-gosho)	PCNA	1998	Ornamental use
Soshu	Japan	Izu × 109-27 (Okitsu-2 × Okitsu-17)	PCNA	2000	Medium-large size and juicy fruits, early ripening
Kanshu	Japan	Shinshu × 18-4 (Fuyu × Okitsu-16) (Oku-gosho × Hana-gosho)	PCNA	2002	Fruit sweetness, early ripeness, no fruit cracking habits, excellent eating quality
Kishu	Japan	Izu × Akitsu-5 (Fuyu × Akitsu-16)	PCNA	2003	Big fruit, no apex cracking

non-PCNA cultivars native of Japan are homozygous dominant: natural intercrosses between PCNA and non-PCNA types were very limited in the evolution of cultivars in Japan, given the recent origin and the narrow area of diffusion of PCNA type. No F₁ PCNA offspring results in from crossing PCNAs with non-PCNAs, including “Nishimura Wase”, which had been thought to be obtained from “Fuyu” (PCNA) × “Akagaki” (non-PCNA) (Yamada and Sato, 2002). Backcrosses between PCNA × (non-PCNA × PCNA) yields around 15% and 20% of PCNA offspring (Ikeda *et al.*, 1985; Yamada and Sato, 2002).

The segregation pattern between PCNA Japanese varieties and the PCNA Chinese cultivar, “Loutian Tianshi”, resulted different, since unexpectedly non-PCNA and PCNA offsprings were obtained in a 1:1 ratio (Ikegami *et al.*, 2004). Further studies indicate that PCNA progenies are obtained also by crossing “Loutian Tianshi” with non-PCNA cultivars and that the PCNA trait of “Loutian Tianshi” is heterozygous and dominant on non-PCNA, this cultivar becoming a relevant tools for obtaining PCNA offspring (Ikegami *et al.*, 2006). Taking into account the hexaploidy of *D. kaki* and the different pathways of evolution of PCNA types

in Japan and China, confirmed also by molecular markers (Luo *et al.*, 1999; Kanzaki *et al.*, 2000), further studies on allele dosage should be carried on to clearly understand inheritance of this relevant trait.

Fruit cracking at the calyx and/or stylar ends, appearing in PCNA Japanese cultivars, and mostly when pollinated, are independent traits; they are quantitatively inherited, noncracking cultivars being homozygous and the others heterozygous (Yamada *et al.*, 1988). Hence, noncracking \times noncracking cultivars give no or little noncracking cultivars, while offsprings from highly cracking cultivars are more susceptible to these disorders: this is recurrent since quite all PCNA cultivars are cracking (Yamada *et al.*, 1988). Degree of cracking fluctuates depending strongly on the year, hence selection of noncracking genotypes is long and time spending (Yamada *et al.*, 1987c).

High sugar content (expressed as soluble solids content (SSC)) is a relevant objective in kaki breeding. Yamada *et al.* (1986, 1993, 1994a) indicated SSC trait to be quantitatively inherited and to strongly depend on environmental factors, to exhibit a low broad-sense heritability (0.47–0.69). Furthermore, estimates of variance components in the offspring populations show that choice of parent has only a small effect on the progeny for SCC (Yamada *et al.*, 1997).

Ripening time is quantitatively inherited. A study by Yamada and coworkers indicates that broad-sense heritability for this trait ranges between 0.84 and 0.92 in a population of 19 PCNA cultivars and selections (Yamada *et al.*, 1993). The coefficient of regression of mean values in a full-sib family on mid parent value was 0.99 and the values of the obtained progenies fitted with those predicted (Yamada *et al.*, 1995; Yamada and Yamane, 1997).

Fruit weight shows a high broad-sense heritability (0.85) (Yamada *et al.*, 1993). Studies carried on 39 families during 1982–1985 analyzing estimates of variance within and between progenies, showed that the expected proportion of offspring with large fruits decreased as mid-parental value decreased and inbreeding coefficient increased. It was also observed that the mean value of fruit weight of offspring in a full-sib family and the expected proportion of F_1 seedlings with higher weight could be predicted (Yamada *et al.*, 1994b; Yamada and Yamane, 1997).

Sex expression is genetically determined but pistillate cultivars can bear male flowers on rare occasions (Kajiura and Blumenfeld, 1989; Yonemori *et al.*, 2000) and sex conversion, staminate towards pistillate flowers, can be induced by applications of benzylamino purine on flower primordia (Yonemori *et al.*, 1990, 1993). Even if the presence of staminate flowers is not a breeding objective, since seedless fruits are mostly preferred, genotypes with male flowers are relevant mostly for cross-breeding. This trait seems to be quantitatively inherited: progenies obtained by crossing genotypes from high to low percentage of staminate flowers, showed a gradual reduction in the percentage of individuals with staminate flowers (Oohata *et al.*, 1964; Bellini and Giordani, 1992).

The inheritance of parthenocarpy is still unknown. Ability of parthenocarpy presents a wide variability among cultivars. Yamada *et al.* (1987b) indicated that the ability of parthenocarpic production, as percentage of fruit drop after bagging flowers to avoid pollination, fluctuates markedly, the year effect being stronger than cultivar–year interaction. On the contrary, ability of seed formation, also depending on the cultivar, shows a lower fluctuation (Yamada *et al.*, 1987b).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional breeding of kaki has not yielded as good results as those of other fruit species, such as peach, apple, and citrus, cross-breeding program of which actively progressed worldwide. The main results achieved by cross-breeding in most of cases have no or little impact on the varietal assortment of kaki, ancestral cultivars being selected from widely cultivated native germplasm (Giordani, 2002).

In general terms, conventional breeding of kaki is hindered by the high ploidy level of *D. kaki*, which is hexaploid or nonaploid, and by its complex sex expression. Monoic cultivars are few, and most of the valuable cultivars are of pistillate type. Obstacles to achieve relevant results depend on the breeding objectives, namely when PCNA cultivars are sought.

As regards PCNA cultivars, Japanese breeding programs have had to face, for the main traits

to be improved, relevant troubles linked mainly to the narrow genetic pool of the PCNA group (Yonemori *et al.*, 2000). The main objectives were to obtain new PCNA cultivars with the following characteristics: early ripening time, large fruit size, high sugar content, high parthenocarpic productivity, limited physiological disorders (apex and styler cracking) (Yamada, 2005). Depression by inbreeding appeared on progenies showing small and susceptible to fruit cracking, and weak and low productive trees, since PCNA progenies were obtained by crossing PCNA \times PCNA, the number of PCNA parents being limited, and very few PCNA cultivars bear staminate flowers (Bellini *et al.*, 1993, 1998; Yonemori *et al.*, 2000; Yamada, 2005). On the other hand, to obtain different PCNA genotypes by crossing PCNA Japanese cultivars \times non-PCNA cultivars, the proportion of PCNA offspring resulted by back crossing F_1 (PCNA \times non-PCNA) with PCNA is very low, given the high level of ploidy of *D. kaki* and the dominance of astringency trait in Japanese native cultivars (Bellini *et al.*, 1998; Yonemori *et al.*, 2000; Giordani, 2002; Yamada, 2005). More efficient results in order to obtain PCNA progenies are expected by using "Loutian Tianshi", since the PCNA progenies are obtained in F_1 by crossing it with any astringent type (Ikegami *et al.*, 2006), although the fruit is small (69 g, against 200–250 g required by the market). The low heritability of sugar content and the yearly fluctuation of susceptibility to fruit cracking hindered cross-breeding for PCNA cultivars.

Problems also occur when the objective is obtaining new non-PCNA astringent varieties with an aim to improve those already widely used. No superior genotypes has been selected among the over 4000 seedlings obtained from different crosses and evaluated in Italy since 1970 (Bellini *et al.*, 1998; Giordani, 2002). Other critical aspects of conventional breeding are associated with the lack of known sources of resistance to the main pests and diseases affecting kaki and of other important traits for kaki industry, such as rooting ability and dwarfism.

During the past 60 years, as mentioned above, several new cultivars have been released from the kaki cross-breeding programs of the Fruit Tree Research Station in Japan (Yamada, 1993). However, rapid breeding progress through conventional cross-breeding is difficult because, as with other

woody plants, kaki has a long juvenile period and large plant size (Tao and Dandekar, 1999). Furthermore, kaki is a polyploid (Tamura *et al.*, 1998b), which makes it difficult to predict segregation of characters. Genetic transformation allows to make small specific changes in the genome of the existing excellent cultivars. Introduction of genes, which produce antibacterial substance and toxin for pests, add substance giving tolerance to environmental stresses, change metabolism of sugar and pigment of fruit, increase nutraceutical substance in fruit, prolong shelf life, improve rooting of cuttings, and make trees dwarf, into the genome of kaki would be expected. Transgenic kaki cultivars thus produced have potential for commercial success and grower acceptance because the genetic constitution of the cultivar was not altered (Dandekar, 1994). Moreover, biotechnology for kaki has intensively developed recently, including micropropagation from shoot tips (Sugiura *et al.*, 1986; Tao and Sugiura, 1992a), organogenesis from callus (Tao *et al.*, 1988; Tamura *et al.*, 1992; Tao and Sugiura, 1992b; Benelli *et al.*, 1999) and protoplasts (Tao *et al.*, 1991; Tamura *et al.*, 1995a), and somatic hybridization (Tamura *et al.*, 1995b, 1998a). Although these techniques are also powerful tools for breeding, the development of biotechnology makes it easy to create genetically modified kaki. Many countries have not approved growing genetically modified crops yet, partly because there is fear that their progeny will naturally spread through their pollens and seeds and damage the realm of nature. If the genetic transformation is applied to seedless and pistillate-type cultivars or rootstocks, the risk will be minimized.

2. DEVELOPMENT OF TRANSGENIC KAKI

2.1 Donor Gene and Transgene

Initially, in the development of genetically transformed kaki, *Agrobacterium* wild strains were used to determine which strains have virulence on kaki. Five wild-type strains of *A. tumefaciens* were used, and inoculation with three strains, A281, C58, and K12, induced tumor formation on kaki shoots *in vitro* (Tao *et al.*, 1995a). These strains successfully introduced transfer-DNA (T-DNA) of their Ti plasmid to kaki. This was evident because most calli from the tumor cell lines



Figure 3 A “Nishimura Wase” kaki shoot with crown gall tumor at the wound site where *A. rhizogenes* was inoculated (Tao *et al.*, 1994)

produced opines and showed hormone-induced autotrophic growth (Tao *et al.*, 1995a). Four wild-type strains of *A. rhizogenes* were also used for inoculation on the shoots and they successfully induced the formation of tumors (Figure 3), rather than roots (Tao *et al.*, 1994; Tamura, 1997).

The first synthetic gene integrated into the kaki genome was the *cryIA(c)* gene (Tao *et al.*, 1997). The T-DNA region of a binary plasmid vector pDU92.710 harbored in a disarmed strain of *A. tumefaciens* EHA101 contained the kanamycin resistance gene (*nptII*), the β -glucuronidase (GUS) gene, and a synthetic reconstruct of *cryIA(c)* encoding the insecticidal crystal protein fragment of *Bacillus thuringiensis* subsp. *kurstaki*.

Nakamura *et al.* (1998) used three types of binary vectors, pBI121 (Jefferson *et al.*, 1987), pTOK233 (Hiei *et al.*, 1994), and pSMAC251 (Yamashita *et al.*, 1995), to improve transformation efficiency in kaki. However, only the last one, which contained 35S promoter-*nptII* and a nopaline synthase (*nos*) promoter-*gus* and was harbored in EHA101, was successfully introduced into the kaki genome. The other vectors harbored in LBA4404 (Hoekema *et al.*, 1983) were not introduced.

The *codA* gene of *Arthrobacter globiformis*, encoding choline oxidase, was contained in the binary vector pGC95.091 (Gao *et al.*, 2000),

which was generated from pDU92.3103 (Tao *et al.*, 1995b). This vector also contains the *nptII* gene and the GUS gene (*gusA*) with the 35S promoter. Choline oxidase catalyzes the oxidation of choline to glycinebetaine (Deshrieum *et al.*, 1995), one of the water-soluble compounds of low molecular weight that are referred to as compatible solutes; it allows them to tolerate certain types of environmental stress (Hellebust, 1976). The binary vector pRS95.101, which contained only the *gusA* and *nptII* genes in the T-DNA region, was used for control transformations (Gao *et al.*, 2000).

To endow kaki with the ability to synthesize sorbitol, a sugar alcohol that may serve as an osmoprotectant in the Rosaceae, including apple, the binary plasmid vector pDU93.0305 (Tao *et al.*, 1995b) was used (Gao *et al.*, 2001). The T-DNA region of this vector contained the *nptII* gene, the *gusA* gene, and a chimeric sorbitol-6-phosphate dehydrogenase (*S6PDH*) gene that was comprised of apple complementary DNA for S6PDH and cauliflower mosaic virus regulatory sequences. Similarly, tobacco (*Nicotiana tabacum* L.), which normally does not produce sorbitol, has been engineered to produce sorbitol by expression of the transgene *S6PDH* using the 35S promoter (Tao *et al.*, 1995b).

The *rolC* gene from *A. rhizogenes* induces dwarfism (Kaneyoshi and Kobayashi, 1999). Therefore, the binary vector pSMAC251-*rolC*, which was developed from pSMAC251 (Yamashita *et al.*, 1995), containing the *rolC* and *nptII* genes was used to produce dwarfed rootstock (Koshita *et al.*, 2002). The promoters were 35S for the *rolC* gene and *nos* for the *nptII* gene.

The polygalacturonase inhibiting proteins (PGIPs) are thought to be a component of plant pathogen defense (Albersheim and Anderson, 1971). *Botrytis cinerea* lesion growth was reduced on the leaves and fruits of transgenic tomato plants expressing the pear fruit PGIP (Powell *et al.*, 2000). Both plasmid pDU94.0928, carrying chimeric genes of *PGIP* cloned from pear fruit (Stotz *et al.*, 1993), *gusA*, and *nptII*, and plasmid pYS95.091 were constructed from a base binary vector (pCGN1559; McBride and Summerfelt, 1990) for a study of the enhancement of disease resistance by transformation of kaki (Tamura *et al.*, 2004). Plasmid pYS95.091 had the same construction as pDU94.0928, but without the chimeric *PGIP* gene.

2.2 Transformation Methods

All transformation experiments of kaki have been carried out using *Agrobacterium* because the system is well developed. The first synthetic gene transfer into the kaki genome was conducted using EHA101 (Hood *et al.*, 1986) because the wild type strain of EHA101, A282, is very virulent on kaki (Tao *et al.*, 1995a). Nakamura *et al.* (1998) found that EHA101 was a more suitable strain for transformation than LBA4044. Hence, EHA101 has been used in most kaki transformation works using *Agrobacterium*.

Leaf discs were used as one of the plant materials (Tao *et al.*, 1997; Gao *et al.*, 2000, 2001; Tamura *et al.*, 2004). Leaf discs of 8 mm in diameter were collected from *in vitro* leaves, precultured on MS (1/2N) medium containing zeatin and α -naphthalene acetic acid for 3 days at 28 °C in darkness, inoculated by immersion for 15 min in low-pH (5.2) virulence-induction medium (James *et al.*, 1993), and then cultivated with *Agrobacterium* for 3 days at 28 °C in darkness. After co-cultivation, the leaf discs were transferred to fresh aliquots of the same medium with an antibiotic to kill the bacterial cells. After 3 days, leaf discs were transferred to a selection medium with kanamycin. Spent medium was replaced by fresh medium every 2 weeks. During selection, calli that had formed at the cut surface of the leaf discs were collected to induce the formation of transformed shoots (Figure 4). The frequency of callus formation varied by study: 2% (Tao *et al.*, 1997), 12–14% (Tamura *et al.*, 2004), and

>20% (Gao *et al.*, 2000). Tamura *et al.* (2004) conducted fluorometric GUS assays on calli to select those that were transformed. They found that the transformation rates with the *gusA* gene of pDU94.0928 and pYS95.091 were 9.1% and 7.7%, respectively.

Hypocotyl segments prepared from embryos in seeds were used as the other plant material (Nakamura *et al.*, 1998; Koshita *et al.*, 2002). The hypocotyl segments were immersed in the bacterial suspension for 15 min, and thereafter placed on co-culture medium consisting of MS (1/2N) medium containing acetosyringone and zeatin. After co-cultivation for 3 days at 28 °C in darkness, segments were transferred to a selection medium with kanamycin. The cultures were maintained at about 26 °C under a 16 h photoperiod and were subcultured every month to induce the formation of adventitious shoots at the cut end of each segment.

Addition of acetosyringone to the co-culture medium was used to increase the virulence of *Agrobacterium* (Nakamura *et al.*, 1998). Cefotaxime effectively removed *Agrobacterium* after inoculation (Tao *et al.*, 1997; Nakamura *et al.*, 1998), but carbenicillin sulfate inhibited bud formation on hypocotyl segments (Nakamura *et al.*, 1998).

To our knowledge, except gene gun (Benelli *et al.*, 1999), there have been no reports of successful transformation of kaki by other methods including electroporation, microinjection, or polyethylene glycol. These methods are worth investigating because plant regeneration systems

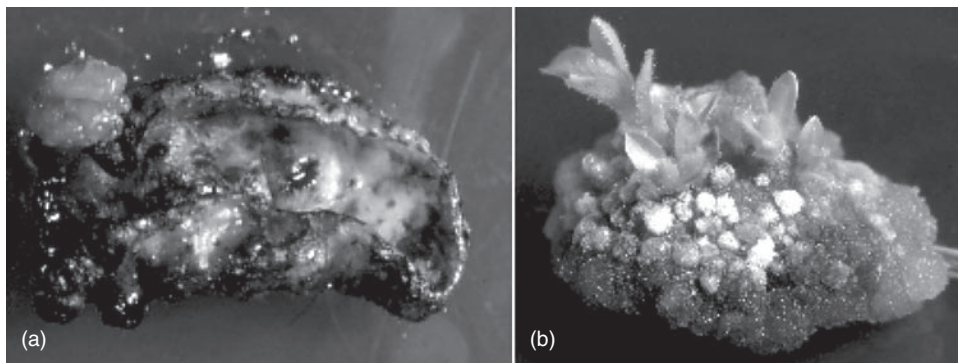


Figure 4 Regeneration of transformed kaki cv. Jiro. (a) a putative transformed callus formed on the inoculated leaf disc cultured with kanamycin; (b) adventitious bud formation from the transformed callus (Tao *et al.*, 1997)

from shoot tips (Sugiura *et al.*, 1986; Tao and Sugiura, 1992a), leaves (Tao *et al.*, 1988), roots (Tetsumura and Yukinaga, 1996), calli (Tao *et al.*, 1988; Tao and Sugiura, 1992b; Tamura *et al.*, 1992; Benelli, *et al.*, 1999), hypocotyls (Yamada *et al.*, 1987a), and protoplasts (Tao *et al.*, 1991; Tamura *et al.*, 1995a) of kaki are well developed.

2.3 Selection of Transformed Tissue

Kanamycin has been used exclusively for the selection of transformed tissue in kaki because plasmid T-DNA regions used in all transformations of kaki have contained the *nptII* gene. All of the control untransformed calli cultured on medium without kanamycin formed adventitious buds, whereas only 20% of the control calli formed buds on medium with kanamycin, and no further growth of the adventitious buds induced from the control calli was observed on shoots in medium with kanamycin (Tao *et al.*, 1997). Although it is possible that other antibiotic-resistant and herbicide-resistant genes will be used as selection makers, kanamycin is a good tool for the selection of transformed tissue, and its application has been improved (Nakamura *et al.*, 1998).

An assay for GUS activity was also conducted to confirm putative transformed lines in all studies. Leaves and leaf discs from the shoots were used in GUS assays and they performed well (Tao *et al.*, 1997; Nakamura *et al.*, 1998). Callus was also used for GUS assays in some cases (Gao *et al.*, 2000; Tamura *et al.*, 2004).

2.4 Regeneration of Whole Plant

Of the 17 kanamycin-resistant calli formed on leaf discs ($n = 720$) after infection by *Agrobacterium*, 15 formed adventitious buds on the regeneration medium (Tao *et al.*, 1997). The frequency reported by Gao *et al.* (2000) was also high (90–100%). These frequencies were comparable to those of the untransformed calli of the same cultivar, “Jiro” (Tamura *et al.*, 1992). However, the regeneration rates were considerably lower for transformed lines than for untransformed calli when the *PGIP* gene was introduced; furthermore, the control transformed line without the *PGIP* gene showed a comparably low regeneration rate compared to the line with the gene (Tamura *et al.*, 2004).

The frequency of bud formation on hypocotyl segments after infection by *Agrobacterium* was 14.9–27.0%. However, the shoots regenerated from the segments directly, not through callus, so transformants were obtained within a relatively short time (Nakamura *et al.*, 1998).

The frequency of transformed GUS(+) shoot formation differentiated from the callus formed on leaf discs was 1.3% (Tao *et al.*, 1997), whereas that from the hypocotyl segments was 11.1% (Nakamura *et al.*, 1998). Clearly, the regeneration ability of the plant material was responsible for the difference in frequency (Nakamura *et al.*, 1998). However, Gao *et al.* (2000) increased the frequency of transformed GUS(+) shoot formation from the callus on leaf discs by modifying the concentration of zeatin during the initial period of transformation. Under those conditions, 18% and 10% of the leaf discs produced transformed shoots by pGC95.091 and pRS95.101, respectively.

A method for the rooting of microcuttings has been established (Tao and Sugiura, 1992a), allowing the development of whole plantlets. When necessary for further investigation, plantlets were obtained from transformed shoots, except for “Fuyu” shoots with introduced Ri T-DNA (Tao *et al.*, 1994) and one “Jiro” shoot line with the *PGIP* gene (Tamura *et al.*, 2004). The rooting ability of transformed shoots varied with the gene introduced; for example, rooting was high in *rolC* (Koshita *et al.*, 2002) and low in Ri T-DNA (Tao *et al.*, 1994) and *PGIP* (Tamura *et al.*, 2004). Because transformed lines, including a control transformed line, had lower rooting rates, Tamura *et al.* (2004) suggested that rooting is affected by the overall transformation process, as well as the regeneration rate from callus.

2.5 Activity and Stability of Gene Inheritance, and Adverse Growth Effects

Tao *et al.* (1997) used kaki plants integrated with synthetic *cryIA(c)*, which produces a 60-kDa insecticidal crystal protein fragment (ICPF; Adang *et al.*, 1985) for screening of insect resistance using the Indian meal moth (IMM), *Plodia interpunctella*. This moth is a pest of stored products worldwide, and it is quite susceptible to ICPF (Vail *et al.*, 1991). The lines producing

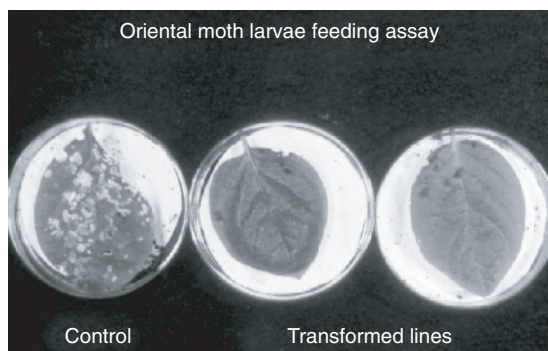


Figure 5 Appearance of the leaves from the transformed kaki with the *cyrIA(c)* gene of *Bacillus thuringiensis* and the control nontransformed plants after 7 days of feeding with oriental moth larvae (Tao *et al.*, 2001)

CryIA(c) protein showed highly significant mortality of IMM when compared to untransformed controls and one transformed line, which did not produce detectable CryIA(c) protein. Using the two transformed lines that showed the highest mortality, they further evaluated insect resistance because IMM is not a pest of kaki. A natural insect pest of kaki, the oriental moth (OM) larvae, *Monema flavescentis*, was used for bioassays, and the lines producing CryIA(c) protein caused significantly higher mortality of OM larvae than those that did not produce the protein (Figure 5). The field test trees were grown at the University of California at Davis (Figure 6).

Of the eight transformed lines producing choline oxidase by the *codA* gene, two lines (PCs) producing high levels of glycinebetaine (0.16



Figure 6 The transformed kaki trees growing at the University of California at Davis

and $0.28 \mu\text{M g}^{-1}$ fresh weight) and the control transformed line (PR) lacking glycinebetaine (Gao *et al.*, 2000) were selected for evaluation of tolerance to salt stress. Transgenic plants showed normal growth, resembling wild type plants both *in vitro* and *ex vitro*. Although the activity of photosystem II in the PCs and PR declined under salt stress, the rate of decline was lower in the PCs than in the PR. This result demonstrated that genetic engineering of kaki for the accumulation of glycinebetaine enhanced its tolerance to salt stress. This has also been accomplished in *Arabidopsis* (Hayashi *et al.*, 1997; Alia *et al.*, 1998) and rice (Sakamoto *et al.*, 1998).

Other salt-tolerant transformants producing sorbitol by the chimeric S6PDH gene were used in the evaluation, including two transformed lines producing high (PS1) and medium (PS6) amounts of sorbitol, one control transformed line (PS7) with no ability to synthesize sorbitol, and an untransformed type ("Jiro"; Gao *et al.*, 2001). As a result of the same salt stress evaluation system tested, PS1 appeared to be more tolerant to salt stress than the other three lines. Moreover, PS1 had reduced stature with shorter internodes and more lateral growth with more branching compared to the other lines, although it grew normally *in vitro* and during the early stage of *ex vitro* growth. Enhanced tolerance to salts and slower growth in transgenic tobacco plants with the ability to produce mannitol, another compatible solute, has also been observed (Karakas *et al.*, 1997). Hence, further investigation of induced dwarfism (Deguchi *et al.*, 2004) in these lines was conducted. Given the fact that glucose 6-phosphate, indole-3-acetic acid, and gibberellin levels did not affect dwarfism in PS1 in comparison with PS7, the control, and the wild type, Deguchi *et al.* (2004) deduced that growth inhibition of PS1 was likely caused by an osmotic imbalance between the cytosol and vacuole owing to high sorbitol accumulation in the plants.

Heights and internode lengths of the transformed kaki with *rolC* were shorter than those of the untransformed plants, and the leaves of transformants were smaller (Koshita *et al.*, 2002), which has also been observed in pear (Bell *et al.*, 1999). Furthermore, a higher rooting rate of *in vitro* shoots was also observed, and this ability, along with dwarfism, is a horticulturally preferred characteristic. Dwarfism should be confirmed by

investigation of the growth of scions grafted on the transformants (Koshita *et al.*, 2002).

Following Taylor and Secor (1988), PGIP activity in shoots transformed with EHA101/pDU94.0928 and EHA101/pYS95.091 was analyzed using a radial diffusion assay (Tamura *et al.*, 2004). Fungal polygalacturonase (PG) activity was inhibited by crude extracts from shoots transformed with the *PGIP* gene. The inhibition was substantially stronger than that in the control transformants obtained from the transformation with pYS95.091 and in the untransformed plants. The fact that the controls showed mild inhibition of PG support the idea that kaki leaf tissue expresses an endogenous PGIP-encoding gene that is relatively dissimilar to the pear *PGIP* transgene because it was not detected in the DNA and protein blotting analysis (Tamura *et al.*, 2004).

All of the studies discussed here considered only the growth and reaction of the plants, not the fruit. It is necessary to investigate the quality of fruit harvested from transformants. For further studies, transformed plants should be planted in a field and allowed to bear fruit. Also, the heredity of the transgene in kaki is an interesting subject of inquiry.

2.6 Regulatory Measures

Field tests of the transformed kaki are now performed only in California (Figure 6). There is no cultivation of transformed plants in Japan, where most research on transformation of kaki has been conducted, partly because there are strict regulations for field tests of transformed crops and public acceptance of GMO foods has not increased. Recently, field tests on the transformed fruit trees were initiated in Japan (Tao *et al.*, 2001). Regulations for cultivating the transformed fruit trees in Japan may be established during the study.

3. FUTURE ROAD MAP

3.1 Expected Products

Previous works have shown how engineering tolerance to salt stress, growth inhibition, and resistance to pests and disease has developed. These genetic improvements are all related to

the culture of kaki, but kaki fruit is so rich in vitamin C, carotenoids, pectic substances, and polyphenols that there may be little interest in improving its nutraceutical quality by introducing foreign genes into the kaki genome.

As mentioned before, the main breeding objective for kaki is to obtain PCNA cultivars with many valuable characteristics, but Japanese breeding programs have had difficulties linked mainly to the narrow genetic pool of the PCNA group (Yonemori *et al.*, 2000). However, if non-PCNA cultivars producing fruit of high quality are transformed into PCNA, the breeding program will be changed dramatically. For example, seedless PCNA cultivars will appear relatively soon by transforming an excellent non-PCNA cultivar ("Hiratanenashi"), although nonaploid PCNA types may result from cross-breeding (Sugiura *et al.*, 2000; Yamada and Tao, 2006).

3.2 Addressing Risks and Concerns

Kaki originated in China and is naturally distributed in East Asia. Hence, the assessment of risk to the environment should begin in East Asia. As for damage to human health, assessment of fruit harvested from trees on transformed rootstocks, as well as fruit harvested from transformed scion cultivars is needed. These assessments, conducted within a country following a set of national guidelines, will enhance the possibility of the cultivation of transformed kaki worldwide.

3.3 Expected Technologies

The efficiency of kaki transformation using *Agrobacterium* is not lower than that of other fruit trees and is higher than that of apple (Kotoda *et al.*, 2006) and grape (Nakajima *et al.*, 2006). However, using another marker such as green fluorescent protein (Yancheva *et al.*, 2006) or using positive selection (e.g., mannose; Ramesh *et al.*, 2006) might improve efficiency.

For the leaf disc transformation system developed by Tao *et al.* (1997), a callus intermediate stage was indispensable to obtain transformants. Earlier work had shown that it is difficult to induce adventitious bud formation directly from leaf discs (Tao *et al.*, 1988). The major drawback in using a callus intermediate stage is

that the regeneration process takes longer and there is a risk of somaclonal variation (Tao and Dandekar, 1999). In contrast, for the hypocotyl segment transformation system developed by Nakamura *et al.* (1998), transformed shoots were regenerated directly from the segments, so that transformants were obtained rapidly. However, this transformation method cannot be used to introduce genes into cultivars directly because it uses hypocotyls that are of cross-bred origin. Hence, *in vitro* root segments of cultivars are promising materials for transformation using *Agrobacterium* because the technique for the induction of adventitious shoots directly on a root segment has been established (Tetsumura and Yukinaga, 1996). However, no adventitious buds formed on “Saijo” roots co-cultivated with *Agrobacterium* EHA105 (Itamura *et al.*, 2006).

All of the promoters used in the transformation of kaki have been 35S, except for the *nos* promoter with the *nptII* gene in pSMK251 (Nakamura *et al.*, 1998; Koshita *et al.*, 2002). Recently, gene expression of kaki has been investigated intensively for ethylene production (Nakano *et al.*, 2003; Zheng *et al.*, 2005, 2006; Ortiz *et al.*, 2006) and tannin biosynthesis (Ikegami *et al.*, 2005a, b) of fruits. These studies might lead to the detection of a promoter specific to the maturation of kaki fruit. A fruit-specific promoter for other species (Yamagata *et al.*, 2002) may be used as an alternative promoter for introducing genes into the kaki genome.

A future improvement in the transformation system of kaki involves the establishment of many valuable cultivars (Tao *et al.*, 2001), although “Jiro,” the only transformed cultivar, is an important PCNA cultivar. The key to success may lie in enhancing adventitious bud formation from callus cultures because more than half of the cultivars tested did not form adventitious buds (Tao and Sugiura, 1992b). Transformants from kaki seedlings can be used as rootstocks (Koshita *et al.*, 2002) and as research models for the juvenile phase (Kotoda *et al.*, 2006).

3.4 Future Release and its Consequences

Although all studies discussed here were experimental, all genes used for the transformation of kaki were eminently practical. Hence, a new

cultivar integrated with the genes considered here might be released after field testing. There are many issues concerning intellectual property, public perceptions, political consequences, and economic consequences that must be discussed and solved.

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Tomato

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1. INTRODUCTION

Tomato is one of the most consumed and widely grown vegetable crops in the world. Wild-type tomato species are thought to be native of western South America, specifically in the dry coastal desert of Peru. Tomato crop is valued at 5–6 billion dollars, with international trade amounting to 3–3.5 billion dollars annually (FAOSTAT, 2005). Tomato is grown on about 4.5 million hectares with a production of 122.6 million metric tons worldwide. In 2005, Asia was the highest tomato-producing continent with over 49% (or 60 243 562 t) of the total production. Americas ranked second in the world with a production of ~20 million metric tons, with North America producing the major quantity. Based on the average world production from 1999 to 2005, the top 10 tomato-producing countries are: China, the United States, Turkey, India, Italy, Egypt, Spain, Iran, Brazil, and Mexico. China contributed over 25% (or 31.6 million metric tons) to the total world production during the year 2005 (FAOSTAT, 2006).

In the global export market of tomatoes, Spain has been the world's leading exporter, with \$972 million export in 2004, Mexico being close second with \$909 million, followed by Canada. The United States ranks fourth in export value with \$233.8 million in 2004 (FAOSTAT, 2006). The

North American Free Trade Agreement (NAFTA) between United States, Canada, and Mexico resulted in higher export quantity of 1 244 568 t with an export value of \$1415 million in 2004 (FAOSTAT, 2006). Canada and Mexico are the largest export destinations for US fresh tomatoes with over 80% of US fresh tomato at a value of \$141.7 million exported to Canada alone in 2004. The summer and fall are the prime seasons for tomato export from the United States to Mexico but the value of shipments to Mexico is variable (Boriss and Brunke, 2005). In spite of the fact that the United States has higher tomato production, it was the leading importer of fresh tomatoes in 2004 (FAOSTAT, 2006).

The tomato was not very popular until the 18th century mainly because it was rumored to be poisonous. The first improved cultivated tomatoes were Tilden, released in 1865, and Trophy, released in 1870 (Bailey, 1949; Robertson and Labate, 2007). Thomas Jefferson grew tomatoes in Virginia. George Washington Carver encouraged introduction of tomatoes in the diet and was known to eat tomatoes in front of crowds. The popularity of the tomato comes not only from its flavor and freshness (fresh market tomato), but also because it can be processed as: ketchup, sun dried, tinned, powder, sauce, soup, puree, juice, canned whole fruit, etc. (processing tomato industry). Tomato processing began in 1847, when

Harrison Woodhull Crosby, the chief gardener at Lafayette College, developed a crude method of canning tomatoes (Boriss and Brunke, 2005). Tomato processing has come a long way since the use of jars in home preserve (Salunkhe and Desai, 1984). Prior to 1890, all tomato canning was done by hand. Industry techniques improved with canning technology, and tomato juice came on the market with the development of the juice extractor in the 1920s (Gould, 1983). Processed tomatoes account for the majority of tomato tonnage in the United States, while the comparatively higher prices of fresh market tomatoes make them higher ranked in terms of value. Today, the United States produces over 10 million tons of processing tomatoes, in which California processes over 90% of the total.

Tomato is a very versatile vegetable for culinary purposes. Unripe green tomatoes are used to make preserves, salsa, be breaded and fried, or be pickled. Tomatoes are used extensively in the Mediterranean and Middle Eastern cuisines, especially Italian. Tomato fruit is acidic, a property that is used to bring out other flavors. This same acidity makes tomatoes especially easy to preserve in home canning as tomato sauce or paste. Such versatility coupled with a growing demand for the fresh fruits, has pushed the development of tomato as a one of the main crops of the last century and the beginning of this new millennium.

Tomato is a popular vegetable/fruit also because it is an important source of vitamins and minerals in diets. One medium-sized tomato provides 57% of the recommended daily allowance (RDA) of vitamin C, 25% RDA of vitamin A, and 8% RDA of iron, yet with only 35 calories. Tomato extract has been used to treat various diseases in traditional medicine in different countries such as Japan, Greece, Peru, and Guatemala. Hot water extract of dried fruits has been used in the treatment of ulcers, wounds, hemorrhoids, and burns (Caceres *et al.*, 1987). Tomatoes are also rich in an antioxidant called lycopene, a carotenoid that has been found to protect cells from oxidants that have been linked to cancer (Gerster, 1997). In laboratory tests, lycopene was found to be twice as powerful as β -carotene in neutralizing free radicals. Lycopene has been linked to risk reduction in a number of cancer types, including prostate, lung and stomach, pancreatic, cervical, colorectal, oral, and

esophageal cancers (Giovannucci, 1999; Rao and Agarwal, 2000). Giovannucci *et al.* (1995) reported that the risk of prostate cancer was reduced nearly 45% among men who ate at least 10 servings a week of tomato-based foods. Compared with carotenoids and other antioxidant compounds including vitamin E, lycopene has been reported to be a more efficient quencher of singlet oxygen *in vitro*. In addition to its antioxidant and anticarcinogenic properties, lycopene shows an array of biological effects including cardioprotective, anti-inflammatory, and antimutagenic activities. The mechanisms underlying the inhibitory effects of lycopene on carcinogenesis could involve reactive oxygen species scavenging, upregulation of detoxification systems, interference with cell proliferation, induction of gap-junctional communication, inhibition of cell cycle progression, and modulation of signal transduction pathways (Bhuvaneswari and Nagini, 2005).

1.1 Botanical Description

Tomato is a member of the dicot family Solanaceae, which contains well-known plant species such as potato, tobacco, eggplant, and pepper. Bailey (1949) classified tomatoes as belonging to two species: *Lycopersicon pimpinellifolium* and *Lycopersicon esculentum*. The latter species is the parent of commercial tomatoes, and practically all belong to the botanical variety *Commune*. Other botanical varieties included in this species are: *Grandifolium*, the large-leaved or potato-leaved type; *Validum*, the upright or dwarf type, with dense, dark green foliage; *Cerasiforme*, the cherrylike type, with normally two-celled globular fruits and standard foliage; and *Pyriforme*, the pearlike type or oblong fruits and standard foliage. In addition to these two species, several more species of tomato are recognized, namely, *Lycopersicon cheesemanii*, *Lycopersicon peruvianum*, *Lycopersicon hirsutum*, and *Lycopersicon glandulosum* (Taylor, 1986). The current range of wild tomato relatives extends from the northern tip of Chile on the south, to Ecuador on the north, and reaching inland from the Pacific 100–200 miles (161–322 km), also including the Galapagos Islands (Gould, 1991).

Miller's classification (Miller, 1754) of tomato under the genus *Lycopersicon* continued to be

used until now. However, the recent molecular studies on phylogenetic relationships within the family of Solanaceae support and firmly re-establish Linnaeus' (1753) earlier classification of tomato under the genus *Solanum* (Peralta and Spooner, 2007; see other chapters in Razdan and Mattoo, 2007). Linnaeus had grouped all the cultivated multilocular tomato species under the name of *Solanum lycopersicum*. Whereas the location of domestication is not well established, the most acceptable explanation is that tomato dispersed to Mexico (and much of the rest of Latin America) as a weedy form and subsequently was domesticated there (Jenkins, 1948). Therefore, our modern tomato cultivars, while not necessarily arising directly in the center of origin, have their root beginnings on the west coast of Latin America (Mills, 2006).

The genus consists of a warm-season perennial plant cultivated as an annual and it is very sensitive to chilling and freezing temperatures. Tomato fruit take about 6–7 weeks from flowering, depending on temperature, to reach full size. Cell division continues for about 2 weeks after flowering, but the bulk of the increase in fruit size is the result of cell expansion (Srivastava and Handa, 2005; Handa *et al.*, 2007). In normal cultivars, the first appearance of red or pink color at the blossom end signals the completion of growth and onset of ripening. Laboratory studies with fruit harvested at mature green stage have shown that ripening actually begins about 2 days before the external color changes (Madhavi and Salunkhe, 1998).

Tomato plants are described as determinate, semi-determinate, or indeterminate. The term “determinate” refers to the plant growth habit. Determinate tomato plants grow outdoors like a bush to a certain size (about 3–5 ft.), set fruit, and then decline. Most of the early ripening tomato varieties are of the determinate type. The primary shoot terminates in a flower cluster, forcing side shoots to develop. The branches terminate their growth at approximately the same distance from the crown, resulting in a compact and symmetrically circular growth. With the indeterminate types, the primary shoot dominates the side shoot development, resulting in a sprawling growth pattern. The primary shoot continues to grow as long as the plants remain healthy and growing conditions are suitable or until frost or disease kills them. Many of the

standard-sized, all-summer tomatoes typical of the home garden are of the indeterminate type. They require physical support of some kind for best results, since the fruit would otherwise be in contact with the soil and thus susceptible to rot. The semi-determinate types have vine characteristics that are intermediate between the other two types (Madhavi and Salunkhe, 1998).

The fruit is an edible, brightly colored (usually red, from accumulation of the pigment lycopene) berry, 1–2 cm diameter in wild plants, commonly much larger in cultivated forms. The fruits vary in shape from a flat round to a true round, square round, oblong, pear, oxheart, and many variations in between. The fruit is a fleshy berry, or, in botanical terms, a swollen ovule. The body of the fruit, developed from the ovary wall that surrounds and encloses the seed, which is known as the pericarp (Gillaspy *et al.*, 1993). The pericarp of tomato fruit is composed of three distinct tissue types: the endocarp, a unicellular layer encasing the locular cavity, the mesocarp, a multicellular layer of large, thin-walled parenchyma cells (>500 μ m diameter) and vascular tissue, and the exocarp (fruit skin), which is composed of an outer epidermis with a thick waxy cuticle (Osman *et al.*, 1999) and two to three layers of thick-walled hypodermal cells (Ho and Hewitt, 1986). The locular cavities occur as gaps in the pericarp and contain the seeds embedded in a jellylike parenchymatous tissue originating from the placenta. The number of locules in normal fruit varies from two upwards, and is more or less characteristic for each variety. Prior to pollination, as well as during a relatively short period after anthesis, the growth of the fruit is mainly by cell division, after which cell enlargement is responsible for the growth of the fruit. During the later phase, vacuoles appear in the cells and differentiation in composition becomes evident. Growth-promoting and growth-inhibiting substances moving into and out of the fruit no doubt play a major part in the maturation process, while respiratory and fermentative mechanisms assume a dominant role in ripening (Hobson and Davies, 1971). The color of mature fruits may be red, pink, orange, yellow, or white. Most commercial cultivars are red or pink.

Tomato fruit is classified as a climacteric fruit, in which onset of ripening is accompanied by an increase in both respiration and ethylene

production. Ethylene plays a central role in both the initiation of early biochemical events during ripening and the integration of subsequent changes (Mattoo and Suttle, 1991). The disappearance of starch, degradation of chlorophyll, and synthesis of carotenoids such as lycopene, flavor components, as well as a considerable increase in polygalacturonase and other cell wall-hydrolyzing enzymes, are processes highly integrated with the increases in respiration and ethylene production (Giovannoni, 2004; Mattoo and Handa, 2004; Srivastava and Handa, 2005). The terminal period of maturation occurs before or after harvest with little loss in edible quality provided that the mature green stage (preclimacteric minimum respiration rate) is reached before harvesting. The physiological and biochemical changes associated with ripening have been previously reviewed (Salunkhe and Desai, 1984; Hobson and Grierson, 1993; Giovannoni, 2004; Razdan and Mattoo, 2007).

1.2 Genome Size

Solanaceae members have differences in genome sizes. However, most, if not all, share the same basic chromosome number of 12 with tomato having the smallest haploid genome size of 953 Mb (Arumuganathan and Earle, 1991). Comparative genetic studies revealed that tomato and potato share very conserved colinearity between their genomes (Bonierbale *et al.*, 1988; Tanksley *et al.*, 1992). Tomato shares conserved gene repertoires with pepper even though their gene order is different (Tanksley *et al.*, 1988). Among geneticists, tomato is considered an ideal model crop plant. Tomato has an excellent classical morphological map, a high-density molecular map containing more than 1000 markers (Rick and Yoder, 1988; Tanksley *et al.*, 1992; Broun and Tanksley, 1996), and a large collection of well-characterized mutants and near-isogenic lines (NILs). In addition, tomato has a relatively small genome size, several large-insert yeast artificial chromosome (YAC) libraries (Martin *et al.*, 1992; Bonnema *et al.*, 1996), a transposon tagging system (Briza *et al.*, 1995), and a routine *Agrobacterium*-mediated transformation system (McCornick *et al.*, 1986).

1.3 Traditional Breeding

Tomatoes to be used either fresh or in processing must have distinct quality characteristics. Fresh tomatoes must have acceptable flavor, color, texture, and taste parameters to satisfy consumer demands and handling requirements. Processing tomatoes, on the other hand, must have intrinsic rheological characteristics that make them suitable for various processing applications, such as juice, ketchup, or sauce production.

The general pattern of the breeding program appropriate to a particular species is determined in part by the reproductive or mating system of the species. Tomato is a self-pollinated species. All varieties are highly inbred populations with no significant genetic diversity within a variety. It has generally been found that self-pollinating plants such as tomatoes, after they have become stabilized, do not change their genetic constitution to any great extent (Kerr, 1969).

Tomato has perfect flowers. Crossing of tomatoes is accomplished by emasculation of the flowers of the seed parent as they begin to open but before pollen is shed. In order to emasculate a flower, the stamens are removed with forceps, either alone or with the petals. Pollen is collected on the tip of a sterile forcep from the pollen parent and placed on the stigma of flowers of the seed parent. When the fruit is ripe, the seeds are extracted, fermented naturally or digested with acid, washed, dried, and packaged. Selfing plants of this first generation cross (F_1) yields the F_2 generation, or crossing F_1 to one of the parents (backcrossing) gives the backcross (BC) generation (George and Berry, 1983).

The basic breeding used following hybridization includes pedigree and BC methods. These methods are used to handle the segregation populations and are based on the fact that selfing or backcrossing to a homozygous parent leads to homozygosity upon self-fertilization. Continued self-pollination causes an increase in homozygosity by one-half per generation. The pedigree method is widely used by tomato breeders. In this system, records are kept of the ancestry or pedigree of each of the progenies (families). Selection is based on productivity and other horticultural characteristics of single plants or progenies. After the F_2 generation, selection is practiced within and between families. Considerable detailed record keeping is required,

and the number of selections under test can quickly become burdensome. Once a degree of uniformity is reached, selections are placed in comprehensive evaluation trials (George and Berry, 1983).

The BC methods are particularly useful in transferring specific genes into an established variety deficient in one or a few characteristics. It is very effective in transferring genes for disease resistance. Continued backcrosses are made to the desirable parent, and selection is practiced for the characters being transferred from the donor parent. Essentially, the new variety will be the same as the original parent except for its improved characteristics. This is an advantage because of the fact that the new variety need not be extensively evaluated. It has the disadvantage in that the new variety will only have the specific improvements transferred. Many programs utilize a combination of the pedigree and BC methods. The BC system is used early in the program to transfer specific genes, but while sufficient heterozygosity exists pedigree selection is undertaken to improve other horticultural characters (George and Berry, 1983).

Traditional breeding requires the selection of a tomato genotype or its wild species that has a desirable trait, such as early ripening, disease resistance, and crossing it with another tomato cultivar that has a good genetic background. The desired result is an earlier ripening tomato that makes it to the market sooner, or varieties that resist the pathogens' attack. In this way, several thousands of tomato cultivars were developed. In the United States, as an example, developed cultivars include: Better Boy F₁, Burpee's Big Boy F₁, Campell 1327, Dombito F₁, Earlypak 707, Glamour, Heinz 722, Heinz 1350, 1439, 2653, Jubilee, Jumbo F₁, New Yorker, Red Cherry, Roma VF, Rutgers 39, Spring Set F₁, Supersonic F₁, Traveler 76, UC 82, UC 204, among others (Madhavi and Salunkhe, 1998).

Since the early days of the 20th century, traditional breeding for disease resistance in plants has been a major method for controlling plant diseases. Varieties that are resistant or tolerant to one or few specific pathogens are already available for many crops. Resistant hybrids with multiple resistances to several pathogens exist and are currently used in vegetable production. For tomato, the genetic control of pathogens is a very useful practice and most of used resistance is monogenic and dominant. So far,

tomato breeding has resulted in varieties with resistance to at least 15 pathogens, although with varying stability and level of expression (Grube *et al.*, 2000). Tomato varieties with some resistance to fungi or oomycetes (*Alternaria alternata* f. sp. *lycopersici*, *Cladosporium fulvum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Phytophthora infestans*, *Pyrenochaeta lycopersici*, *Verticillium dahliae*), bacteria (*Corynebacterium michiganense*, *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *tomato*), virus (beet curly top hybrigeminivirus, tomato mottle bigeminivirus, tomato spotted wilt tospovirus, and several variants of the tomato yellow leaf curl bigeminivirus), and nematodes (*Meloidogyne* spp.) are available (Lacerrot, 1996). Many open-pollinated varieties presently cultivated possess genetic resistance to three or four pathogens. With the increasing use of F₁ hybrids it is possible to use varieties combining from four up to six resistances (Grube *et al.*, 2000).

In processing tomatoes, the major breakthrough had been the advent of the once-over machine for harvesting in the late 1960s. The breeding and development of tomato varieties for machine harvest adapted to any region is complex. For once-over machine harvest, concentrated fruit setting and maturity are important considerations; however, there are many sources of plant material that can produce variations in plant growth habit, concentrated flowering, fruit setting ability, concentrated maturity, and ability of the ripe fruit to store on the vine, than presently available in commercial varieties (see Razdan and Mattoo, 2007). In general, the materials that have variations in concentrated plant habit and higher degree of earliness have been of limited use due to low yield potential and deficiencies in quality, such as small fruit size, lack of firmness, crack susceptibility, low solids, and poor color (Gould, 1991).

1.4 Rationale for Transgenic Technology Application

Conventional plant breeding has had a significant impact on improving tomato breeding for fruit quality and resistance to important diseases. However, the progress made thus far has been confounded by: the time-consuming process of making crosses and backcrosses; difficulty in

combating the evolution of new virulent pathogens or breakdown of resistance; saturation of the genetic pools available for improvement; and time required, 10–15 years, to release a new tomato variety. Besides, traditional breeding methods used may not impart the desired characteristics (Gould, 1983).

Genetic engineering or “recombinant-DNA technology” is a radical new avenue for transplanting genetic characteristics of one species into another. This technology is based on identifying the molecular unit(s) responsible for the desired agronomic trait and then isolating and determining the structure and organization of such a unit or units. The unit(s) can then be engineered to transform and modify any existing variety of a crop plant. This technology provides a continuum between plant breeding-based varietal development and manipulation of beneficial genes by genetic engineering to develop transgenics. Moreover, the cloned gene can be introduced not only into the plant species from which it was isolated, but also to other agriculturally important species (Peleman and Rouppe van der Voort, 2003). Using this approach, the time needed to release a new tomato variety can be halved as compared with the time taken using traditional breeding methods.

In order to establish a successful program of practical plant genetic engineering, it is important to develop a system for the recovery of large number of whole plants from explant tissues. It is also important to optimize the procedure for inserting foreign or modified host genes into the targeted plant genomes. A combination of these two approaches is required for plant genetic engineering. Early experiments involving gene transfer used *Agrobacterium tumefaciens* for introducing foreign genes (Barton and Brill, 1983; Herrera-Estrella *et al.*, 1983). *Agrobacterium* is a very effective tool for introduction of transformed genes into solanaceous species. The limitation of host specificity that made it difficult to transform crops like legumes and cereals has been partially tackled. The reproducibility of this approach has made it a method of choice for solanaceous species. On the other hand the biolistic method has emerged as a simple and promising alternative for transformation. Particle bombardment offers opportunities for successful transformation of many plant tissues without

limitation. Particle bombardment is a technique through which metal particles coated with DNA are rapidly accelerated into plant tissues, often resulting in stable transformation (Christou *et al.*, 1988; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Bower and Birch, 1992; Vasil *et al.*, 1992). Because DNA is being shot into cells, it represents a type of biological ballistics (Sanford, 1990).

Using tools derived from the recombinant-DNA technology, several genes involved in disease resistance in tomato plants and fruit development and ripening have been cloned and characterized for the creation of new transgenic tomatoes. So far, over 40 genes (including many single genes and quantitative trait loci, QTL) that confer resistance to major classes of plant pathogens have been mapped on the tomato molecular map and/or cloned from solanaceous species. The map provides a basis for further identification and mapping of genes and QTLs for disease resistance and other attributes, and should be useful for marker-assisted selection (Grube *et al.*, 2000).

Also, several genes involved in fruit development and tomato ripening have been identified, cloned, and characterized for the creation of genetically modified new tomato varieties. Of the various genes identified, those encoding the enzymes polygalacturonase (PG) and pectinesterase were utilized for targeted single-gene modification studies. Genetically modified tomatoes with reduced levels of these cell wall modifying enzymes have been successfully developed (Tieman *et al.*, 1992; Schuch, 1994). Based on these studies, an improved quality low-PG fresh market tomato named Flavr SavrTM was developed. This tomato contains an antisense version of the PG gene as well as a bacterial marker gene that confers resistance to the antibiotic kanamycin. The presence of this gene attracted opposition to Flavr Savr from critics of genetic engineering and consumer-activist groups, who claimed that the gene might jeopardize the use of kanamycin by transferring antibiotic resistance to gut-living bacteria. But, after years of scrutiny, Flavr Savr tomato (Redenbaugh *et al.*, 1992) was approved by the US Food and Drug Administration (FDA) in May 1994, thus making it the world's first genetically engineered whole food to reach US consumers (Holden, 1994). Some of the important groups of genetically modified tomato cultivars are: Earliana, Bonny Best, Marglobe, Stone, and Pink. Earliana var.

Table 1 Nonselectable markers or reporter genes used to create transgenic plants^(a)

External substrates	Genes	Enzymes	Source
o-Nitrophenol- β -D-galactoside	<i>LacZ</i>	β -galactosidase	<i>E. coli</i>
5-Bromo-4-chloro-3-indolyl- β -D-galactoside			
5-Bromo-4-chloro-3-indoxyl- β -D-glucuronide cyclohexylammonium salt	<i>gusA</i> (<i>gusA</i>)	β -glucuronidase	<i>E. coli</i> , <i>Bacillus</i> sp.
4-Methylumbelliferyl β -D-glucuronide			
Luciferin	<i>Luc</i>	Luciferase	<i>Photinus pyralis</i>
Decanal	<i>luxA</i> , <i>B lux F</i>		<i>Vibrio harveyi</i>
None	<i>Gfp</i>	Green fluorescence protein (GFP)	<i>Aequorea victoria</i>
None		Phytoene synthase	<i>Erwinia herbicola</i>
None	R, C1, B	Anthocyanin pathway regulatory factors	Maize
None		Thaumatococcal II	<i>Thaumatococcus danielli</i>
Oxalic acid		Oxalate oxidase (OxO)	Wheat

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Table 2 Selectable marker genes used for the conditional-positive selection of transgenics^(a)

Antibiotics	Genes	Enzymes	Genetic sources
Aminoglycosides	<i>aacC3</i>	Aminoglycoside-N-acetyl transferases	<i>Serratia marcescens</i>
Gentamycin	<i>aacC4</i> <i>6'gat</i>		<i>Klebsiella pneumoniae</i> <i>Shigella</i> sp.
Bleomycin/ Phleomycin	<i>Ble</i>	Bleomycin resistance	<i>E. coli</i> Tn5 <i>Streptoalloteichus hindustanus</i>
Chloramphenicol	<i>cat</i>	Chloramphenicol acetyl transferase	<i>E. coli</i> Tn5, Phage p1 cm
Kanamycin neomycin	<i>nptII</i>	Neomycin phosphotransferases	<i>E. coli</i> Tn5
Paramomycin G418	<i>aphA1</i> <i>aphA2</i>		<i>E. coli</i> Tn601
Hygromycin B	<i>aphIV</i>	Hygromycin phosphotransferase	<i>E. coli</i>
Spectinomycin	<i>aadA</i> ^(b)	Aminoglycoside-3'-adenyl transferase	<i>Shigella</i> sp.
Streptomycin	SPT	Streptomycin phosphotransferase	Tn5
Streptothricin	<i>sat3</i>	Acetyl transferase	<i>Streptomyces</i> sp.
Sulfonamides	<i>sulI</i>	Dihydropteroate synthase	<i>Escherichia coli</i> pR46

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^(b)Aldehyde-alcohol dehydrogenase gene

is characterized by ripening early and its bright red fruits. Bonney Best has medium-sized, round to oblate, deep red fruit with a high incidence of cracking and scalding. The Marglobe variety is tolerant to *Fusarium* wilt, heavy yielding, with bright red, medium to large fruits. It is considered good for canning in the United States. Stone is grown extensively for canning and has scarlet-red, flattened, and firm fruits. The fruits of Pink are large, globose or heart shaped to irregular in shape, purplish pink to pinkish red with few seeds, low acidity, and have a pleasant flavor.

Selectable marker genes are used to discriminate between transgenic and nontransgenic cells and facilitate the identification of plants containing

co-transformed transgenes. The most common selectable markers and reporter genes are listed in Tables 1 and 2.

2. DEVELOPMENT OF TRANSGENIC TOMATOES

2.1 Transgenic Tomatoes *vis-a-vis* Ripening Phenomenon

A summary of studies conducted to manipulate ripening, fruit texture, flavor, and nutritional quality of tomato fruit using transgenic approach is presented in Table 3.

Table 3 Genetic manipulation of *Solanum lycopersicum* L. to improve fruit quality

Protein/enzyme	Gene source	Promoter:gene	Cultivar	Phenotype	References
ACC oxidase	Apple Tomato	CaMV 35S:AP4 antisense D35S ω :ACC oxidase (RNAi)	UC82B Heinz 906	Reduced ethylene production Prolonged shelf life for more than 120 days	Bolitho <i>et al.</i> , 1997 Xiong <i>et al.</i> , 2005
ACC synthase	Tomato	CaMV 35S:LeACC2 antisense	VF36	Impaired ethylene production, fruit ripening, and extended shelf life	Oeller <i>et al.</i> , 1991
Amino acid decarboxylases	Tomato	FMV:LeAADC1A FMV:LeAADC2 antisense	M82	Changes in flavor and aroma volatiles	Tieman <i>et al.</i> , 2006
Deoxyhypusine synthase	Tomato	CaMV 35S:DHS antisense	UCT5	Pleiotropic effects on growth and development. Delayed postharvest softening and senescence	Wang <i>et al.</i> , 2005
Endo-1,4- β - glucanase (EGase)	Pepper	CaMV 35S:CaCell1	Bonny Best	No change in xyloglucan depolymerization and fruit softening	Harpster <i>et al.</i> , 2002
Ethylene receptor	Tomato	CaMV 35S:LeETR1 antisense	Ailsa Craig	Delayed abscission, shorter internode length and reduced auxin transport. No effect on pigmentation and fruit softening	Whitelaw <i>et al.</i> , 2002
Expansin	Cucumber	CaMV 35S:CExp1	Ailsa Craig	Up to 20-fold increase in expansin activity in cell walls	Rochange and McQueen- Mason, 2000
Expansin and polygalactur- onase	Tomato	CaMV 35S:LeExp1 and FMV:LePG	T52 and T53	Increased juice viscosity, consistency, and pectin molecular size	Kalamaki <i>et al.</i> , 2003a
Fructose-1,6- bisphosphatase	Potato	PPatatin B33:cp-FBPase	Money maker	Decreased fruit size, slight changes in carbohydrate metabolism	Obiadalla-Ali <i>et al.</i> , 2004
β -galactosidase	Tomato Tomato	CaMV 35S:TBG1 antisense CaMV 35S:TBG4 antisense	Ailsa Craig Rutgers	Up to 90% down- regulation of TBG1 mRNA did not alter the degree of galactan degradation in the ripening fruits	Carey <i>et al.</i> , 2001 Smith <i>et al.</i> , 2002
β -glucuronidase	<i>Escherichia coli</i>	Pp-ACO1:GUS Pp-ACO2:GUS ppACO1:GUS	Microtom Pixie	Decrease in fruit softening Identification of ERE and AUXre	Rasori <i>et al.</i> , 2003 Moon and Callahan, 2004
Hexokinase	<i>Arabidopsis</i>	CaMV 35S:AtHXK1	MP-1	Effect on softening Hexose phosphorylation diminishes during fruit development	Roessner-Tunali <i>et al.</i> , 2003 Dai <i>et al.</i> , 1999
Lycopene β -cyclase	<i>Arabidopsis</i>	Tomato Pds: β -Lcy	Money maker	Sense: increase in β -carotene Antisense: decrease in β -Lcy expression with slight increase in lycopene	Rosati <i>et al.</i> , 2000

(Continued)

Table 3 Genetic manipulation of *Solanum lycopersicum* L. to improve fruit quality (*Continued*)

Protein/enzyme	Gene source	Promoter:gene	Cultivar	Phenotype	References
NADP-dependent glutamate dehydrogenase	<i>Aspergillus nidulans</i>	P35S:gdhA	Mini tomato	Higher total amino acids including glutamate	Kisaka and Kida, 2003
Pectin methylesterase	Tomato	CaMV 35S:PME2 antisense	Ohio 8245	Increased juice viscosity and serum viscosity; higher total solids, decreased pectin hydrolysis	Tieman <i>et al.</i> , 1992 Thakur <i>et al.</i> , 1996
Peroxidase (basic)	Tomato	CaMV 35S: <i>Tpx1</i>	Pera	Role of <i>tpx1</i> in lignin synthesis shown	Mansouri <i>et al.</i> , 1999
Phytoene desaturase (carotenoid)	<i>Erwinia uredovor</i>	CaMV 35S: <i>crt1</i>	Ailsa Craig	Increased β -carotene with up to 45% of the total carotenoids	Romer <i>et al.</i> , 2000
Phytoene synthase	<i>Erwinia uredovora</i>	Tomato PG Fruit specific:CrtB	Ailsa Craig	Increased fruit carotenoids: phytoene, lycopene, β -carotene, and lutein	Fraser <i>et al.</i> , 2002
Polygalacturonase and expansin	Tomato	CaMV 35S:LePG antisense CaMV 35S:LeEXP1 sense suppression	Ailsa Craig	Firmer fruits, better in storage, increase juice viscosity	Powell <i>et al.</i> , 2003
SAM decarboxylase	Yeast	<i>E8:SAMDC</i>	Ohio 8245	Accumulation of polyamines: spermidine and spermine. High lycopene, improved juice quality, and longer vine life	Mehta <i>et al.</i> , 2002
Sucrose-phosphate synthase	<i>Zea mays</i> L.	CaMV 35S:SPS pRubisco small subunit:SPS	UC82B	Affects acclimation to elevated CO ₂ , flowering and fruiting due to increase in sucrose synthesis	Micallef <i>et al.</i> , 1995
Thaumatococin	<i>Thaumatococcus daniellii</i> Benth	CaMV 35S: thaumatocin	β	Increased fruit sweetness and a liquorices after taste lasting for a few minutes	Bartoszewski <i>et al.</i> , 2003

2.1.1 Cell wall carbohydrases, deoxyhypusine synthase, and fruit softening

Textural properties of tomato fruits are important contributors to the overall quality of fresh market fruits and to the properties of products processed from tomatoes (Barrett *et al.*, 1998). Because cell wall disassembly in ripening fruit contributes to fruit texture, modification of cell wall proteins and enzymatic activity during ripening can impact cell wall polysaccharide metabolism and influence texture. Transgenic tomato lines in which the expression of single or multiple genes

was altered have been developed (Carrington *et al.*, 1993; Brummell *et al.*, 1999a, b). A major focus on altering ripening characteristics arose with the isolation of the tomato fruit PG gene whose transcripts represent ~1% of ripening fruit messenger-RNA (mRNA). Both antisense repression (Smith *et al.*, 1988) and ectopic expression in unripe fruit (Giovannoni *et al.*, 1989) indicated that PG alone is not sufficient for softening. Nevertheless, a reduction in the susceptibility of ripe fruit to postharvest pathogens in antisense PG fruit led to the commercialization, though transiently, of PG antisense tomatoes.

Removal by pectin methylesterase (PME) of the methyl groups from pectins located in the fruit cell walls facilitates access of PG to its substrate. PME is expressed early in ripening and is down-regulated as ripening advances. Repression of tomato fruit PME via antisense technology resulted in increased juice viscosity attributable to the retention of the pectin chain length; however, softening was not altered significantly as compared with the wild type, nontransgenic tomato fruit (Tieman *et al.*, 1992; Tieman and Handa, 1994; Tieman *et al.*, 1995). The molecular weights of pectins isolated from processed juice were much higher from the transgenic fruit than the control fruit even under a cold break, indicating a role of pectin demethoxylation in pectin breakdown. Depending on the processing conditions, juice from these transgenic fruits contained 30–50% higher amounts of total uronic acids and had 25–250% higher degree of methoxylation (DOM) compared to juice from control fruits (Handa *et al.*, 1996; Thakur *et al.*, 1996).

In addition to PG and PME enzymes, several hemicellulose-metabolizing enzymes have been characterized in ripening fruit. Repression of the ripening-related endo- β -1,4-glucanases (also known as EGases or cellulases), *CEL1* and *CEL2*, altered pedicel and fruit abscission, respectively, but did not influence fruit softening (Lashbrook *et al.*, 1998a; Brummell *et al.*, 1999a). However, a ripening-related and ethylene-inducible tomato β -galactosidase gene, *TBG4*, was shown to have a role in fruit softening when its expression was repressed via antisense (Smith *et al.*, 2002). Similar role for the ripening-related expansin, *LeExp1*, was elucidated using similar approach (Rose *et al.*, 1997; Brummell *et al.*, 1999b).

Overexpression of the ripening-related *LeExp1* in tomato resulted in enhanced fruit softening (Barrett *et al.*, 1998; Powell and Bennett, 2002; Kalamaki *et al.*, 2003a). In contrast, suppression of expansin protein accumulation to about 3% of wild-type levels resulted in fruits with a firmer texture (Brummell *et al.*, 1999b; Kalamaki *et al.*, 2003a). Kalamaki *et al.* (2003b) showed that *LeExp1* overexpression resulted in higher juice viscosity and paste prepared from the transgenic fruits as compared to the control tomato fruit or the transgenic fruits with reduced *LeExp1* expression. These data indicated that *LeExp1*

overexpression enhanced depolymerization of water-soluble pectins as well as tightly bound matrix glycans.

Genes encoding β -galactosidase belong to a family of at least seven members of glycosyl hydrolases that reduce the levels of cell wall galactosyl residues in ripening tomato fruit (Carey *et al.*, 2001). Among these β -galactosidase (*TBG*) genes, *TBG6* shows the highest expression levels, but only at the mature green stage, prior to the development of visible signs of ripening (Smith and Gross, 2000). Moctezuma *et al.* (2003) reported that antisense suppression of *TBG6* in tomato increases fruit cracking, suggesting an important role for these enzymes in early tomato fruit growth and development.

Invertases (β -fructofuranosidase) are enzymes that hydrolyze Suc to Glc and Fru. In tomato, acid invertase activity is active during fruit ripening, and hexose sugars stored in the vacuole are the primary storage carbohydrate (Manning and Maw, 1975). Primary transformants with a chimeric, antisense acid invertase gene (*TIV1*) resulted in Suc accumulation in ripe fruit (Ohyama *et al.*, 1995). The constitutive expression of the *TIV1* antisense resulted in elevated levels of Suc but smaller fruit size and elevated ethylene production rates in T₁ transgenic plants (Klann *et al.*, 1996). These reports suggest that genetic modification of a single invertase gene has the potential of altering soluble sugar composition in tomato fruit. Interestingly, Chengappa *et al.* (1999) expressed the antisense sucrose synthase gene under the control of a fruit-specific promoter. In the young fruit of the transgenic tomato plants, sucrose synthase activity was reduced by up to 99% without affecting starch or sugar accumulation.

Deoxyhypusine synthase (DHS) mediates the first of two sequential enzymatic reactions that post-translationally modify eukaryotic translation initiation factor-5A (eIF-5A) with spermidine that becomes the unusual amino acid, deoxyhypusine. Constitutively suppressing *DHS* gene in tomato resulted in transgenic plants whose fruit ripened normally but exhibited delayed postharvest softening and senescence (Wang *et al.*, 2005). The transgene expression caused pleiotropic effects: male sterility, larger and thicker leaves that accumulated chlorophyll, higher activity of photosystem II, and enhanced starch deposition in the stems.

2.1.2 Targeting ethylene biosynthesis or its receptors

Ethylene is a simple gaseous plant hormone that has profound effects on plant growth and development (Mattoo and Suttle, 1991; Abeles *et al.*, 1992). Its role in fruit ripening has been genetically established (Theologis, 1992). Enzymes that regulate ethylene biosynthesis in plants are: S-adenosylmethionine (SAM) synthase, 1-aminocyclopropane-1-carboxylate (ACC) synthase, and ACC oxidase (Fluhr and Mattoo, 1996; Mattoo and Handa, 2004). The genes encoding these enzymes (Hamilton *et al.*, 1990; Oeller *et al.*, 1991) as well as those that metabolize SAM or ACC (Klee *et al.*, 1991; Good *et al.*, 1994) have been targeted in order to manipulate ethylene biosynthesis and thereby to regulate fruit ripening (Figure 1; see also Table 3). From these studies, it has been elegantly demonstrated that ethylene is the ripening hormone and that modulation of ethylene biosynthesis using genetic engineering can yield fruits with predictable characteristics.

To investigate the transcriptional regulation of two ACC oxidase genes in peach, Rasori *et al.* (2003) created constructs in which the ACC oxidase promoters, *PpACO1* and *PpACO2*, were used to drive the expression of β -glucuronidase (*GUS*) gene. These constructs were introduced into tomato (cv. Microtom). It was found that both promoters were active in tomato. *PpACO1-GUS* promoter activity was localized in leaf blade, ovary, leaf, fruit abscission zones, and pericarp, being up-regulated by propylene and wounding. Using a transient activity assay with peach fruit and constructs created by deleting different sections of the *PpACO1* promoter region, it was possible to find a region located between -716 and -346 bp, containing an ethylene-responsive element. Furthermore, two auxin responsive elements located upstream of the ethylene responsive elements were found. *PpACO2-GUS* promoter activity was found in vascular bundles of small and ripe fruit, senescent leaf blade, fruit and leaf abscission zones. The transformation of tomato for the expression of an apple antisense ACC oxidase led to reduced production of ethylene in the transgenic tomato fruit. Thus, tomato is a good crop model for testing specific ripening-related genes from heterologous species (Bolitho *et al.*, 1997).

The first characterization of an ethylene receptor gene from *Arabidopsis*, *AtETR1*, responsible for a dominant ethylene insensitivity showing many similarities with the two-component regulators in yeast and bacteria (Chang *et al.*, 1993), was another landmark discovery that virtually opened the area of research on ethylene signaling for genetic dissection as well as for biotechnological manipulations. Subsequently, the ethylene receptor gene family in tomato was found to consist of six members *LeERT1-LeETR6* (Wilkinson *et al.*, 1995; Zhou *et al.*, 1996a, b; Lashbrook *et al.*, 1998b; Tieman and Klee, 1999; Klee and Tieman, 2002). Each of the receptor genes has a distinct pattern of expression throughout development and in response to external stimuli. For instance, *LeERT1* and *LeETR2* are constitutively expressed in all tissues throughout development with *LeETR1* expression is about five times that of *LeETR2*. Results of a study that employed *LeERT1* antisense expression in tomato showed that the negative regulation model of ethylene receptors in ethylene signaling may not apply to ethylene-regulated abscission (Whitelaw *et al.*, 2002). The amino acid sequences of *LeERT1* and *LeERT2* protein are more closely related to *ERT1* of *Arabidopsis*, are highly similar to each other, and are constitutively expressed in all tomato tissues throughout development (Zhou *et al.*, 1996a, b; Lashbrook *et al.*, 1998b). However, their functional significance in ethylene receptor family is still unclear. With the goal to determine the effect of *LeETR1* and *LeETR2* specifically in the ethylene receptor family in tomato on ethylene signaling, Wang *et al.* (2006) generated two transgenic lines of tomato, ale1 and ale2, by introducing the antisense copy of *LeERT1* or *LeERT2* separately. A cross of ale1 and ale2 lines produced a line that had a phenotype similar to the ale1 in many aspects, which suggested that *LeERT1* plays a role in ethylene signaling of tomato growth and development.

In *Arabidopsis*, ethylene-insensitive 3 (*EIN3*) gene encodes a transcription factor that functions downstream from the ethylene receptors in the ethylene signal transduction pathway. Homologs of the *Arabidopsis EIN3* gene are present in tomato: *LeEIL*, *LeEIL1*, *LeEIL2*, and *LeEIL3*. Chen *et al.* (2004a) fused *EIL1* with green fluorescence protein (GFP) and induced its expression constitutively in the ethylene-insensitive

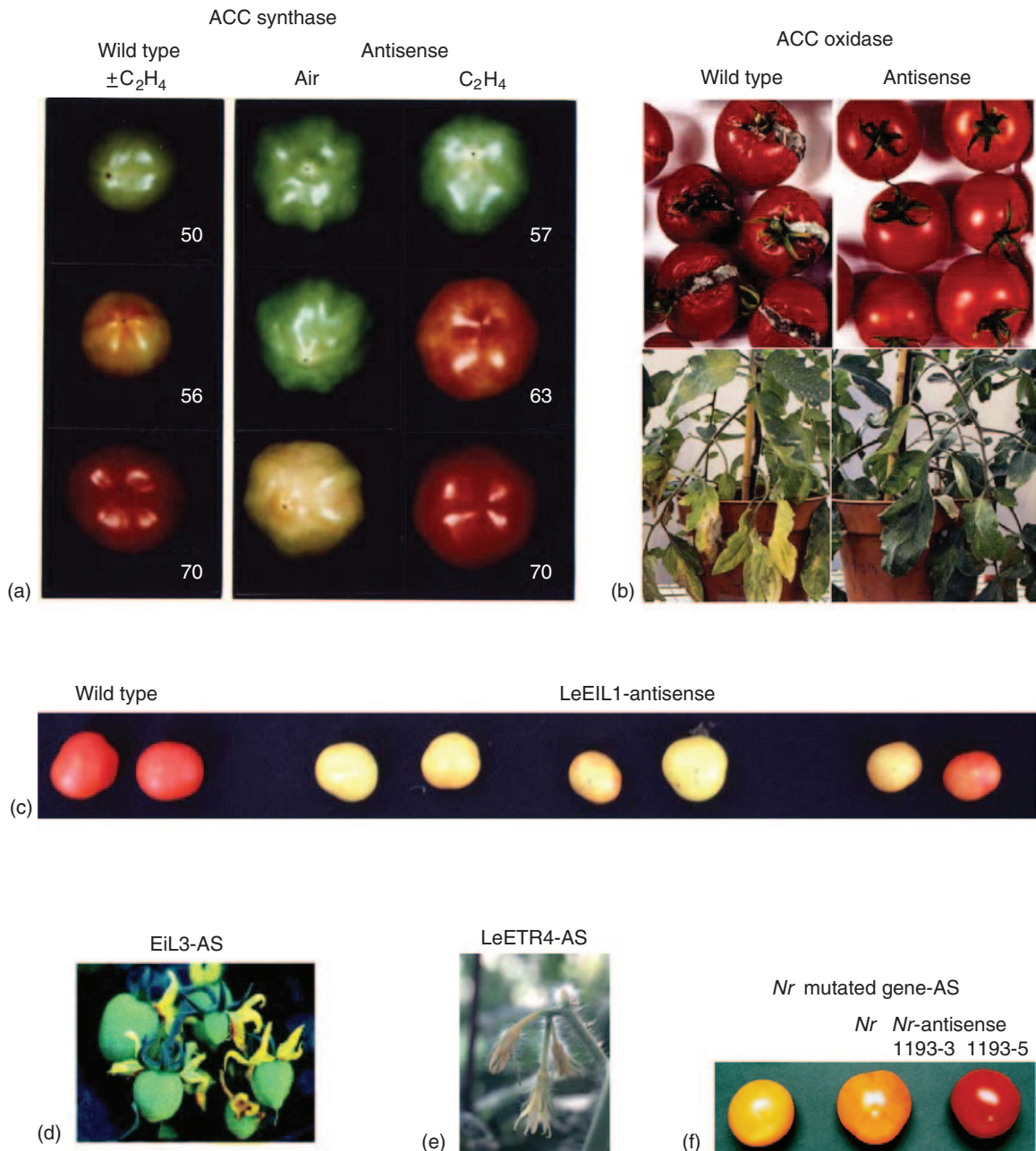


Figure 1 Reverse genetics studies that established the role of ACC synthase, ACC oxidase, ethylene-insensitive 3 like tomato homologs (LeEIL1 and LeEIL3) transcription factors, ethylene receptor LeETR4, and ethylene receptor NR in fruit ripening. Shown are the phenotypes obtained: (a) fruit ripening in the presence and absence of ethylene treatment after introduction of an antisense gene for ACC synthase (Oeller *et al.*, 1991), (b) senescence of tomato fruits and leaves following inhibition of ACC oxidase by its antisense gene (Picton *et al.*, 1993; John *et al.*, 1995), (c) fruit ripening in LeEIL1 antisense plants (Tieman *et al.*, 2001), (d) flower abscission in LeEIL3 antisense plants (Tieman *et al.*, 2001), (e) flower senescence in LeETR4 antisense (Tieman *et al.*, 2000), and (f) restoration of fruit ripening in Never-ripe (*Nr*) mutant by knocking out expression of the mutated ethylene receptor by its antisense (Hackett *et al.*, 2000). We thank Prof. Athanasios Theologis, Prof. Don Grierson, Prof. Harry Klee, and Dr. Denise Tieman for the original photographs of the illustrations shown here

tomato mutant Nr. The chimeric protein was found localized in the nuclei of the transgenic tomato fruit cells, and its overexpression induced a phenotype similar to the wild-type plants. Thus, this work emphasized the importance of using the Nr genetic background in studies to understand the ethylene-signaling pathway in tomato.

2.1.3 Tomato fruit quality

Tomato fruit and its processed products are the principal dietary sources of carotenoids such as lycopene. Lycopene is a potent antioxidant with the potential to prevent epithelial cancers and improve human health. Therefore, there is considerable interest to elevate the levels of carotenoids in tomato fruit by genetic manipulation and thereby improve the nutritional quality of the crop (Fraser *et al.*, 2002; Mehta *et al.*, 2002). It was shown that the constitutive expression of phytoene synthase 1 (*Psy-1*) in transgenic tomato resulted in a number of pleiotropic effects, including dwarfism due to diversion of geranylgeranyl diphosphate (GGPP) away from gibberellin biosynthesis (Fray *et al.*, 1995). Further work demonstrated that the use of regulatable promoters with heterologous genes could override the endogenous regulation and enable accumulation of higher levels of carotenoids in a fruit-specific manner. Thus, Mehta *et al.* (2002) developed transgenic tomatoes by introducing yeast SAM decarboxylase fused to the ripening-inducible promoter E8, and the transgenic fruits accumulated polyamines spermidine (Spd) and spermine (Spm) at the cost of the diamine putrescine. The independent homozygous lines accumulated lycopene levels that were two to three times higher than the nontransformed or azygous control and the fruit juice quality of the transgenics was improved by 50%. In another study, bacterial phytoene synthase was fused to the tomato PG promoter and overexpressed in Ailsa Craig (Fraser *et al.*, 2002). The fruits from the resulting transformants showed higher levels of carotenoids, phytoene, lycopene, β -carotene, and lutein. The biosynthetically related isoprenoids, tocopherols, plastoquinone, and ubiquinone, and the activities of isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, and incorporation of isopentenyl diphosphate into phytoene were not significantly altered.

Engineering the accumulation of higher polyamines, Spd and Spm, in tomato fruit enabled nuclear magnetic resonance (NMR) spectroscopic analysis for profiling distinct metabolite trends in the transgenic and wild-type/azygous fruits ripened off the vine (Mattoo *et al.*, 2006). Gln, Asn, choline, citrate, fumarate, malate, and an unidentified compound A also accumulated in the red transgenic fruit while the levels of Val, Asp, sucrose, and glucose were significantly reduced as compared to the control (wild type and azygous) red fruit. Specificity of these changes was apparent since the levels of Ile, Glu, γ -aminobutyric acid (GABA), Phe, and fructose remained similar in the nontransgenic and transgenic fruits. Mattoo *et al.* (2006) suggested that the pathways involved in the nitrogen sensing/signaling and carbon metabolism are targeted when higher levels of Spd/Spm accumulate in the fruit. These results also demonstrated that fruit cells can metabolize amino acid pathways late in fruit ripening independent of the parent plant.

Flavonoids are polyphenols whose dietary intake has the potential to prevent chronic diseases. Schijlen *et al.* (2006) introduced heterologous, flavonoid pathway genes—stilbene synthase, chalcone synthase, chalcone reductase, chalcone isomerase, and flavone synthase, to produce novel flavonoids in tomato fruit. These novel flavonoids—flavones and flavonols—increased threefold, mostly in the peel that was found to have higher total antioxidant capacity. These findings add further credence to the potential of engineering tomato fruit for accumulation of high levels of beneficial nutrients.

The first successful study conducted to engineer the taste of tomato fruit involved transformation of tomato with the thaumatin gene from the African plant “katemfe” (Bartoszewski *et al.*, 2003). Thaumatin is a sweet-tasting protein. Fruit from T₂ transgenic plants tasted sweeter than the control plants, leaving a unique and sweet-specific aftertaste.

The volatile aroma compounds are essential for good flavor in tomato (Baldwin *et al.*, 2000). The tomato genome contains two genes potentially encoding carotenoid cleavage dioxygenases, *LeCCD1A* and *LeCCD1B*; *LeCCD1B* being highly expressed in the ripening fruit. Antisense expression of *LeCCD1B* gene in tomato reduced transcripts for both the genes in leaves

and fruits. Further, the transgenic lines were found to have reduced levels of volatiles β -ionone and geranylacetone, suggesting the importance of these genes in the production of tomato volatiles (Simkin *et al.*, 2004). A similar approach was used by Tieman *et al.* (2006) to overexpress *LeAADC1A* and *LeAADC2* in tomato, which led to a 10-fold increase in the emission of volatiles, 2-phenylacetaldehyde and 2-phenylethanol. The antisense-mediated reduction of *LeAADC2* gene significantly decreased the emission of these volatiles.

Chen *et al.* (2004b) reported the identification of a specific isoform of tomato lipoxygenase, *TomloxC*, involved in the generation of fatty acid-derived flavor components. Sense and antisense constructs of *TomloxC* were designed and introduced in tomato plant. The transgenic plants in which *TomloxC* transcripts were reduced showed a reduction in the flavor volatiles including hexenal, hexanal, and hexanol. Thus, this gene is another candidate for manipulation of aroma/flavor of tomato fruit.

2.2 Transgenic Tomatoes Resistant or Tolerant to Biotic Stresses

The increasing importance of integrated pest management, largely as a result of concerns over environmental quality and food safety, together with the failure of conventional chemicals due to the development of increasing number of insecticide-resistant species has provided a major niche for deploying the potential of insecticidal proteins such as *Bacillus thuringiensis* (*Bt*) in protection against insects (Dent, 1993; Marron, 1993; Forrester, 1994). The cry protein toxins have enormous commercial potential as safe, biodegradable pesticides. What is particularly important is that a biopesticide is specific to the insect and does not affect aquatic or forest ecosystems where other beneficial and nontarget insect must be conserved (May, 1993). In 1909, Berliner had isolated a sporulating bacterium from diseased mediterranean flour moth larvae he received from a mill in Thuringen. He named the bacterium, *Bt*, from which the toxin protein called *Bt* was first used as an insecticide in the early 1930s, primarily against the European corn borer (*Ostrinia nubilalis*) in Southeast Europe.

The first commercial product (spreine) was available in 1938 in France (Weiser, 1986) for the control of the flour moth (Jacobs, 1951). In 1960, stimulated by the growing concern over the use of chemical insecticides, the first *Bt* strain was commercialized and was marketed as "Thuricide."

Genetic engineering of disease resistance by transferring defence-related genes or pathogen-origin genes into crops has economic value as well as maintaining an eco-friendly environment by cutting the costs and reducing the pesticide usage (Shah, 1997; Salmeron and Vernooij, 1998; Rommens and Kishore, 2000; Stuiver and Custers, 2001). In cotton alone the introduction of *Bt* has resulted in reduction of over 95 million kilograms of pesticide use worldwide in the first 10 years of commercial use (Brookes and Barfoot, 2006). Since insect infestation causes significant losses in tomato, it is likely that the genetic engineering approaches have the potential to substantially reduce pesticide use in tomato and other vegetable crops. Likewise, much more reduction in the overall pesticide use is anticipated by engineering vegetable crops to resist various viral, fungal, and bacterial pathogens. Significant progress has been made in developing resistance to biotic stress in all major crops including tomato.

2.2.1 Insect infestation

Table 4 lists genes that represent a variety of functional activities and which have been used to engineer insect resistance in tomato. These include *Bt* (Cry1Ac), proteinase inhibitors, expansin, arginase, threonine deaminase, sarcotoxin1 A, plant lectin; Mi-1.2 protein, a member of the nucleotide-binding leucine-rich repeat (NB-LRR) protein, and carboxypeptidases. However, only in limited cases efficacy of the introduced genes has been field evaluated for resistance trait (Rhim *et al.*, 1995). Until such studies are performed, the utility of these genes or the putative "insect-resistant" plants will remain obscure.

Tomato plants transformed with a crystal δ -endotoxin gene encoding a coleopteran insect-specific toxin from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) were shown to express a 74 kD protein that cross-reacted with *Bt* toxin antibodies. These transgenic plants exhibited a

Table 4 Genetic manipulation of *Solanum lycopersicum* L. for insect resistance

Gene product	Source	Promoter:gene	Phenotype (tolerance)	References
Arginase	Tomato	CaMV 35S:ARG2 overexpression	Increased resistance to <i>M. sexta</i> larvae Identification of JA-inducible host protein in insect midgut	Chen <i>et al.</i> , 2005
Bt toxin	<i>Bacillus thuringiensis</i> subsp. <i>tenebrionis</i>	CaMV 35S:B.t.t.	Colorado potato beetle larvae	Rhim <i>et al.</i> , 1995
Chitinase	Poplar	CP:win6	Inhibition of development of Colorado potato beetle	Lawrence and Novak, 2006
Chymotrypsin inhibitor	Tomato	CaMV 35S:JIP21	Increased mortality and delayed growth of Egyptian cotton worm (<i>Spodoptera littoralis</i>)	Lison <i>et al.</i> , 2006
Cry1Ac	<i>Bacillus thuringiensis</i> (Bt)	CaMV 35S:cry1Ac	Tomato fruit borer (<i>Helicoverpa armigera</i>). Resistant leaves and fruits	Mandaokar <i>et al.</i> , 2000
Lectin <i>Galanthus nivalis</i> agglutinin	Snow drop	:GNA	Significant increases in the mean larval weight and in the amount of food consumed by <i>L. oleracea</i> larvae feeding on transgenic plants	Wakefield <i>et al.</i> , 2006
Nucleotide-binding, leucine-rich repeat PR protein	Tomato	CaMV 35S:Mi-1.2	Resistance to nematode (<i>Meloidogyne</i> spp.) and aphid (<i>Macrosiphum euphorbiae</i>)	Goggin <i>et al.</i> , 2006
Pin and carboxypeptidase inhibitors	Potato	<i>StLS1</i> :PI-II and <i>rbcs-1A</i> :PCI	<i>Heliothis obsoleta</i> and <i>Liriomyza trifolii</i>	Abdeen <i>et al.</i> , 2005
Sarcotoxin1 A	Fleshfly (<i>Sarcophaga peregrine</i>)	Tob:Sarc	Parasitic weed (<i>Orobancha aegyptiaca</i>)	Radi <i>et al.</i> , 2006
Systemin	Tomato	CaMV 35S:prosystemin antisense	Reduced resistance toward <i>Manduca sexta</i> (tobacco hornworm) larvae	Orozcocardenas <i>et al.</i> , 1993

significant insecticidal activity when challenged with Colorado potato beetle larvae (Rhim *et al.*, 1995). A synthetic *cry1Ac Bt* gene was introduced into tomato plants using *Agrobacterium*-mediated transformation (Mandaokar *et al.*, 2000) and high levels of *Bt* expression in the leaves of transgenic plants were ascertained by enzyme-linked immunosorbent assay (ELISA). The transformed plant leaves and fruits were found resistant to the larvae of tomato fruit borer (*Helicoverpa armigera*). The limited field trail of T₁ generation was successful in demonstrating resistance against *H. armigera*.

Constitutive expression of a prosystemin antisense gene in tomato resulted in about three times higher growth rates of *Manduca sexta* (tobacco hornworm) larvae feeding on transgenic plants

compared with nontransformed control plants (Orozcocardenas *et al.*, 1993). In the leaves of antisense transgenic tomato plants, proteinase inhibitors I and II were undetectable until the 6th day of larval feeding before exhibiting an increase throughout the plants reaching over 100 mg g⁻¹ of leaf tissue after 14 days. In the nontransformed control plants, in comparison, a rapid increase in both proteinase inhibitors occurred from the second day of larval feeding reaching over 225 mg g⁻¹ of leaf tissue by the 8th day. These results offered the first evidence showing that plant resistance against an insect pest can be genetically modulated by molecular engineering.

In order to evaluate whether or not a combination of two anti-insect genes will be

synergistic in preventing insect infestation, Abdeen *et al.* (2005) overexpressed two different potato protease inhibitors, the serine-proteinase inhibitor PI-II and the carboxypeptidase inhibitor (PCI) in tomato plants. Serine proteases (trypsin and chymotrypsinlike) are the main midgut proteases of *H. armigera* and *Heliothis virescens* while carboxypeptidases have also been identified (Katherine, 1995; Bown *et al.*, 1998). Leaf-specific overexpression of the PI-II and PCI resulted in increased resistance to *Heliothis obsoleta* and *Liriomyza trifolii* larvae in homozygous tomato lines expressing high levels (>1% the total soluble proteins) of the transgenes. However, leaf damage in hemizygous lines for these transformants was more severe than in controls. The experiment suggested that the combined expression of defence genes with different mechanisms of action rather than combination of inhibitors may be more effective in overcoming the general adaptive response of the insects. Tomato proteinase inhibitor II genes (*tin2*, *tin2i*) when transformed into tobacco showed that the tobacco transformants carrying *tin2i* sequence were more resistant to 2-instar larvae of *Heliothis armigera* Hubner than those carrying the *tin2* sequence. Therefore, the intron of *tin2i* sequence may contribute to the insecticidal activity of the transgenic tobacco. A proteinaceous aspartic proteinase inhibitor, designated as tomato chymotrypsin inhibitor 21 (*TCI21*), was expressed in tomato and found to increase mortality and delay growth of Egyptian cotton worm (*Spodoptera littoralis*) (Lison *et al.*, 2006).

Chen *et al.* (2005) used a mass spectrometry-based approach to identify host proteins that accumulate in the midgut of *M. sexta* larvae reared on tomato plants. They showed that two jasmonate-inducible proteins, arginase and threonine deaminase, act in the *M. sexta* midgut to catabolize the essential amino acids arginine and threonine, respectively. Transgenic plants overexpressing arginase were more resistant to *M. sexta* larvae, and this effect correlated with reduced levels of midgut arginine. This work demonstrated that the jasmonate signaling pathway strongly influences the midgut protein content of phytophagous insects and further supports the hypothesis that amino acid catabolism in the insect digestive tract by host enzymes plays a role in plant protection against herbivores.

The parasitism of root-knot nematode (*Meloidogyne javanica*) was controlled when the antisense expansin *LeEXPPA5* was expressed in tomato gall cells adjacent to the nematode feeding cells. It was concluded that the reduced expansin expression reduced the ability of the nematode to complete its life cycle in transgenic roots (Gal *et al.*, 2006). The effect of ingestion of transgenic tomato leaves expressing the plant lectin *Galanthus nivalis* agglutinin (GNA) on development of larvae of *Lacanobia oleracea* (Linnaeus) was studied by Wakefield *et al.* (2006). They reported that when *L. oleracea* larvae were fed on tomato line 14.1H expressing approximately 2.0% GNA, significant increases in the mean larval weight and in the amount of food consumed were found. Examination of the effects of ingestion of tomato leaves expressing GNA on the development of the tomato moth, *L. oleracea*, showed that larvae grew larger, developed more quickly, and consumed more plant material than larvae fed on untransformed tomato leaves. Also, survival of *L. oleracea* to the adult stage was much reduced on untransformed tomato leaf compared to previously published figures for survival on untransformed potato leaf and artificial diet (Bell *et al.*, 2001). In a further series of experiments the effects of GNA ingestion by the host larvae on the development and survival of the solitary endoparasitoid *Meteorus gyrator* were evaluated. Life history parameters examined for development of *M. gyrator* in hosts reared on transgenic or nontransgenic diets were not significantly altered, regardless of the time period until when the host was maintained on the diet. The authors suggested that *M. gyrator* is not particularly sensitive to GNA present in the host.

Transformation of a susceptible tomato cv. Moneymaker with the *Mi-1.2* gene resulted in resistance to nematodes and aphids (Goggin *et al.*, 2006). The *Mi-1.2* locus confers resistance against root-knot nematodes (*Meloidogyne* spp.), the potato aphid (*Macrosiphum euphorbiae*), and the sweet potato whitefly (*Bemisia tabaci*). This locus was introgressed into cultivated tomato about 60 years ago through a cross with a wild tomato species, *Solanum peruvianum* (syn. *L. peruvianum*) (Smith, 1944). The *Mi-1.2* protein is a member of the NB-LRR class of plant resistance genes. Interestingly, when the same chimeric gene construct was used to transform a susceptible

variety of eggplant, the transgenic lines displayed resistance to the root-knot nematode *M. javanica* but were fully susceptible to the potato aphid indicating involvement of additional genes in aphid resistance.

Transgenic tomato plants expressing the sarco-toxin IA gene were grown either in polyethylene (PE) bags or in pots inoculated with *Orobanche aegyptiaca* seeds. Transgenic plants exhibited strong inhibition of parasite growth and significantly increased yield as compared with nontransgenic ones. In both PE and pot systems, most of the parasite tubercles attached to the transgenic root plants turned necrotic and developed abnormally. These results indicate that the insect gene produced in the plant cells was selectively toxic to the parasite and not the host plant (Radi *et al.*, 2006).

Lawrence and Novak (2006) used a recombinant potato virus X (PVX), carrying a gene for chitinase, WIN6, to infect tomato plants. The leaves from infected plants were tested for insecticidal properties on Colorado potato beetle (CPB; *Leptinotarsa decemlineata* (Say)). Less than half of CPB neonates feeding on leaves containing >0.3% w/w WIN6 developed to 2nd instar compared to 93% on control uninfected leaves. These results confirmed the role of chitinases in providing resistance against insects and other pathogens (Kumar *et al.*, 2004).

2.2.2 Fungal pathogens

Plants are constantly exposed to various kinds of microbes, which derive nutrition from them. To overcome the pathogen infestation, plants have evolved both broad-spectrum and species-specific mechanisms. As a part of defence strategy plants produce low-molecular-weight compounds and proteins that inhibit the growth of phytopathogenic fungi. In recent years, significant understanding of host–pathogen interactions has led to the characterization of specific genes that play a role in disease establishment. A number of antimicrobial proteins (typically consisting of ~100 amino acid residues) and polypeptides (around 30–60 amino acid residues) have been identified (Veronese *et al.*, 2003). Additionally, pathways leading to production of various antifungal compounds, such as phytoalexins, have been characterized (Morrissey and Osbourn, 1999).

Although some of these genes have been expressed in transgenic tomato to enhance tolerance to fungal pathogens (see a summary in Table 5), new targets will emerge from further understanding of the interactions between plants and their fungal pathogens (Maor and Shirasu, 2005).

Phytoalexins are a class of chemicals that are implicated in plant's defence responses by acting as toxins to invading phytopathogens. They represent several classes of chemicals including alkaloids, terpenoids, and glycosteroids. This plant defensive arsenal is generally induced in plants when they confront a pathogen attack. Stilbene synthase produces the phytoalexin transresveratrol. Thomzik *et al.* (1997) transformed tomato with two stilbene synthase genes from grapevine (*Vitis vinifera*) and characterized the transgenic plants for stable integration and expression of the transgene, and finally tolerance of the transgenics to *P. infestans*. The authors reported that both the introduced genes were stably maintained and expressed in transgenic regenerants. Upon fungal inoculation, transgenic plants accumulated the phytoalexin transresveratrol, the product of stilbene synthase, and exhibited increased resistance to *P. infestans*. Inoculation of transgenic tomato with *Botrytis cinerea* and *Alternaria solani* also caused accumulation of resveratrol but plants did not show significant resistance to these fungi.

Schaefer *et al.* (2005) introduced genes coding for an iris ribosomal-inactivating protein (I-RIP), a maize β -glucanase (M-GLU), and a *Mirabilis jalapa* antimicrobial peptide (Mj-AMP1) into tomato. Selected transgenic lines were inoculated with a suspension containing $2\text{--}3 \times 10^4$ conidial spores/ml of the fungal pathogen *A. solani*, the causal agent of tomato early blight. Compared to parental control, two transgenic lines carrying either M-GLU or Mj-AMP1 showed enhanced resistance to early blight disease. None of the four lines carrying the I-RIP transgene showed resistance to early blight.

A synergistic effect of a tobacco class I chitinase and β -1,3-glucanase genes in planta was first demonstrated by Jongedijk *et al.* (1995). The double transformants showed a 36–58% reduction in disease severity when infected with *F. oxysporum* f. sp. *lycopersici*. The transgenic tomato plants expressing either one of these genes were not protected against fungal infection. Tabaeizadeh

Table 5 Genetic manipulation of *Solanum lycopersicum* L. for fungal resistance

Gene product	Source	Promoter:gene	Phenotype (tolerance)	References
<i>Arabidopsis</i> thionin	<i>Arabidopsis</i>	Fruit inactive <i>RB7</i> : <i>Thi2.1</i>	Dual resistance-BW (<i>R. solanacearum</i>) and FW (<i>F. oxysporum</i>)	Chan <i>et al.</i> , 2005
Chitin-binding domain and PR protein	Rubber tree (<i>Hevea brasiliensis</i>)	CaMV 35S:HEV1	Retardation of growth of <i>Trichoderma hamatum</i>	Lee and Raikhel, 1995
Chitinases and β -1,3-glucanase	Tobacco	CaMV 35S:Chi-I,II and Glu-I,II	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> /synergistic effect	Jongedijk <i>et al.</i> , 1995
Endochitinase	Tomato (<i>Lycopersicon chilense</i>)	CaMV 35S:pcht28	Tolerance to <i>Verticillium dahliae</i>	Tabaeizadeh <i>et al.</i> , 1999
Nonexpresser of PR genes	<i>Arabidopsis</i>	CaMV 35S:NPR1	Broad-spectrum resistance toward ToMV, BW, FW, gray leaf spot (GLS, BS)	Lin <i>et al.</i> , 2004
Oxalate decarboxylase	<i>Collybia velutipes</i>	CaMV 35S:OXDC	<i>Sclerotinia sclerotiorum</i>	Kesarwani <i>et al.</i> , 2000
Pear fruit PG inhibitor protein	Pear	CaMV 35S:pPGIP	Delayed disease lesions and tissue maceration/inhibition of fungal PGs	Powell <i>et al.</i> , 2000
PR-S, PR-1a (elicitor)	<i>Cladosporium fulvum</i> /fungus in tomato	P35S-ALMV:pAVIR1, P35S-ALMV-PRs:pAVIR2 and P35S-ALMV-PR1a:pAVIR21	AVR9 peptide in plants with Cf9 gene is deleterious	Honee <i>et al.</i> , 1995
Ribosomal inactivating, β -glucanase and antimicrobial peptide	Iris, maize, <i>Mirabilis jalapa</i>	CaMV 35S :I-RIP, M-GLU, Mj-AMP1	Early blight- <i>Alternaria solani</i> in M-GLU or Mj-AMP1 transgenic but not in I-RIP	Schaefer <i>et al.</i> , 2005
Stilbene synthase (phytoalexin)	Grapevine (<i>Vitis vinifera</i>)	CaMV 35S:vst1 and vst2	Increased resistance to <i>P. infestans</i>	Thomzik <i>et al.</i> , 1997

et al. (1999) observed the effect of constitutive expression of an acidic endochitinase gene, *pcht28*, from *Lycopersicon chilense* for resistance against *V. dahliae*. The R_1 plants evaluated in the greenhouse for tolerance to *V. dahliae* race 1, 2 and R_2 plants against race 2 showed a significantly ($P < 0.05$) higher level of tolerance to the fungi compared to the nontransgenic plants. The resistance was demonstrated by foliar disease symptoms and vascular discoloration index.

Honee *et al.* (1995) reported the potential use of avirulence genes in tomato and tobacco. They reconstructed Avr9 from the tomato fungal pathogen *C. fulvum*, pAVIR1, pAVIR2, and pAVIR21, respectively encoding the wild-type AVR9 protein and two hybrid AVR9 proteins containing the signal sequences of the

pathogenesis-related proteins PR-S and PR-1a. The Cf0 genotypes of tomato, with construct pAVIR21, showed a normal phenotype.

Hevein, a protein from rubber tree (*Hevea brasiliensis*), is composed of one chitin-binding domain and a C-terminal polypeptide homologous to PR proteins such as tobacco PR-4 and tomato P2 proteins. Constitutive expression of mature hevein in tomato resulted in retardation of growth of *Trichoderma hamatum* in transgenic plants (Lee and Raikhel, 1995).

Heterologous expression of pear fruit polygalacturonase inhibitor protein (pPGIP) resulted in abundant accumulation of (PGIP) in all tissues and did not alter the expression of an endogenous tomato fruit PGIP (Powell *et al.*, 2000). The growth of *B. cinerea* on ripe tomato

Table 6 Genetic manipulation of *Solanum lycopersicum* L. for bacterial resistance

Gene product	Source	Promoter:gene	Phenotype (tolerance)	References
Cys-2/His-2 zinc finger protein-TF	Pepper (<i>Capsicum annuum</i>)	CaMV 35S:CaPIF1 overexpression	Tolerance to cold stress and to the bacterial pathogen <i>P. syringae</i> pv. <i>tomato</i> DC 3000	Seong <i>et al.</i> , 2007
Glycoprotein antibacterial protein	Human	:lactoferrin (LF)	Partial resistance to <i>Ralstonia solanacearum</i> Smith (bacterial wilt)	Lee <i>et al.</i> , 2002
Heveinlike protein	<i>Pharbitis nil</i>	CaMV35S:Pn-AMP2	Resistant against <i>Phytophthora capsici</i> and <i>Fusarium oxysporum</i>	Lee <i>et al.</i> , 2003
Magainin-antimicrobial peptide	Synthetic	ECaMV 35S:MSI-99	Resistance to <i>Pseudomonas syringae</i> (bacterial Speck)	Alan <i>et al.</i> , 2004
Polyphenol oxidases	Potato Potato	CaMV 35S:PPO CaMV 35S:PPO antisense	<i>P. syringae</i> /PPO-mediated phenolic oxidation Increased susceptibility to <i>P. syringae</i>	Li and Steffens, 2002 Thipyapong <i>et al.</i> , 2004
Serine/threonine protein kinase	Tomato	CaMV 35S: <i>Pto</i> overexpression	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> and <i>Cladosporium fulvum</i>	Tang <i>et al.</i> , 1999

fruit expressing pPGIP was reduced, and tissue breakdown was diminished by as much as 15% as compared with nontransgenic fruit. However, in an earlier investigation Desiderio *et al.* (1997) characterized different PGIPs from *Phaseolus vulgaris* and reported that *P. vulgaris* produces several PGIPs with different specificities for various phytopathogenic fungi. These authors also reported that the high-level expression of *PGIP-1*, a member of the *PGIP* gene family, in transgenic tomato plants is not sufficient to confer enhanced resistance to either *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, or *A. solani*. More work is needed to characterize PGIP produced in different species to identify genes with generalized resistance to various phytopathogenic fungi.

Oxalic acid is used by several phytopathogenic fungi in their attack on plants. Therefore, research was done to overexpress oxalate decarboxylase from *Collybia velutipes* in transgenic tobacco and tomato both of which developed resistance to fungal infection. These transgenic tobacco and tomato plants showed remarkable resistance to phytopathogenic fungus *Sclerotinia sclerotiorum* that utilizes oxalic acid during infestation (Kesarwani *et al.*, 2000).

The *Arabidopsis* thionin (*Thi2.1*) gene was used to genetically engineer enhanced resistance to multiple diseases in tomato. A construct was created in which the fruit-inactive promoter RB7

was used to control the expression of the *Thi2.1* gene. In transgenic lines containing *RB7/Thi2.1*, constitutive *Thi2.1* expression was detected in roots and a little in leaves, but not in fruits. Disease assays revealed that the transgenic lines tested showed enhanced resistance to bacterial wilt (BW) and *Fusarium* wilt (FW). It was found that BW disease progression in transgenic lines was delayed by a systemic suppression of bacterial multiplication (Chan *et al.*, 2005).

2.2.3 Bacterial pathogens

Table 6 summarizes seminal studies on developing resistance against bacterial pathogens. The tomato disease resistance (*R*) gene *Pto* encodes a serine/threonine protein kinase that is postulated to be activated by a physical interaction with the *AvrPto* protein. It also specifies race-specific resistance to the bacterial pathogen *P. syringae* pv. *tomato* carrying the *avrPto* gene. Overexpression of *Pto* under the cauliflower mosaic virus (CaMV) 35S promoter resulted in microscopic cell death, salicylic acid accumulation, and increased expression of PR genes indicating activation of defence responses in the absence of the *Pto-AvrPto* interaction. Only the palisade mesophyll cells exhibited cell death that required light for induction. Mesophyll

cells expressing *Pto* showed accumulation of autofluorescent compounds, callose deposition, and lignification. The transgenic tomato plants exhibited significant resistance to *P. syringae* without *avrPto* and reduced bacterial growth than did nontransgenic lines. These transgenics also showed more resistance to *Xanthomonas campestris* pv. *vesicatoria* and *C. fulvum*. Taken together, these results provided evidence that overexpression of an *R* gene can activate defence responses and general resistance to bacterial pathogens (Tang *et al.*, 1999).

A cationic iron-binding glycoprotein, lactoferrin (LF), was tested for developing transgenic tomatoes resistant to bacterial-wilt-causing *Ralstonia solanacearum* Smith. R_1 and R_2 transgenic lines inoculated with race 1 exhibited early resistance and subsequent susceptibility, while 44–55% of plants survived until fruit maturity (Lee *et al.*, 2002).

Thipyapong *et al.* (2004) introduced a chimeric antisense of the polyphenol oxidase enzyme (PPO) complementary-DNA (cDNA) into tomato. It was found that all members of the *PPO* gene family were down-regulated. Down-regulation of *PPO* in antisense plants did not affect growth, development, or reproduction of greenhouse grown plants. However, antisense *PPO* expression dramatically increased susceptibility to *P. syringae*, suggesting a critical role for PPO-catalyzed phenolic oxidation in limiting disease development in tomato.

Seong *et al.* (2007) have previously isolated a *Capsicum annuum* pathogenesis induced factor (CaPIF1) from pepper leaves after infection with the soybean pathogen *Xanthomonas axonopodis* pv. *glycines* 8ra. Overexpression of *CaPIF1*, which encodes a Cys-2/His-2 zinc finger transcription factor, resulted in major transcriptional modulation without exhibiting any visual morphological abnormality. The up-regulated genes included those from various metabolic pathways, and stress and defence response categories. The transgenic plants exhibited tolerance to cold stress and to the bacterial pathogen *P. syringae* pv. *tomato* DC 3000, tolerance being correlated with the expression levels of *CaPIF1*. The authors suggested that *CaPIF1*, a plant-specific unique Cys-2/His-2 transcription factor, regulates expression of genes both directly and indirectly, leading to increased tolerance to biotic and abiotic stresses.

2.2.4 Viral pathogens

Substantial efforts have been made to engineer virus resistance in tomato plants. Table 7 lists some of the studies conducted and phenotypes obtained.

Whitefly transmitted geminiviruses are widely distributed in tropical and subtropical regions around the world, causing yield losses in important crops such as cassava, tomato, bean, cotton, and pepper (Brown and Bird, 1992; Polston and Anderson, 1997; Moriones and Navas-Castillo, 2000; Morales and Anderson, 2001). Fuentes *et al.* (2006) developed transgenic tomato plants transformed with an intron–hairpin construct to induce post-transcriptional gene silencing in the geminivirus tomato yellow leaf curl virus (TYLCV) replication-associated protein gene (*CI*). The intron–hairpin RNA produced involved 726 bp of the 3' end of the TYLCV *CI* gene as the arms of the hairpin, and the castor bean catalase intron. Transgenic tomato plants belonging to line 126, harboring a single transgene copy, showed immunity to TYLCV, even in extreme conditions of infection such as 4-leaf-stage plants and from 300 up to many hundreds viruliferous whiteflies per plant during 60 days. In an earlier study, Brunetti *et al.* (1997) had shown that high expression of a truncated version of the *CI* gene of tomato yellow leaf curl geminivirus, encoding the first 210 amino acids of the multifunctional *rep* protein, confers resistance to TYLCV in transgenic tomato plants.

Motoyoshi and Ugaki (1993) reported the transformation (with a chimeric tobacco mosaic virus (TMV) coat protein gene under the control of the CaMV 35S promoter) of an F_1 hybrid between *L. esculentum* and *L. peruvianum*. Transgenic line 8804-150 accumulated 2.5 mg coat protein per gram fresh weight in fully developed fresh leaves and exhibited the strongest resistance to tomato mosaic virus (TOMV) among the plants examined. The transgenic plants did not show any morphologic or physiologic differences when compared to the nontransgenic control plants. The accumulation of the coat protein and associated higher resistance to TMV were found to be transmissible to the next generation. Xue *et al.* (1994) also successfully developed transgenic tomato plants expressing a high level of resistance to cucumber mosaic virus strains of subgroup-I and subgroup-II. These transgenic tomatoes

Table 7 Genetic manipulation of *Solanum lycopersicum* L. for viral resistance

Gene product	Source	Promoter:gene	Phenotype (tolerance)	References
<i>rep</i> protein (T-Rep)	TYLCV	Enhanced CaMV 35S:CI Sense and antisense	Sense: Tomato yellow leaf curl virus T-Rep accumulation is required for resistance Cross between sense and antisense produced normal phenotype but was susceptible	Brunetti <i>et al.</i> , 1997
Chimeric TMV coat protein	TMV	CaMV 35S:TMV-CP	Lt1b1, a specific TMV mutant strain was used to assay the resistance	Motoyoshi and Ugaki, 1993
CMV satellite RNA	CMV	CaMV 35S:S-CARNA 5	Tolerance in transgenics, which produced mature unit-length satellite RNA after CMV infection	McGarvey <i>et al.</i> , 1994
Coat protein	CMV-WL CMV (subgroup II)	CaMV 35S:CP	Resistance to infection by CMV-WL and CMV-China, resistance to isolates from both subgroups of CMV	Xue <i>et al.</i> , 1994
Replicase	CMV	CaMV 35S:rep Defective rep gene	Replicase-mediated resistance affects long-distance movement	Gal-On <i>et al.</i> , 1998
C4 of TLCV encodes a protein	Tomato leaf curl geminivirus (TLCV)	CaMV 35S:C4 and C4 with frame shift version	No disease. TLCV encodes a protein involved in the development of disease symptoms during viral infection	Krake <i>et al.</i> , 1998
Nucleoprotein	Tobacco	:N POCA 28 vector	A hypersensitive response/components necessary for N-mediated resistance are conserved in tomato	Whitham <i>et al.</i> , 1996
Coat protein	CMV-D strain and Italian CMV isolates	FWMV:CMV-22, FWMV:CMV-PG	Resistance to CMV infection	Kaniewski <i>et al.</i> , 1999
Coat protein	Physalis mottle tymovirus (PhMV)	CaMV 35S:CP	Partial resistance to PhMV	Vidya <i>et al.</i> , 2000
Capsid protein	TYLCV	CaMV 35S:V1	Delayed disease symptoms	Kunik <i>et al.</i> , 1994
Movement proteins	Bean dwarf mosaic virus (BDMV)	pSK+:BV1 and:BC1	Significant delay in ToMV infection	Hou <i>et al.</i> , 2000
Truncated replicase	(CMV) strain GT, subgroup II	CaMV 35S:T-rep	T ₁ generation was resistant to viral inoculation	Nunome <i>et al.</i> , 2002
CMV satellite RNA	CMV	CaMV 35S:CMV Tfn-sat RNA	RNA silencing as the second mechanism determining resistance of transgenic tomato lines	Cillo <i>et al.</i> , 2004

that are resistant to isolates from both subgroups of cucumber mosaic virus (CMV) have practical significance for controlling this serious disease.

Transgenic tomato plants exhibiting a broad commercial resistance to CMV infection have been developed by expressing coat protein (*CP*) genes from the CMV-D strain and two Italian subgroups CMV-22 of subgroup I and CMV-PG of subgroup II (Kaniewski *et al.*, 1999). Transgenic plants were selected based on *CMV CP* expression, resistance to CMV in segregating progenies, and presence of single transgene copy. Transgenic plants generated

using *CP* from any of the strains showed broad resistance against CMV strains from both the subgroups, I and II. These transgenic lines were field tested to assess the level of resistance and agronomic performance (Tomassoli *et al.*, 1999). Field evaluation was performed at multilocations in Italy where the target virus spread naturally by the indigenous aphid populations. Although these trials showed CMV resistance of the transgenic tomatoes under field conditions, resistance was less effective in the field than what was observed in growth chamber experiments. Transgenic tomato

plants expressing the *CP* gene of *Physalis mottle* tymovirus (PhMV) showed delay in symptom development indicating partial resistance to the virus (Vidya *et al.*, 2000).

Whitham *et al.* (1996) tested if resistance to pathogen found in other plant species can be transferred by transformation across interspecies barriers. These authors transformed tomato with *N* gene from tobacco. *N* genes possess a putative nucleotide binding site and leucine-rich repeats and confer a gene-for-gene resistance against the viral pathogen TMV and most other members of the tobamovirus family (Whitham *et al.*, 1994). Tomato transgenics expressing the *N* gene exhibit a hypersensitive response and effectively restricts TMV to the sites of inoculation, as in tobacco. These results showed that all the components necessary for *N*-mediated resistance are conserved in tomato. Thus, the utility of using resistance genes from a heterologous species to protect crop plants from diseases was demonstrated for the first time. A viral nucleoprotein (N) was shown to impart resistance to tomato spotted wilt virus when expressed in tomato (Fedorowicz *et al.*, 2005). These authors further reported that transgenic plants exhibited resistance to virus infection even in the absence of detectable levels of the translational product of the introduced gene.

The interspecific tomato hybrid, *L. esculentum* × *Lycopersicon pennellii* (F₁), sensitive to the TYLCV disease was successfully transformed with *TYLCV* gene encoding capsid protein (V1). The R₁ plants inoculated with *TYLCV* using whiteflies showed delayed disease symptoms and increased recovery from the disease (Kunik *et al.*, 1994). Transgenic (R₁ progeny) tomato plants expressing CMV satellite RNA fused to *GUS* gene when inoculated with virion or RNA preparations of CMV or tomato aspermy virus (TAV) showed mild disease symptoms in the first 2 weeks followed by a decrease in symptoms (McGarvey *et al.*, 1994). Subsequently, the transgenic and control plants were same by the 4th week. Along with *CP* genes, the role of movement protein (BVI and BCI) and truncated replicase (T-rep) in conferring viral resistance seems apparent (Gal-On *et al.*, 1998; Hou *et al.*, 2000; Nunome *et al.*, 2002; Antignus *et al.*, 2004). Likewise, *N* genes from tobacco and tomato spotted wilt virus (TSWV) have been used for viral tolerance (Whiteman

et al., 1996; Fedorowicz *et al.*, 2005). Field trials of transgenic tomato plants expressing an ameliorative satellite RNA of CMV exhibited mild or no CMV symptoms and low viral titers relative to nontransformed plants. When infected with CMV, the transgenic lines showed 40–84% greater total marketable yield compared to parent lines. A significant negative correlation between satellite RNA levels and disease severity was found in transgenic lines (Stommel *et al.*, 1998).

Tomato plants transformed with the *Arabidopsis NPR1* (nonexpresser of PR genes) gene showed heat tolerance and resistance to TMV. These transgenic lines had significant level of enhanced resistance to BW and FW, and moderate degree of enhanced resistance to gray leaf spot (GLS) and bacterial spot (BS). The enhanced disease-resistance was stably inherited. These transgenics open the possibility of transferring these characteristics into tomato cultivars with good agronomic and organoleptic characteristics (Lin *et al.*, 2004).

The D satellite RNA (satRNA) is a strain of CMV that induces an epidemic lethal disease by triggering programmed cell death (PCD). With the goal to control the disease, Xu *et al.* (2004) created transgenic tomato plants expressing animal antiapoptotic genes *bcl-xL* and *ced-9*. T₁ and T₂ generations of transgenic plants showed delayed cell death symptoms and absence of symptoms. The degree of symptom suppression was correlated with increased expression levels of the transgenes. More than 80% of the *bcl-xL* and *ced-9* T₁ transgenic lines showed higher survival rates than the average for the line G138A, generated by a loss of function mutation of a transgenic line carrying the *bcl-xL* gene. Total RNA extracted from surviving plants contained D satRNA, indicating systemic accumulation of D satRNA. Thus, expression of *bcl-xL* and *ced-9* improved tolerance, rather than resistance, to CMV/D satRNA infection. In addition, it was found that expression of *bcl-xL* and *ced-9* specifically abrogated the formation of necrotic lesions, but not the symptoms due to chilling at 4°C (abiotic stress). At 7°C, temperature-induced leaf senescence was dramatically delayed in *bcl-xL* and *ced-9* transgenic plants, and high levels of anthocyanins accumulated, possibly limiting oxidative stress. It was concluded that heterozygous expression of *bcl-xL* and *ced-9*

in tomato plants enhanced plant survival by inhibiting PCD induced by both biotic and abiotic stress, including virus infection and chilling. These transgenic plants not only provide a good system for plant PCD research but also have a potential value for agricultural applications.

2.3 Transgenic Tomatoes Resistant or Tolerant to Abiotic Stresses

Plants are sessile and because they are constantly exposed to vagaries of nature, they have developed fine responses to environmental extremes that in turn may impact their growth, productivity, and quality. Much work has been done on plant responses to environmental extremes such as high salinity, drought, cold and heat, heavy metals, hypoxia due to flooding, and oxidative stress. To confront these different abiotic stresses, plants have developed an elaborate signaling network that perceives these signals and modulates the expression of select genes (Zhang *et al.*, 2004). Significant progress has been made in understanding these pathways using *Arabidopsis* as a model system. However, a great effort has gone into developing tomato plants that can withstand different abiotic stresses. Table 8 summarizes some of these studies.

2.3.1 Drought tolerance

Plant response to water deficit is articulated in a complex network of morphological, physiological, and molecular changes. As plant's relative water content decreases, plant growth is slowed, and stomatal closure occurs, which is paralleled by a decreased photosynthetic rate (Lawlor, 2002). Emerging knowledge of processes involved in drought-stress adaptation will likely translate into crop varieties that can withstand drought without impacting crop productivity. A number of drought-responsive genes have been identified in various genetic screens using *Arabidopsis* as a model system. It is anticipated that development of tomato varieties tolerant to water deficits will not only increase the productivity of tomato in many regions of the world but also help in regaining more arable land. However, significant more work is needed before this knowledge can be translated

into developing desirable tomato varieties that can tolerate water stress.

Park *et al.* (2005) constitutively overexpressed the vacuolar H⁺-pyrophosphatase in commercial tomato varieties. The resulting transgenic plants exhibited greater pyrophosphate-driven cation transport into root vacuolar fractions, increased root biomass, and enhanced survivability under soil water deficit stress. Constitutive expression of *Arabidopsis CBF1* (C-repeat/dehydration-responsive element-binding factor 1) in tomato resulted in enhanced plant tolerance to cold, drought, and salt stresses. However, this enhanced tolerance came at a cost, causing reduced plant growth and yield (Hsieh *et al.*, 2002a, b). When the same gene (*CBF1*) was expressed using an abscisic acid (ABA)/stress-inducible promoter from barley *HAV22* gene, the transgenic tomatoes showed enhanced tolerance to chilling, water deficit, and salt stresses as compared to untransformed plants and the use of the inducible promoter eliminated the deleterious effects of the ectopic expression of *CBF1* on plant growth and yield (Lee *et al.*, 2003).

Boiling stable proteins (BSP) have been implicated in desiccation tolerance against water stress by protecting proteins. Transgenic tomato plants transformed with a novel 66-kD BSP from *Populus tremula* were tested for drought tolerance by polyethylene glycol (PEG) test, biomass analysis, proline estimation, and electrolyte leakage measurement. These plants exhibited slightly increased tolerance to water stress compared to the parental genotype (Roy *et al.*, 2006).

Linker histone proteins are induced by environmental conditions in many plant species playing a role as yet not elucidated. Two possible roles for H1-S histone have been proposed: a structural role in DNA protection from damage occurring during water deficit and a functional role in the regulation of gene expression (Scippa *et al.*, 2000). Scippa *et al.* (2004) developed transgenic tomato plants overexpressing antisense histone *H1-S*, which is a drought-induced linker histone of tomato. *H1-S* antisense transgenic plants developed normally indicating that H1-S does not play an important role in the basal functions of tomato development. However, the stem height of the transgenic lines was reduced by 30% compared with the wild type by a shortening in the stem internodes. Later in development, transgenic plants appeared to have a higher number of leaves than the wild type at the

Table 8 Genetic manipulation of *Solanum lycopersicum* L. for abiotic stress tolerance

Gene product	Gene source	Promoter:gene	Phenotype	References
Water deficit/drought stress				
Boiling stable protein—66 kD	<i>Populus tremula</i>	<i>Enh</i> CaMV 35S: <i>bspA</i>	Slightly increased tolerance to water stress	Roy <i>et al.</i> , 2006
CBF1	<i>Arabidopsis</i>	CaMV 35S:CBF1	Tolerance to cold, drought, and salt loading, but impaired growth under normal conditions	Hsieh <i>et al.</i> , 2002a, b
CBF1	<i>Arabidopsis</i>	ABRC1:CBF1	Tolerance to chilling, water deficit and salt stress with normal growth and yield	Lee <i>et al.</i> , 2003
H ⁺ -pyrophosphatase	<i>Arabidopsis</i>	CaMV35S:AVP1D	Greater pyrophosphate-driven cation transport into root vacuolar fractions, increased root biomass, and enhanced survivability during water deficit stress	Park <i>et al.</i> , 2005
H1-S, drought-induced linker histone	Tomato	CaMV 35S:H1-s antisense	Normal growth of antisense plants, modulation of mechanisms related to the stomatal function	Scippa <i>et al.</i> , 2004
High salt stress				
Betaine aldehyde dehydrogenase	<i>Atriplex hortensis</i>	CaMV 35S:BADH (two genes)	Enhanced tolerance to salt stress	Jia <i>et al.</i> , 2002
Betaine aldehyde dehydrogenase	Sorghum	CaMV 35S:BADH-1	Maintenance of the osmotic potential under salt stress	Moghaieb <i>et al.</i> , 2000
<i>HAL1</i> (K ⁺ transport regulation) gene	Yeast	CaMV 35S:HAL1	Tolerance to high levels of salt. Maintains high levels of K ⁺ and low levels of intracellular Na ⁺	Gisbert <i>et al.</i> , 2000
HAL2	Yeast	CaMV 35S:HAL2	Salt-tolerant plant	Arrillaga <i>et al.</i> , 1998
NHX1, vacuolar Na ⁺ /H ⁺ antiporter	<i>Arabidopsis</i>	:NHX1 overexpressed	Salt tolerance up to 200 mM NaCl; High Na ⁺ concentrations in leaves, but very low levels in the fruits	Zhang and Blumwald, 2001
Trehalose-6-phosphate synthase	Yeast	CaMV 35S:TPS1	Improved tolerance to drought, salt, and oxidative stress	Cortina and Culianez-Macia, 2005
Polyphenol oxidases	Potato	CaMV 35S:PPO sense and antisense	PPO-suppressed plants show more favorable water relations and decreased photoinhibition compared to PPO-overexpressing or untransformed plants Higher chlorophyll levels in PPO-suppressed transgenic compared to PPO-overexpressed and untransformed plants after water stress	Thipyapong <i>et al.</i> , 2004
Temperature stress				
Heat shock factor, hsfA1	Tomato	CaMV 35S:HsfA1 cosuppression CaMV 35S:HsfA1 overexpression	No effect on growth and development. Reduced tolerance to elevated temperatures No effect on growth and development	Mishra <i>et al.</i> , 2002
Heat shock factor, hsfA1b	<i>Arabidopsis</i>	CaMV 35S: <i>AtHsfA1b</i>	Heat shock-induced chilling tolerance	Li <i>et al.</i> , 2003
LeHSP100/ClpB	Tomato	CaMV 35S:LeHSP100 antisense	Reduced thermotolerance	Yang <i>et al.</i> , 2006

(Continued)

Table 8 Genetic manipulation of *Solanum lycopersicum* L. for abiotic stress tolerance (*Continued*)

Gene product	Gene source	Promoter:gene	Phenotype	References
sHSP21 (chloroplast)	Tomato	CaMV 35S:Chlo sHsp	Protected PSII from temperature-dependent oxidative stress; no effect on thermotolerance, conversion of chloroplasts to chromoplasts, carotenoids accumulation in cold temperature stored fruits	Neta-Sharir <i>et al.</i> , 2005
sHSPs (ER)	Tomato	CaMV 35S:LeHSP21.5	Attenuated the lethal effect of tunicamycin (a potent inducer of ER stress)	Zhao <i>et al.</i> , 2007
sHSP (mitochondrial)	Tomato	CaMV 35S:MT-sHSP	Thermotolerance	Nautiyal <i>et al.</i> , 2005
vis1 (an sHSP)	Tomato	CaMV 35S:vis1 (antisense) CaMV35S:vis1 (overexpression)	Early accumulation of carotenoids in field-grown plants Delayed accumulation of carotenoids but increased juice viscosity	Ding and Handa (unpublished, results)
CBF1-induced protein	Tomato and <i>Arabidopsis</i>	CaMV 35S:LeCBF1 and <i>AtCBF3</i>	No increase in freezing tolerance; tomato CBF regulon differs from <i>Arabidopsis</i>	Zhang <i>et al.</i> , 2004
Choline oxidase	<i>Arthrobacter globiformis</i> I	CaMV 35S:CodA	Improved chilling and oxidative stress tolerance	Park <i>et al.</i> , 2004
Catalase	Tomato	CaMV 35S:CAT1 antisense	Susceptibility to oxidative stress and chilling injury	Kerdnaimongkol and Woodson, 1999
Cys-2/His-2 zinc finger protein-TF	Pepper (<i>Capsicum annuum</i>)	CaMV 35S:CaPIF1 overexpression	Tolerance to cold stress and to the bacterial pathogen <i>P. syringae</i>	Seong <i>et al.</i> , 2007
Hypoxia				
ACC deaminase	Bacterial	CAMV, rolD, and PRB-1b: ACC deaminase	Tolerance to flooding stress, rolD promoter protects to greater extent	Grichko and Glick, 2001
Hexokinase	<i>Arabidopsis</i> <i>Saccharomyces Cerevisiae</i>	CaMV 35S:AtHXK1 CaMV 35S:ScHXK2	No decline of the adenylate energy status or increase in sucrose synthase after hypoxic treatment. Decline similar to wild-type lines of the adenylate energy status and increase in sucrose synthase activity after hypoxia	Gharbi <i>et al.</i> , 2007
Oxidative stress				
CBF1	<i>Arabidopsis</i>	CaMV 35S:CBF1	Considerable tolerance against oxidative damage	Hsieh <i>et al.</i> , 2002a, b
CBF1	<i>Arabidopsis</i>	ABRC1:CBF1	Tolerance to chilling, water-deficit, and salt stress with normal growth and yield	Lee <i>et al.</i> , 2003
Choline oxidase	<i>Arthrobacter globiformis</i> I	CaMV 35S:CodA	Improved oxidative stress tolerance	Park <i>et al.</i> , 2004
sHSP21 (chloroplast)	Tomato	CaMV 35S:Chlo sHsp	Protected PSII from temperature- dependent oxidative stress	Neta-Sharir <i>et al.</i> , 2005
Polyphenol oxidases	Potato	CaMV 35S:PPO sense or antisense	More favorable water relations, decreased photoinhibition, and higher chlorophyll levels in PPO-suppressed transgenic compared to overexpressed and untransformed plants after water stress indicating reduced photo-oxidative stress in PPO-suppressed lines	Thipyapong <i>et al.</i> , 2004
Catalase (katE)	<i>Escherichia coli</i>	RbcS3C:katE	Enhanced photo-oxidative stress tolerance	Mohamed <i>et al.</i> , 2003

same age. Also, differences between the wild type and antisense plants were observed in leaf anatomy and physiological activities. No differences were detected in chromatin organization, excluding a structural role for H1-S in chromatin organization. It was concluded that likely H1-S histone modulates mechanisms related to the stomatal function.

2.3.2 Salt stress tolerance

About one-fifth of the irrigated, agriculture land is impacted by salinity and it is rapidly expanding due to the increased use of underground water. Throughout its ontogeny, tomato, like most other crop plants, is sensitive to salinity (Foolad, 2004, 2007). Our limited understanding of molecular bases regulating salt tolerance in plants has hampered progress in developing salt-tolerant crops. Nonetheless, several genes with a potential role in imparting tolerance to salinity stress have been identified. These include calcium sensor protein, SOS3, which binds to and activates a Ser/Thr protein kinase SOS2 that regulates activities of a plasma membrane Na^+/H^+ antiporter SOS1, and a tonoplast Na^+/H^+ antiporter NHX1 (Sahi *et al.*, 2006). A putative osmosensory histidine kinase (AtHK1)-MAPK (mitogen activated protein kinase) cascade is another signaling pathway hypothesized to regulate osmotic homeostasis and reactive oxygen species (ROS) scavenging. It is expected that understanding of ion transporters and their regulators, and of the CBF (C-repeat-binding factor) regulons, will help in the future development of salt-tolerant crops (Chinnusamy *et al.*, 2005).

Zhang and Blumwald (2001) overexpressed the *Arabidopsis* *NHX1* vacuolar Na^+/H^+ antiporter in tomato plants. These transgenic plants were able to grow, flower, and produce fruit in the presence of 200 mM sodium chloride. Interestingly, their leaves accumulated high concentrations of sodium, but the transgenic fruits contained very low levels of sodium. Salt and other osmotic stresses have been shown to increase levels of glycine betaine, which is an important quaternary ammonium compound in many organisms. Betaine aldehyde dehydrogenase encoded by *BADH* gene catalyzes conversion of betaine aldehyde into glycine betaine. Overexpression of *BADH* gene from

sorghum contributed to maintenance of osmotic potential under salt stress (Moghaieb *et al.*, 2000). The tomato hairy root lines transformed with *BADH-1* gene using root inducing plasmid (pRi) plasmid accumulated betaine. Constitutive expression of *Atriplex hortensis* *BADH* gene in tomato led to enhanced tolerance of transgenic tomato to salt stress (Jia *et al.*, 2003).

Overexpression of yeast *HAL1* in transgenic tomatoes, which is involved in the regulation of K^+ transport, also imparted tolerance to high levels of salt. Like the yeast, these transgenic tomatoes were able to maintain higher levels of K^+ and decreased levels of intracellular Na^+ than the control (Gisbert *et al.*, 2000), suggesting similarities between these transgenic plants and yeast as far as the mechanisms that regulate salt tolerance are concerned. Accumulation of a nonreducing disaccharide of glucose, trehalose, has been implicated in plant tolerance to different stresses. Transgenic tomato plants transformed with yeast trehalose-6-phosphate synthase (*TPS1*) gene exhibited improved tolerance to drought, salt, and oxidative stress indicating a great potential of this gene to impart stress tolerance to plants (Cortina and Culianez-Macia, 2005).

2.3.3 Temperature stress tolerance

Plants vary in their responses to temperature extremes. Apart from stress due to freezing temperatures, nonfreezing low temperatures cause chilling injury while high temperatures impair crop productivity (Iba, 2002). Tomato is highly sensitive to all these. Freezing tolerance of tomato is limited to -2 to -3°C whereas extended storage at temperatures below 12°C results in severe chilling injury-related physiological disorder that also affects fruit ripening. Tomato fruit kept at a temperature of 30°C and above show abnormal ripening including lack of lycopene accumulation, slow down in chlorophyll degradation and tissue softening, and decrease in ethylene production (Biggs *et al.*, 1988; Picton and Grierson, 1988). Heat-exposed fruit upon return to moderate temperatures recover and start ripening, at least partially, but there is a delay in the overall ripening process (Biggs *et al.*, 1988; Kagan Zur *et al.*, 1995). Steady-state transcript levels and enzyme activity of several ripening-related genes, including

ACC synthase, ACC oxidase, and PG, decrease in tomato fruit stored at 35°C (Biggs *et al.*, 1988; Picton and Grierson, 1988; Kagan Zur *et al.*, 1995). Expression of PG, an enzyme implicated in fruit softening, is gradually and irreversibly impaired in fruit at elevated temperatures (Kagan Zur *et al.*, 1995). Symptoms of chilling injuries are reduced after heat treatment, and this reduction is correlated with persistence of several heat shock proteins, HSPs, in the fruit tissue (Sabeheh *et al.*, 1998).

In response to a sudden exposure to heat, most organisms including plants synthesize a set of proteins, designated as HSPs. Based on molecular size, HSPs have been classified into five classes (Hsp100, Hsp90, Hsp70, Hsp60, and a family of small HSPs ranging between 17 and 43 kD). HSPs act as molecular chaperones to facilitate protein folding, transport and maturation, and protect other proteins from heat-induced aggregation. The conservation of the primary structure of HSPs and ubiquitous expression in all organisms led to the hypothesis that these proteins play protective roles against heat stress by maintaining homeostasis. A short exposure to temperatures of 38–40°C causes transient induction of HSPs transcription in plants. A number of heat shock activated transcription factors (HSFs) are present in plant genomes and are characterized by binding to the *cis*-elements (heat shock elements; HSEs) present at the 5'-flanking regions of the HSPs. More work is needed to understand the role of HSPs in plant adaptation to high temperatures in order to use them for developing heat-tolerant plants (Iba, 2002).

To evaluate the role of chloroplast small HSPs, Neta-Sharir *et al.* (2005) overexpressed tomato *HSP21* under the CaMV 35S promoter. Characterization of the resulting transgenic plants revealed that chloroplastic *HSP21* protected photosystem II (PSII) from temperature-dependent oxidative stress but did not as such affect the thermotolerance. These authors also provided evidence of the protein's role in fruit reddening and the conversion of chloroplasts to chromoplasts. Interestingly, the expression of chloroplastic *HSP21* substituted for the heat treatment via the accumulation of carotenoids in fruits stored for 2 weeks at 2°C and then transferred to room temperature. These authors suggested that in addition to a protective role under

stress conditions, *HSP21* plays a role in plant development under normal growth conditions.

Ramakrishna *et al.* (2003) have cloned from tomato a novel small HSP gene designated as viscosity 1 (*vis1*). This protein plays a role in pectin depolymerization and juice viscosity of ripening fruits. *vis1* is regulated by the fruit ripening process and high temperature. It exhibits a typical HSP chaperone function when expressed in bacterial cells. Interestingly, in nontransgenic tomato genotypes, expression of *vis1* is negatively associated with juice viscosity. Deng and Handa (unpublished results) developed tomato lines expressing both antisense and sense chimeric genes under the control of CaMV 35S. Field evaluation of these plants revealed that reduction in *vis1* expression accelerated accumulation of carotenoids while its overexpression delayed their accumulation. Also, ectopic expression of *vis1* increased viscosity of the processed tomato fruit juice (Deng and Handa, unpublished results).

Transgenic tomato plants that express antisense of *LeHSP100* exhibited reduced tolerance to heat stress (Yang *et al.*, 2006). These transgenic plants were not able to express *LeHSP100/ClpB* upon exposure to heat and the plants withered within 21 days of the recovery period at normal temperature following a "lethal" heat shock at 46°C for 2 h. In contrast, the untransformed control plants and the vector-transformed plants survived. These results show that *LeHSP100/ClpB* contributes to the acquisition of thermotolerance in higher plants. Nautiyal *et al.* (2005) developed transgenic tomato lines overexpressing tomato *MT-sHSP* and showed that vegetative tissues of T₀ and T₁ lines exhibited enhanced thermotolerance.

Yi *et al.* (2006) have characterized 1.9 kb of 5'-flanking region of *Lehsp23.8* by expressing GUS under its control in transgenic tomato plants. These authors reported that GUS expression was seen in the roots, leaves, flowers, fruits, and germinated seeds after heat shock but the heat inducibility was different at various developmental stages. In fruit tissues, the heat-induced GUS activity was higher in the pericarp followed by placenta and lowest in the locular gel. Unlike *vis1* (a ripening-induced shsp) that showed much higher heat inducibility in ripening fruit (Ramakrishna *et al.*, 2003), maximum heat inducibility of GUS under the *Lehsp23.8* promoter was observed in green fruit. The promoter of

Lehsp23.8 was also induced by cold, exogenous ABA, and heavy metals (Cd^{2+} , Cu^{2+} , Pb^{2+} , or Zn^{2+}).

Characterization of transgenic tomato plants over and underexpressing the heat stress transcription factor *HsfA1* revealed no difference in their major developmental parameters under normal growth conditions compared to wild-type plants (Mishra *et al.*, 2002). However, *HsfA1* underexpressing plants as well as their fruits exhibited extreme sensitivity to elevated temperatures. Co-silencing of *HsfA1* by its transgenes resulted in reduced heat stress-induced accumulation of chaperones and Hsfs suggesting a unique function of *HsfA1* as a master regulator for inducing thermotolerance. The effect of heat shock factor (Hsf) on chilling tolerance has also been studied by expressing *AtHsA1b* and *gusA* under CaMV 35S promoter. The transformed tomato showed more accumulation of heat shock-induced gene transcripts and enzymes including a twofold increase in the specific activity of soluble isoforms of ascorbate peroxidase (Li *et al.*, 2003).

The function of endoplasmic reticulum (ER)-located small heat shock proteins (ER-sHSPs) in ER stress was tested by Zhao *et al.* (2007) by overexpressing *LeHSP21.5* in tomato plants. ER stress is basically an imbalance between the cellular demand for protein synthesis and the capacity of the ER to promote protein maturation and transport, which leads to an accumulation of unfolded or misfolded proteins in the ER lumen. Gene expression in the transgenic lines greatly attenuated the lethal effect of tunicamycin (a potent inducer of ER stress) on tomato seedlings. Moreover, tunicamycin treatment led to lower levels of the chaperone-binding protein, protein disulfide isomerase, and the chaperone calnexin transcripts in transgenic tomato plants than the nontransgenic tomato plants. These results suggest that the HSP *LeHSP21.5* can alleviate the tunicamycin-induced ER stress by promoting proper protein folding.

Plants from tropical regions, such as tomato, maize, and rice, are unable to tolerate freezing and suffer chilling injury when exposed to temperatures in the range of 0–12°C (Zhang *et al.*, 2004). Expression of a number of genes is altered in plants exposed to low temperatures. A variety of stress-responsive transcriptional factors that regulate the signal transduction network

underlying perception of stress response have been identified (Yamaguchi-Shinozaki and Shinozaki, 2006). Some of the components of the complex cascade of gene expression under cold stress have been engineered into tomato to determine their effect on cold tolerance including freezing.

Transformation of tomato (cv. Moneymaker) with a chloroplast targeted *codA* gene of *Arthrobacter globiformis*, which encodes choline oxidase that catalyzes the conversion of choline to glycine betaine, improved chilling and oxidative stress tolerance of transgenic plants (Park *et al.*, 2004). Transgenic tomato plants accumulated up to 1.2 mM of glycine betaine per gram of fresh weight with the chloroplasts containing up to 86% of total leaf glycine betaine. These transgenic tomato lines produced 10–30% more fruit compared to untransformed plant. As mentioned above, overexpression of *Arabidopsis CBF1/DREB1B*, especially driven by three copies of an ABA-responsive complex (*ABRC1*) from the barley *HAV22* gene, resulted in enhanced tolerance in tomato to chilling, water deficit, and salt stress (Hsieh *et al.*, 2002a; Lee *et al.*, 2003). Seong *et al.* (2007) reported that overexpression of *CaPIF1* enhanced tolerance to cold stress and bacterial pathogen *P. syringae* pv. *tomato* DC 3000. These authors further reported that the enhanced tolerance was correlated with *CaPIF1* expression levels in transgenic plants. However, underexpression of a catalase gene or overexpressing an *Escherichia coli* glutathione reductase did not alter chilling sensitivity of transgenic tomatoes (Bruggemann *et al.*, 1999; Kerdnaimongkol and Woodson, 1999).

Although significant progress has been made in identifying components of freezing tolerance in *Arabidopsis*, limited success has been obtained in engineering any tolerance to this stress in tomato (Zhang *et al.*, 2004). Constitutive overexpression of either *LeCBF1* or *AtCBF3* in transgenic tomato plants was ineffective in increasing freezing tolerance. Transcript analysis using a tomato microarray with 8000 genes revealed only 4 genes whose expression was over 2.5-fold up-regulated in the *LeCBF1* or *AtCBF3* overexpressing transgenic tomato lines. Three of these genes were up-regulated in response to low temperature. These authors reported that although tomato has a CBF cold response pathway, it is much smaller and less diverse in function compared to that of

Arabidopsis. Overexpression of *DEAL*, a circadian-regulated tomato gene that showed sequence similarity to an *Arabidopsis* cold protection gene, *EARLII*, did not alter cold susceptibility of transgenic tomato (Weyman *et al.*, 2006).

2.3.4 Tolerance to hypoxia

Anaerobic conditions (anaerobiosis) occur during waterlogging, flooding, poor drainage, or even during irrigation causing oxygen deficiency, anoxia with bare minimum oxygen availability, and hypoxia with only some oxygen availability in the rooting zone. Plants respond to anaerobiosis in a variety of ways. Increase in the rate of glycolysis and fermentation occurs to compensate for the decrease in energy and lack of nicotinamide adenine dinucleotide (NADH) regeneration. In the root apical meristem, cell survival is important for the plant development. Metabolic changes under anoxia help maintain cell survival by generation of adenosine 5'-triphosphate (ATP) anaerobically and minimization of cytoplasmic acidosis associated with cell death. Hypoxia causes loss in crop productivity by depressing growth and yield of dryland species (Drew, 1997). In the fully expanded cells behind the root apex, hypoxic conditions result in the formation of continuous, gas-filled channels (aerenchyma) carrying O₂ from the leaves. A bacterial ACC deaminase under the transcriptional control of double CaMV 35S, the *rolD* promoter from *Agrobacterium rhizogenes* (root-specific expression), enabled tomato plants with increased tolerance to flooding stress and lesser deleterious effects of root hypoxia on plant growth than the nontransformed plants (Grichko and Glick, 2001). Plant attributes evaluated in this study included shoot height, fresh and dry weight, epinasty, ACC deaminase activity, ethylene production, and leaf chlorophyll and protein contents.

To determine if increased hexokinase (HXK) activity alters hypoxic metabolism in roots, Gharbi *et al.* (2007) overexpressed *Arabidopsis* and *Saccharomyces cerevisiae* HXKs in tomato. Wild type and *ScHXX2* transgenic tomatoes showed a decline in the adenylate energy status after 2 days of hypoxic treatment, with a further decrease occurring by 7 days in roots, but these did not happen in *AtHXX1* transgenic lines. Increase in

sucrose synthase activity that occurs in wild-type plants under hypoxia was not seen in transgenic tomato overexpressing *AtHXX1*.

2.3.5 Oxidative stress tolerance

Most environmental stresses including high salinity, drought, and temperature extremes result in generation of ROS causing oxidative stress in plants. Molecular engineering of plants by altering expression of genes involved in these stress responses also resulted in their altered response to oxidative stress. The genes used include: *CBF* (cor-binding-factors) that regulate expression of *cor* (cold-regulated) genes (Hsieh *et al.*, 2002b; Lee *et al.*, 2003), *HSP21* (Neta-Sharir *et al.*, 2005), and *choline oxidase* (Park *et al.*, 2004).

The role of PPO has also been examined by its overexpression and suppression using transgenic approach (Thipyapong *et al.*, 2004). PPOs have been implicated in photoreduction of molecular oxygen by photosystem I (PSI) in the Mehler reaction. Transgenic tomato plants with suppressed PPO showed more favorable water relations and decreased photoinhibition compared to transgenic plants overexpressing PPO or the untransformed control plants. Higher chlorophyll levels were observed in water-stressed, PPO-suppressed transgenics compared to PPO-overexpressed and untransformed plants indicating reduced photooxidative stress in PPO-suppressed lines.

Transgenic tomato overexpressing an *E. coli* catalase (*katE*) gene under tomato *Rubisco* small subunit gene promoter showed approximately threefold higher catalase activity than that the wild-type plants (Mohamed *et al.*, 2003). The transgenic plants exhibited higher tolerance to 100 μ M paraquat under high light illumination than wild-type plants. Leaf discs from transgenic lines remained green and had reduced ion leakage at 24 h after treatment with paraquat than the discs from nontransgenic plants. Photosynthesis (determined by the Fv/Fm ratio) and catalase activity were less impaired by paraquat in transgenic compared to wild-type tomato plants. Collectively, the transgenic plants exhibited increased tolerance to the oxidative damage caused by drought stress or chilling stress under high light intensity. Previously, the expression of antisense catalase

gene (*ASTOMCAT1*) in “Ohio 8245” enhanced susceptibility to oxidative stress and chilling injury in transgenic tomato plants (Kerdnaimongkol and Woodson, 1999). Leaf catalase activity was lowered by twofold to eightfold with a concomitant twofold increase in H_2O_2 levels. Treatment of these transgenic plants with 3% H_2O_2 resulted in visible damage during first 24 h leading ultimately to death while the wild type and azygous control plants recovered from the treatment. These studies indicate that overexpression of catalase is a promising way to develop transgenic plants that can withstand photo-oxidative stress.

2.4 Vaccines and Clinical Applications

The potential use of transgenic plants as bioreactors is now well established. Plants offer several advantages in comparison to other expression systems, including low-cost inputs, feasibility of scaling-up, reduction of health risks deriving from contamination with human pathogens, simplification of downstream or upstream processing and, most importantly, plant cells are able to perform complex post-translational modifications (Daniell *et al.*, 2001; Walmsley and Arntzen, 2003). A wide variety of pharmaceutically valuable proteins that fall into three major categories have been produced in plants: antibodies (Vaquero *et al.*, 1999; Stoger *et al.*, 2000), antigens (Chikwamba *et al.*, 2002; Jani *et al.*, 2002; Bouche *et al.*, 2003), and biopharmaceuticals that fully retain their activity (Magnuson *et al.*, 1998; Mor *et al.*, 2001; Zhang *et al.*, 2003).

Research for using plants to express and deliver oral vaccines has attracted much attention. This is mainly because this strategy offers several advantages over vaccine delivery by injection. Oral vaccines also offer the hope of more convenient immunization strategies and a more practical means of implementing universal vaccination programs worldwide. Oral vaccines acts by stimulating the immune system at effector site (lymphoid tissue) located in the gut. Curtiss and Cardineau (1990) first reported the expression of *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco. Since then, a great progress has been made (Ma *et al.*, 2001). So far, more than 10 viral epitopes and subunits of bacterial toxins have been successfully expressed in plants,

including hepatitis B surface antigen (HBsAg) (Mason *et al.*, 1992; Gao *et al.*, 2003), *E. coli* heat-labile enterotoxin B subunit (LTB) (Haq *et al.*, 1995; Streatfield *et al.*, 2001), cholera toxin B subunit (CT-B) (Arakawa *et al.*, 1998), Norwalk virus capsid protein (Mason *et al.*, 1996; Tacket *et al.*, 2000), and rabies virus glycoprotein (McGarvey *et al.*, 1995).

Tomato is one of the fruits tested for expression of vaccines. McGarvey *et al.* (1995) engineered tomato (cv. UC82b) plants to express a gene encoding a glycoprotein (G-protein), which coats the outer surface of the rabies virus. The recombinant constructs contained the G-protein gene from the environmental risk assessment (ERA) strain of rabies virus. The G-protein was expressed in leaves and fruit of the transgenic plants and it was found localized in Golgi bodies, vesicles, plasmalemma, and cell walls of vascular parenchyma cells.

Ma *et al.* (2003) overexpressed hepatitis E virus (HEV) open reading frame 2 partial gene in tomato plants, to investigate its expression in transformants, the immunoactivity of expressed products, and explore the feasibility of developing a new type of plant-derived HEV oral vaccine. The recombinant protein was produced at 61.22 ng g^{-1} fresh weight in fruits and $6.37\text{--}47.9 \text{ ng g}^{-1}$ fresh weight in the leaves of the transformants. It was concluded that the *HEV-E2* gene was correctly expressed in transgenic tomatoes and that the recombinant antigen derived had normal immunoactivity. These transgenic tomato plants are a valuable tool for the development of edible oral vaccines.

Gutierrez-Ortega *et al.* (2005) reported the expression of functional interleukin-12 (*IL-12*) from mouse in transgenic tomato plants with a construct in which the expression of the single-chain mouse *IL-12* was controlled by the CaMV 35S promoter. They showed that the *IL-12* accumulated up to 7.3 and $3.4 \mu\text{g g}^{-1}$ of fresh weight in leaves and fruits, respectively. The mouse *IL-12* expressed in tomato was shown to have immunomodulatory function *in vitro*, as determined by interferon- γ (IFN- γ) secretion by T cells.

Vaccination against anthrax is considered the most promising strategy to combat the disease. The expression in plants of the protective antigen (PA) encoded by the gene *pagA* is thought

to be a good approach to generate an edible vaccine against anthrax. Aziz *et al.* (2005) reported the successful expression of a functional PA in tomato. The integration of the *pagA* gene in the tomato nuclear genome was verified by polymerase chain reaction (PCR) and Southern analysis. The fidelity of PA protein expression in transgenic tomato plants was confirmed by the detection of a single protein of 83 kD by anti-PA polyclonal antibodies at a maximum dilution of 1:200. Transgenic tomato plants did not show phenotypic aberrations although they were found to be more sensitive to fluctuations in temperature and humidity when compared with the wild type.

The Gram-negative bacterium *Yersinia pestis* is the causative agent of plague, affecting human health since ancient times. It is still endemic in Africa, Asia, and the Americas. Due to the increasing reports of incidence, antibiotic resistance strains and concern with the use of *Y. pestis* as an agent of biological warfare, there is the urgent need for a safe and cheap vaccine. Out of all the *Y. pestis* antigens tested, only F1 and V induce a good protective immune response against a challenge with the bacterium (Benner *et al.*, 1999). The F1 (Fraction 1) antigen is the major capsular protein. It forms a polymer composed of a protein subunit and plays an important role in inhibiting phagocytosis by macrophages (Du *et al.*, 2002). The V antigen is a secreted protein that regulates the translocation of the cytotoxic effector proteins from the bacterium into the cytosol of mammalian cells. Alvarez *et al.* (2006) reported the expression in tomato of the *Y. pestis* F1-V antigen fusion protein. The immunogenicity of the F1-V transgenic tomatoes was confirmed in mice that were injected subcutaneously with bacterially produced F1-V fusion protein and boosted orally with transgenic tomato fruit. Expression of the plague antigens in the tomato fruit allowed producing an oral vaccine candidate without protein purification and with minimal processing technology, offering a good system for a great scale vaccination program.

The vaccinia virus B5 protein is a component of a subunit vaccine against smallpox but the employment of a live vaccine displays some side effects. Golovkin *et al.* (2007) successfully tested two plant expression systems to produce recombinant B5 antigenic domain. The plant-derived B5 antigen was purified and administered

parenterally and intranasally to mice. The mice thus vaccinated showed protection when challenged with a live vaccinia virus.

2.5 Improving the Environment by Using Transgenic Tomatoes

Transgenic plants can be used to have positive effects on the environment through bioremediation. Bioremediation is attracting importance as an alternative technology for the elimination of pollutants in soil and water. This is an effective, environmentally nondestructive and cheap method based on the use of green plants to remove, contain, or transform toxic chemicals into harmless one (Tengerdy and Szakacs, 1998; Suresh and Ravishankar, 2004). This technique has been tested with some success to remove polynitrated aromatic compounds, which are part of explosives (French *et al.*, 1999; Rosser *et al.*, 2001; Ramos *et al.*, 2005), toxic heavy metals (Kramer and Chardonnens, 2001; Singh *et al.*, 2003; Eapen and D'Souza, 2005), and arsenic (Alkorta *et al.*, 2004).

Cover crop mulches, particularly of plants capable of fixing nitrogen (e.g., *Vicia villosa* Roth) (HV), have been shown to enhance productivity and quality of the crop subsequently grown on them and these benefits include: retention of longevity and greenness in the leaves (Teasdale and Abdul-Baki, 1997; Kumar *et al.*, 2004), less pest infestation, and enhanced tolerance or resistance to disease (Mills *et al.*, 2002). Economically, this alternative system is sustainable and superior over the conventional system (Kelly *et al.*, 1995; Lu *et al.*, 2000). The desirable performance of hairy vetch (HV) mulch-grown vegetables has presented an opportunity to explore the kind of genes regulated by this production system. Tomatoes grown in a HV cover crop possess a highly organized and specific network system of genes that remain actively expressed for longer duration than the plants grown in black PE or rye mulch. These genes include: *rubisco* (ribulose-1, 5-bisphosphate carboxylase/oxygenase), a protein essential for photosynthesis and N remobilization; glutamine synthetase (*GS*), which plays a major role in N conversion and C utilization for the synthesis of amino acids, the building blocks for all proteins; and ATPase, chlorophyll-binding proteins in the chloroplast, *cytb₅₅₉*, and plastocyanin,

all important for photosynthesis and converting harvested chemical energy into food. These studies validate the use of alternative production system based on leguminous cover crop mulches, making it possible for the same or reduced inputs to produce greater outputs, something that agriculturalists generally desire (Kumar *et al.*, 2004; Mattoo and Abdul-Baki, 2006). These results also bring to the fore the importance of genotype \times environment ($G \times E$) interactions in determining beneficial attributes in plants. It opens possibilities for evoking from crop plants a more productive response not by adding external inputs but by altering their growing conditions.

An integrated model has been developed in our laboratory. This model is based on comparative performance/metabolism of transgenic tomato genotypes grown in N-fixing leguminous HV mulch, nonleguminous rye mulch, and black polyethylene (BP) mulch. We suggest that this integration holds the promise of value addition to crops, benefit to the environment by decreasing chemical input, preserving agricultural land as well as reducing economic burden on the farmer. Significant genotype \times mulch-dependent interactions affecting plant phenotype and fruit characteristics were revealed and exemplified by 20 fruit metabolites profiles, and expression patterns of the *ySAMdc* transgene and tomato *SAMdc* and *E8* genes in fruits, between a nontransgenic (556AZ) and transgenic (579HO) line accumulating higher polyamines. These studies have demonstrated that HV mulch enables a metabolic system in tomato akin to the one in higher polyamines-accumulating transgenic fruit that have higher phytonutrient content (Neelam *et al.*, 2008).

2.6 Utilization of Transgenic Tomatoes to Improve Human Health

Transgenic plants can potentially bring several positive effects and improve human health by:

1. Reducing the use of chemicals, herbicides, and pesticides, can improve the environment and make the ecosystem friendlier.
2. Development of functional foods with better nutritional composition:
 - a. Increased concentration of the phenolic chlorogenic acid (Niggeweg *et al.*, 2004).
 - b. Transgenics with long chain polyunsaturated fatty acids such as linolenic acid,

arachidonic, eicosapentaenoic acid, and docosahexaenoic acid (Huang *et al.*, 2004; Wu *et al.*, 2005; Truksa *et al.*, 2006), thochromanols (Kinney *et al.*, 2006), conjugated linoleic acid (Khono-Murase *et al.*, 2006).

- c. Enhanced provitamin A β -carotene (Sautter *et al.*, 2006), vitamins A, E, and C (Herbers, 2003), and trace elements (Sautter *et al.*, 2006).
- d. Increased amount of the antioxidant resveratrol (Lim *et al.*, 2005).
- e. Increase in the levels of ascorbate and glutathione, the soluble antioxidants of primary metabolism, as well as in the total antioxidant activity (Giovinazzo *et al.*, 2005).
- f. Foods with high amounts of potent antioxidants: flavonoids, lycopene (Mehta *et al.*, 2002; Ravanello *et al.*, 2003; Davuluri *et al.*, 2005).
- g. Antibodies-producing transgenics for diagnostics, prevention, and treatment of diseases (Fischer *et al.*, 2003).
- h. Recombinant proteins with correct pharmacological characteristics such as human LF protein (Rachmawati *et al.*, 2005; Taylor and Ramsay, 2005).

2.7 Intellectual Property and Regulatory Requirements for the Release of Genetically Modified Tomato Varieties

Development and release of genetically modified crops are complex issues involving diverse players including inventors, institutions/industry, and government agencies that control releases. Additionally, biologists and general public play important roles in consumer acceptance and successful use of a new transgenic plant variety. Multiple technologies involving various biological discoveries are needed to develop a new crop variety. The cost of acquiring technology tied up in intellectual properties has become a substantial barrier to the development and commercialization of genetically engineered crops. A long list of regulatory hurdles had to be crossed for the historic release of "Flavr Savr", a tomato variety that harbored an antisense gene of a PG (Kramer and Redenbaugh, 1994). The benefits and concerns of development and release of transgenic

Table 9 Biotechnology patents—issued for various improvements in tomato^(a)

Term	Number of patents
Tomato	
Tomato	12064
Tomato fruit	5223
Tomato gene	4796
Tomato gene AND chimeric	3130
Tomato transgenic	3926
Tomato polynucleotide sequence	1561
Tomato polynucleotide sequence construct	1183
Biotic stress	
Tomato polynucleotide sequence construct biotic stress (tolerance or resistance)	74
Tomato polynucleotide sequence construct disease (tolerance or resistance)	713
Tomato polynucleotide sequence construct bacterial (tolerance or resistance)	1008
Tomato polynucleotide sequence construct fungal (tolerance or resistance)	543
Tomato polynucleotide sequence construct viral (tolerance or resistance)	871
Tomato polynucleotide sequence construct insect (tolerance or resistance)	777
Abiotic stress	
Tomato polynucleotide sequence construct salt stress (tolerance OR resistance)	759
Tomato polynucleotide sequence construct salt stress tolerance	207
Tomato polynucleotide sequence construct drought stress (tolerance OR resistance)	256
Tomato polynucleotide sequence construct drought stress tolerance	189
Tomato polynucleotide sequence construct abiotic stress (tolerance OR resistance)	117
Tomato polynucleotide sequence construct abiotic stress tolerance	68
Fruit quality	
Tomato polynucleotide sequence construct fruit	583
Tomato polynucleotide sequence construct fruit quality	316
Tomato polynucleotide sequence construct fruit nutrition	62
Tomato polynucleotide sequence construct fruit softening	34
Tomato polynucleotide sequence construct fruit texture	46
Tomato polynucleotide sequence construct fruit shelf life	88
Selected commercial enterprises	
Tomato polynucleotide sequence construct Monsanto	134
Tomato polynucleotide sequence construct Zeneca	21
Tomato polynucleotide sequence construct ICI	10
Selected public university	
Tomato polynucleotide sequence construct Purdue	17
Tomato polynucleotide sequence construct Cornell	46
Tomato polynucleotide sequence construct UC Davis	280
Selected countries	
Tomato polynucleotide sequence construct USA	1118
Tomato polynucleotide sequence construct Britain	41
Tomato polynucleotide sequence construct China	24
Tomato polynucleotide sequence construct India	28

^(a)Data are based on US Patent Collection database search conducted on April 23, 2007 using indicated terms for each search. All terms were used with “AND” except tolerance and resistance that were used with “OR” as indicated in the table. These searches should be interpreted with caution as some entries may show up due to nonspecific search term(s) present in the text, especially in the cited references. In general, the number of patents is expected to be less than shown in this table

horticulture crops have been reviewed (Delmer *et al.*, 2003; Clark *et al.*, 2004; Redenbaugh and McHughen, 2004; Bradford *et al.*, 2005).

Despite the availability of many genes and regulatory sequences to improve various desirable attributes, commercialization of genetically modified crops has seriously lagged world over.

This situation also applies to the development of GM tomato. As discussed in this chapter, impressive numbers of genes with potential to enhance tomato crop productivity and nutritional aspects have been identified and characterized. Table 9 presents a partial list of the numerous patents granted to studies for improvement of both

crop productivity and fruit quality in tomato using genetic engineering.

Pioneering work done by Calgene to evaluate safety of transgenic “Flavr Savr” tomato variety for its unregulated release status has greatly contributed to testing of transgenic tomato under field conditions (Redenbaugh *et al.*, 1992). For most tomato transgenic lines, submission of a Letter of Notification is sufficient. However, a full Animal and Plant Health Inspection Service (APHIS) permit is required for transgenic tomatoes that express any plant pest structural sequences: http://www.aphis.usda.gov/biotechnology/permits_main.shtml.

3. THE FUTURE: EXPECTED TECHNOLOGIES

Transgenic crops including tomato have the potential to promote revolutionary change in agriculture, industry, nutrition, and even medicine. By manipulating plant genomes, crops can be engineered to provide enhanced nutritional value with in-built resistance to biotic and abiotic stresses, plant raw materials adapted to the requirements of industry, and “green factories” to produce a host of novel products, including pharmaceuticals, in an environmentally benign and sustainable manner (Halpin, 2005).

Progress toward “output trait” products with nutritional, environmental, or other benefits that consumers can directly appreciate has been slow. It can be enhanced and time shortened by removing or working around the bottlenecks of developing technologies for the coordinated manipulation of multiple genes or traits. This bottleneck is perhaps not widely appreciated, but is amply evidenced by the huge body of literature describing the manipulation or expression of single, useful genes in plants, and the comparative paucity of publications dealing with the manipulation of multiple genes or master genes. Yet, the advantages of “stacking” or “pyramiding” existing GM traits in crops are obvious, and offer the potential for providing durable multiresistance to particular pests or for engineering multiple resistance to different types of pathogen, perhaps in crops that are also herbicide tolerant. Similarly, the potential for sophisticated metabolic engineering in plants is enormous, and could lead to the development of

plants able to grow in inhospitable environments, which might provide healthier foodstuffs or improved raw materials.

The recent inroads toward systems biology incorporating features from transcriptome, proteome, lipidome, glycome, and metabolome should provide novel “nodes” whose modification may result in multiple attributes being affected by manipulations involving controls via master switches for major or minor secondary metabolic pathways. It is recognized that most metabolic processes that are targets for manipulation depend on the interaction between numerous genes, and flux through biochemical pathways often coordinated with that of competing pathways; therefore, effective metabolic engineering will only be achieved by controlling multiple genes on the same, or interconnected, pathways. The current predominant stacked trait crops are insect-resistant and herbicide-tolerant maize and cotton. Over the past few years significant advances in multigene manipulation have been made using a variety of conventional and novel techniques that, despite imperfections, provide a framework for future improvements (Halpin, 2005).

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Eggplant

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1. INTRODUCTION

The eggplant, *Solanum melongena* L. ($2n = 24$), belongs to the family Solanaceae (the nightshade or potato family), which also includes other important vegetables like potato, tomato, and red peppers. The eggplant has different names in different countries. For example, it is known as “aubergine” in France, “melanzana” in Italy, “eggfruit” in Australia, and “brinjal” in India. It is an important nontuberous vegetable and widely cultivated in many parts of the world, especially Asia, Africa, central America, Mediterranean area, and South of the United States (Sihachakr *et al.*, 1993; Collonnier *et al.*, 2001a). About 1.75 million hectares were cultivated in 2005 in the world for a total production of 30.4 million tons, of which 91.5% of the world production was contributed by Asia (FAO, 2005). The crop requires a relatively long season of warm weather to give good yields, and thus the tropical and subtropical climate is ideal for its cultivation, although it is grown in some parts of the temperate region as well.

The eggplant is generally considered as a “low calorie vegetable”. However, eggplant’s nutritional value is comparable to most of the other common vegetables. It contains vitamins, minerals, proteins, fiber, and also important phytonutrients like phenolic compounds and flavonoids, many of which have antioxidant activity. Besides, eggplant has been used in the traditional medicines. Its tissue extracts are used for the treatment of asthma, bronchitis, cholera, and dysuria and fruits and leaves are beneficial in lowering blood cholesterol. Recently, it has been shown that eggplants also possess antimutagenic properties (Khan, 1979; Hinata, 1986; Kalloo, 1993; Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003; Rajam and Kumar, 2006)

The cultivated eggplant is morphologically similar to a number of taxa (Bhaduri, 1951). It belongs to the nontuberiferous group of species of *Solanum*. In addition to the primitive cultivars and the wild species of *Solanum incanum*, a number of other putative wild varieties and species have been described one time or another. Cultivated varieties of eggplant can usually be easily distinguished

from the remainder of the taxa comprising the eggplant complex by their enlarged and often purple fruit and almost complete lack of prickles on the stem, leaves, and calyx. It is also easy to distinguish members of the eggplant complex from other *Solanum* species. It is an erect or prostrate, branched herb or shrub, about 1 meter tall, wooly or scurfy and spiny, but the spines are inconspicuous in some cultivated varieties. The leaves are large, ovate or oblong ovate, shallowly sinuate lobed, becoming nearly glabrous above but remaining densely tomentose beneath, the flowers are large and mostly in clusters. The calyx is wooly and often spiny, persistent, and accrescent. The spreading, lobed, purplish corolla is 2.5 cm or more in diameter. Heterostyly is a common feature. The fruit is a large berry, showing great varieties in size, shape, and color. It may be oblong, obovoid, ellipsoid, egg shaped, more or less spherical, cylindrical, elongate, etc. The color may be almost black, various shades of purple from dark to very light, blue, green, yellowish, striped variously, or white, ripening to brown, or orange.

Germplasm resources and collections have been well documented, evaluated, and conserved throughout the world (Sarathbabu *et al.*, 1999). Based on the fruit shape, eggplant has been divided into three main types, namely, egg shaped (*S. melongena* var. *esculentum*), long slender shaped (*S. melongena* var. *serpentium*), and dwarf type (*S. melongena* var. *depressum*) (Kalloo, 1993).

Although the genus *Solanum* is predominantly central and South American, the eggplant is probably a native of Asia. De Candolle, in his "Origin of Cultivated Plants" published in 1886, states that the species *S. melongena* has been known in India from a very remote epoch. He further writes, "Thunberg does not mention it in Japan though several varieties are now cultivated in that country. The Greeks and Romans did not know the species, and no Botanist mentions it in Europe before the beginning of the 17th century, but its cultivation must have spread toward Africa before the middle ages". The Arab physician, Ebn Baithar, who wrote in the 13th century, speaks of it, and he quotes Rhasis who lived in the 9th century "A sign of antiquity in Northern Africa is the existence of a name, tabendjalts, among the Berbers or Kabyles of the province of Algiers." Hooker (1885), in his Flora of British India, states

as follows: "De Candolle says it is a native of Asia, not America, and Sendtner fixes its origin in Arabia: all this appears uncertain."

Vavilov (1951) mentions the occurrence of *S. melongena* L. in the Indian Center of origin of cultivated plants, which excludes Northwest India, Punjab and Northwest Frontier but includes Assam and Burma. He also mentions the occurrence of a special form of *S. melongena* with small fruits in the Chinese center of origin of cultivated plants. According to him the place of origin of *S. melongena* is the Indo-Burma region.

The cultivated eggplants have been shown to be susceptible to a variety of biotic and abiotic stress factors, which cause considerable loss of fruit yield and quality. The most common pests are Colorado potato beetle, shoot and fruit borer, *Epilachna* beetle, and stem borer. Among the diseases, fungal wilts caused by *Verticillium* spp. and *Fusarium oxysporum*, bacterial wilt caused by *Ralstonia solanacearum*, damping off caused by *Pythium* spp., *Phomopsis* blight caused by *Phomopsis vexans* and little leaf caused by mycoplasma are important. The abiotic stresses, particularly frost damage, drought, and salinity are important in eggplant. The conventional approaches have been used for the improvement of eggplant with special reference to fruit size, weight, and shape and resistance to diseases and pests (Kalloo, 1993), and in fact these have contributed for the development of several improved varieties of eggplant. There are also many wild species of eggplant that are resistant to pests and pathogens, and are important sources of agronomically important genes for its improvement (Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003; Rajam and Kumar, 2006). Efforts to impart disease and pest resistance to the cultivated varieties have achieved limited success due to sexual incompatibilities with the wild relatives (Daunay and Lester, 1988; Kashyap *et al.*, 2003; Rajam and Kumar, 2006). However, the recent developments in eggplant biotechnology, including *in vitro* regeneration, protoplast culture and fusion, androgenesis and transgenics have facilitated the production of useful somatic hybrids and cybrids as well as transgenic plants with added values.

This chapter reviews the progress made in eggplant improvement using both conventional and nonconventional (biotechnological) techniques.

1.1 Traditional Strategies of Genetic Improvement in Eggplant

1.1.1 Interspecific hybridization

1.1.1.1 Traits of resistance in wild relatives

Eggplant is susceptible to numerous diseases and pests, particularly bacterial and fungal wilts, nematodes, and insects (Sihachakr *et al.*, 1994). The eggplant displays a great variability in morphological (color, shape, and size of plants, leaves and fruits), physiological, and biochemical characteristics. Moreover, resistance to most pathogens is also found in the cultivated eggplant, but its levels are partial and often insufficient for effective utilization in breeding programs (Messiaen, 1989; Daunay *et al.*, 1991). Resistance to bacterial wilt (*R. solanacearum*) (Li, 1988; Daunay *et al.*, 1991; Ali *et al.*, 1992b; Hanudin and Hanafiah Gaos, 1993) in some eggplant varieties has become ineffective during hot planting seasons or in poorly drained fields (Ano *et al.*, 1991). Only partial resistance against root-knot nematodes (*Meloidogyne* spp.), *Verticillium* (*V. dahliae*) and *Fusarium* (*F. oxysporum* f. sp. *melongena*) wilts, and to *Phomopsis* blight (*P. vexans*) is found in some varieties of eggplant (Yamakawa and Mochizuki, 1979; Messiaen, 1989; Ali *et al.*, 1992a). Likewise, some varieties have been reported to be only tolerant to insects, such as *Leucinodes orbonalis* (shoot and fruit borer), *Amrasca biguttula* (leaf hopper), *Aphis gossypii*, and *Epilachna vigintioctopunctata* (Bindra and Mahal, 1981; Chelliah and Srinivasan, 1983; Sambandam and Chelliah, 1983; Rotino *et al.*, 1997a). Therefore, it is greatly needed, through conventional breeding methods and biotechnological approaches, to introgress traits of high resistance to diseases and pests from eggplant landraces and wild relatives, displaying a great variability, into the cultivated eggplant. However, in Europe and some Asian countries like China and Japan, the release of F₁ hybrids, displaying higher productivity but with poor phenotypic variability has contributed to the losses of eggplant landraces, thus inevitably leading to the occurrence of genetic erosion of *S. melongena* (Daunay *et al.*, 1997). Moreover, some African cultivated eggplants also met genetic erosion following social, economic, and political changes (Lester *et al.*, 1990). Therefore, the cultivated

eggplants were considered priority species for the preservation of genetic resources since 1977, and several studies have been carried out in Asia and Africa (Lester *et al.*, 1990; Collonnier *et al.*, 2001a; Gousset *et al.*, 2005), and collections build up (Bettencourt and Konopka, 1990), particularly in China (Mao *et al.*, 2008).

Many wild relatives carrying traits of resistance to most diseases, pests, and environmental stresses affecting eggplant are summarized in Table 1. *Solanum sisymbirifolium* Lam. and *Solanum torvum* SW have been identified as resistant to the most serious diseases of eggplant, bacterial, and fungal wilts (Mochizuki and Yamakawa, 1979a, b; Messiaen, 1989; Daunay *et al.*, 1991). Resistance to *L. orbonalis* and to *Phomopsis* blight has been found in *Solanum khasianum* Clarke and *S. sisymbirifolium* Lam. (Kalda *et al.*, 1977). Interestingly, *Solanum aethiopicum* has been reported to be resistant to *L. orbonalis*, bacterial and *Fusarium* wilts (Ano *et al.*, 1991; Daunay *et al.*, 1991). Resistance to frost damage has been found in *Solanum grandiflorum* Ruiz and Pavon, *Solanum mammosum* L., and *S. khasianum* Clarke (Baksh and Iqbal, 1979), and tolerance to salinity in *Solanum linnaeanum* Hepper and Jaeger, and drought in *Solanum macrocarpon* L. (Daunay *et al.*, 1991) has been reported.

1.1.1.2 Sexual hybridization and its limitations

The use of conventional breeding methods has resulted in the improvement of agronomic and horticultural characteristics of eggplant, through mainly intraspecific crosses, and sporadically interspecific crosses because of sexual barriers between *S. melongena* and related wild species (Ano *et al.*, 1991; Bletsos *et al.*, 1998). The capability of eggplant to cross to species of other genera or subgenera is very low (Daunay *et al.*, 1991). This may result from the lack of genetic information about crossing partners, due to evolutionary divergence, which is known as incongruity (Franklin *et al.*, 1995). Attempt at crossing species from two species, *S. melongena* and *Solanum lycopersicum*, has resulted in sterile hybrids (Rao, 1979).

The interspecific hybridization from conventional crosses between eggplant and its wild

Table 1 Sources of resistance against diseases and pests in wild relatives of eggplant

Pathogens/pests	Resistant wild species	References
Fungi		
<i>Phomosis vexans</i>	(1) <i>S. viarum</i> Dun., (2) <i>S. sisymbirifolium</i> Lam., (3) <i>S. aethiopicum</i> L. gr gilo, (4) <i>S. Nigrum</i> L., (5) <i>S. violaceum</i> Ort., (6) <i>S. incanum</i> agg.	(1,2,3,4,5) Kalda <i>et al.</i> , 1977; (6) Rao, 1981
<i>Fusarium oxysporum</i>	(1) <i>S. violaceum</i> Ort., (2) <i>S. incanum</i> agg. (3) <i>S. mammosum</i> L., (4) <i>S. aethiopicum</i> L. gr <i>aculeatum</i> and gr (5) <i>gilo</i>	(1,2,4) Yamakawa and Mochizuki, 1979; (3) Telek <i>et al.</i> , 1977; (5) Rizza <i>et al.</i> , 2002
<i>Fusarium saloni</i>	(1) <i>S. aethiopicum</i> L. gr <i>aculeatum</i> , (2) <i>S. torvum</i> SW.	(1,2) Daunay <i>et al.</i> , 1991
<i>Verticillium dahliae</i> , <i>V. alboatrum</i>	(1) <i>S. sisymbirifolium</i> Lam., (2) <i>S. aculeatissimum</i> Jacq., (3) <i>S. linnaeanum</i> Hepper and Jaeger, (4) <i>S. hispidum</i> Pers., (5) <i>S. torvum</i> SW, (6) <i>S. scabrum</i> Mill.	(1) Fassuliotis and Dukes, 1972 (1,2) Alicchio <i>et al.</i> , 1984; (3) Pochard and Daunay, 1977; (1,2,3,6) Daunay <i>et al.</i> , 1991; (5) McCammon and Honma, 1982
<i>Colletotrichum coccoides</i>	<i>S. linnaeanum</i> Hepper and Jaeger	Daunay <i>et al.</i> , 1991
<i>Phytophthora parasitica</i>	(1) <i>S. aethiopicum</i> L. gr <i>aculeatum</i> , (2) <i>S. torvum</i> SW.	(1,2) Beyries <i>et al.</i> , 1984
<i>Cercospora solani</i>	<i>S. macrocarpon</i> L.	Madalageri <i>et al.</i> , 1988
Bacteria		
<i>Ralstonia solanacearum</i>	(1) <i>S. capsicoides</i> All., (2) <i>S. sisymbirifolium</i> Lam. (3) <i>S. sessiliflorum</i> Dun., (4) <i>S. stramonifolium</i> Jacq., (5) <i>S. virginianum</i> L., (6) <i>S. aethiopicum</i> gr <i>aculeatum</i> , (7) <i>S. grandiflorum</i> Ruiz and Pavon, (8) <i>S. hispidum</i> Pers., (9) <i>S. torvum</i> SW., (10) <i>S. nigrum</i> L., (11) <i>S. americanum</i> Mill., (12) <i>S. scabrum</i> Mill.	(1,2,3,4,9,12) Beyries, 1979, (2,10) Mochizuki and Yamakawa, 1979a; (3,9) Messiaen, 1989; (4) Mochizuki and Yamakawa, 1979b; (5,8,9,10) Hebert, 1985; (6) Sheela <i>et al.</i> , 1984; (7,11) Daunay <i>et al.</i> , 1991
Nematodes		
<i>Meloidogyne</i> spp.	(1) <i>S. ciarum</i> Dun., (2) <i>S. sisymbirifolium</i> Lam., (3) <i>S. elagnifolium</i> Cav., (4) <i>S. violaceum</i> Ort., (5) <i>S. hispidum</i> Pers., (6) <i>S. torvum</i> SW.	(1,4) Sonawane and Darekar, 1984; (2) Fassuliotis and Dukes, 1972; Di Vito <i>et al.</i> , 1992; (3) Verma and Choudhury, 1974; (5,6) Daunay and Dalmasso, 1985; (6) Messiaen, 1989; Shetty and Reddy, 1986
Insects		
<i>Leucinodes orbonalis</i>	(1) <i>S. mammosum</i> L., (2) <i>S. viarum</i> Dun., (3) <i>S. sisymbirifolium</i> Lam., (4) <i>S. incanum</i> agg., (5) <i>S. aethiopicum</i> gr <i>aculeatum</i> , (6) <i>S. grandiflorum</i> Ruiz and Pavon	(1,4,6) Baksh and Iqbal, 1979; (2,3) Lal <i>et al.</i> , 1976; (4,5) Chelliah and Srinivasan, 1983; (5) Khan <i>et al.</i> , 1978
<i>Epilachna vigintioctopunctata</i>	(1) <i>S. mammosum</i> L., (2) <i>S. viarum</i> Dun. (3) <i>S. torvum</i> SW.	(1) Beyries, 1979; (2,3) Sambandam <i>et al.</i> , 1976
<i>Aphis gossypii</i>	<i>S. mammosum</i> L.	Sambandam and Chelliah, 1983
<i>Tetranychus cinnabarinus</i>	(1) <i>S. mammosum</i> L., (2) <i>S. sisymbirifolium</i> Lam., (3) <i>S. pseudocapsicum</i> L.	(1,2,3) Shalk <i>et al.</i> , 1975
<i>Tetranychus urticae</i>	<i>S. macrocarpon</i> L.	Shaff <i>et al.</i> , 1982
Viruses:		
Potato virus Y	<i>S. linnaeanum</i> Hepper and Jaeger	Horvath, 1984
Eggplant mosaic virus	<i>S. hispidum</i> Pers.	Rao, 1980
Others		
Mycoplasma (little leaf)	(1) <i>S. hispidum</i> Pers., (2) <i>S. aethiopicum</i> L. gr <i>aculeatum</i> , (3) <i>S. viarum</i> Dun., (4) <i>S. torvum</i> SW.	(1) Rao, 1980; (2) Khan <i>et al.</i> , 1978; Chakrabarti and Choudhary, 1974; (3,4) Datar and Ashtaputre, 1984

Table 2 The interspecific crosses between the cultivated eggplant (*S. melongena* L.) and other *Solanum* species within the subgenus *Leptostemonum*

Species for crosses	Hybrid status	References
Section <i>Melongena</i>		
<i>S. melongena</i>	Fertile F ₁ plants	Pearce, 1975
<i>S. incanum</i> (gr C)	Fertile F ₁ plants	Pearce, 1975
<i>S. campylacanthum</i>	Partially fertile F ₁ plants	Pearce, 1975
<i>S. linnaeanum</i>	Partially fertile or sterile F ₁ plants	Pearce, 1975; Pochard and Daunay, 1977
<i>S. macrocarpon</i>	Partially fertile F ₁ plants	Pearce, 1975
<i>S. marginatum</i>	Partially fertile F ₁ plants	Pearce, 1975
<i>S. verginianum</i>	Partially fertile F ₁ plants or no F ₁ plants	Pearce, 1975; Rao, 1979
<i>S. sodomium</i>	Fertile F ₁ plants	Tudor and Tomescu, 1995
<i>S. xanthocarpon</i>	Sterile F ₁ plants	Rajasekaran, 1971; Hiremath, 1952
Section <i>Oliganthes</i>		
<i>S. aethiopicum</i> (= <i>integrifolium</i> , <i>zuccagnianum</i>)	Partially fertile F ₁ or sterile F ₁ plants	Pearce, 1975; Rao and Baksh, 1979
<i>S. anguivi</i>	Partially fertile F ₁ plants	Rajasekaran and Sivasubramanian, 1971
<i>S. cinereum</i>	Sterile F ₁ plants	Pearce, 1975
<i>S. pyracanthos</i>	No F ₁ plants	Pearce, 1975
<i>S. rubetorum</i>	Partially fertile F ₁ plants	Hassan, 1989
<i>S. tomentosum</i>	Partially fertile F ₁ plants	Pearce, 1975
<i>S. trilobatum</i>	No F ₁ plants, no seeds	Rao and Rao, 1984
<i>S. violaceum</i>	Partially fertile F ₁ plants	Bulinska, 1976
Section <i>Torva</i>		
<i>S. hispidum</i>	Partially fertile F ₁ plants	Khan, 1979; Magoon <i>et al.</i> , 1962; Rao, 1980
<i>S. torvum</i>	Partially fertile or sterile	Pearce, 1975; Bulinska, 1976
	F ₁ plants after embryo rescue	McCammon and Honma, 1982
		McCammon and Honma, 1982; Daunay <i>et al.</i> , 1991
Section <i>Acanthophora</i>		
<i>S. capsicoides</i>	No F ₁ plants	Pearce, 1975
<i>S. mammosum</i>	No F ₁ plants or abnormal seeds	Sambandam <i>et al.</i> , 1976
<i>S. viarum</i> = <i>khasianum</i>	Sterile F ₁ plants after embryo rescue	Pearce, 1975; Sharma <i>et al.</i> , 1980
Section <i>Stellatipilum</i>		
<i>S. grandiflorum</i>	Partially fertile F ₁ plants	Rao, 1979
Section <i>Nycterium</i>		
<i>S. lidii</i>	Partially fertile F ₁ plants	Hassan, 1989
Section <i>Cryptocarpum</i>		
<i>S. sisymbriifolium</i>	Sterile F ₁ plants after embryo rescue	Sharma <i>et al.</i> , 1984
Section <i>Campanulata</i>		
<i>S. campanulatum</i>	No F ₁ plants or abnormal seeds	Pearce, 1975
Section <i>Lasiocarpa</i>		
<i>S. stramonifolium</i>	No F ₁ plants, no seeds	Nishio <i>et al.</i> , 1984

relatives is listed in Table 2. Among 19 species used throughout the world in attempts at crossing with *S. melongena* with a view to its genetic improvement, only four (*S. incanum* L., *S. linnaeanum* Hepper and Jaeger, *S. macrocarpon* L. and *S. aethiopicum* L.) have successfully resulted in developing partially fertile F₁ hybrids, giving rise to fertile progenies. The other 15 species, particularly *S. sisymbriifolium*, *S. torvum*, and *S.*

khasianum (Figure 1) with traits of resistance to the most serious diseases of eggplant, gave only partially fertile hybrids or no hybrids at all (Daunay and Lester, 1988). Some eggplant lines showed a variable ability to cross with a given wild species, and successful crosses may depend on one-way incompatibility (Rao, 1979). The low fertility of interspecific hybrids of *S. melongena* with other species of *Solanum* may be related to problems of



Figure 1 Flowers and fruits of *Solanum melongena* (a, e, i–l), *S. aethiopicum* Gilo group (b, f), *S. torvum* (c, g), *S. sisymbirifolium* (d, h)

self-incompatibility brought by the wild parent, and not by the eggplant being self-compatible (Daunay *et al.*, 1991). In the family Solanaceae, self-incompatibility was shown to be gametophytic and mainly controlled by a multiallelic *S*-locus (Franklin *et al.*, 1995).

In several interspecific crosses, *in vitro* embryo rescue was successfully used to recover sexual hybrids. Crosses between *S. melongena* and *S. khasianum* were successful through embryo rescue, but only when the wild species was selected as the female parent (Sharma *et al.*, 1980). Hybrid

plants were successfully obtained from crosses between eggplant and *S. sisymbirifolium*, when the latter was taken as the male parent and that the torpedo-staged embryos were excised and cultured. However, the resulted plants could not survive for very long time and died (Sharma *et al.*, 1984). In other studies, hybrids of *S. melongena* with *S. sisymbirifolium* and *S. torvum* have also been developed through embryo rescue (Daunay *et al.*, 1991; Bletsos *et al.*, 1998). In attempts at interspecific hybridization, the resulted hybrids have either been sterile or have had very low pollen fertility (Sharma *et al.*, 1980, 1984; McCammon and Honma, 1982; Bletsos *et al.*, 1998). Hybrid fertility can however be restored when the diploid hybrids ($2x$) were brought to the allotetraploid status ($2n = 4x = 48$ (AABB)) by application of colchicine, such as the sexual hybrids between eggplant and *S. macrocarpon* (Gowda *et al.*, 1990), *S. torvum* (Daunay *et al.*, 1991), and *S. aethiopicum* (Isshiki and Taura, 2003).

Although wild relatives are valuable and useful for genetic improvement of the cultivated eggplant through conventional breeding methods and biotechnology, the exploitation of such wild species in breeding programs must cautiously be undertaken. Some of them may carry undesirable traits, such as the very high susceptibility to *Colletotrichum gloeosporioides* found in *S. torvum*, and the bitter taste, due to a high level of steroid saponin in *S. linnaeanum* (Collonnier *et al.*, 2001a).

1.1.2 Anther culture

Spontaneous haploid induction has never been reported in eggplant. The first haploids in eggplant were obtained by the Research Group of Haploid Breeding (1978) in China and by Isouard *et al.* (1979), although previously Raina and Iyer (1973) reported plant regeneration from anther culture. The efficiency of the technique was greatly increased by Dumas De Vault and Chambonnet (1982) using a protocol similar to that applied to pepper (Dumas de Vault *et al.*, 1981), which is based on a high temperature (35 °C) treatment during the first period of anther culture. According to this method, excised anthers are cultured in the induction medium and placed in dark at 35 °C for the first 8 days of culture and then transferred to

growth chamber with normal dark/light cycles; on the 30th day, anthers are transferred to the differentiation medium. Embryos become visible from the anthers 1 month from the beginning of culture and the embryo production lasts for 3–4 months. Well-formed embryos easily form complete plantlets in a growth regulator-free medium. Minor modifications of this method have resulted in reliable protocols to produce androgenetic plants and enabled a successful incorporation of this technique in eggplant breeding programs to rapidly obtain homozygous lines and to release F_1 cultivar (Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003). The most important factors influencing the yield of androgenetic plants are genotype, the environmental growth conditions of anther donors, culture media, and heat treatment of freshly cultured anthers (Rotino, 1996). Valuable genetic variation was displayed by doubled haploid lines obtained from anther donors represented by both inbred cultivar and highly heterozygous materials (Rotino *et al.*, 1991, 1992b). Plants were regenerated from isolated microspore culture following either anthers preculture (Gu, 1979) or direct culture of microspores (Miyoshi, 1996). *In vitro* androgenesis was successfully utilized to bring back the ploidy level to the diploid status of tetraploid interspecific hybrids between eggplant and the allied species *Solanum integrifolium* and *S. aethiopicum* gr *gilo*, and the “double somatic hybrid” obtained by sexual cross of the two somatic hybrids ((eggplant + *S. aethiopicum*) \times (eggplant + *S. integrifolium*)) (Rotino *et al.*, 2001, 2005; Kashyap, 2002; Rizza *et al.*, 2002). Dihaploids resistant to *F. oxysporum* f. sp. *melongenae* have been successfully backcrossed to eggplant and incorporated in the breeding program to develop new eggplant breeding lines with introgressed resistance to *Fusarium* wilt (Rotino *et al.*, 2001, 2005).

2. DEVELOPMENT OF TRANSGENIC EGGPLANTS

2.1 Regeneration *In Vitro*

The success of plant genetic engineering is determined by several factors, among which the availability of efficient plant regeneration from cultured cells/tissues is a crucial one. Eggplant

being a Solanaceae member is quite amenable for cell and tissue culture manipulations. Plant regeneration via organogenesis (both direct and indirect organogenesis) and somatic embryogenesis in cultivated as well as wild species of eggplants have been reported by a number of research groups, and such regeneration procedures have been successfully utilized in developing transgenic eggplants with new agronomic traits (Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003; Kantarajah and Golegaonkar, 2004; Magioli and Mansur, 2005; Rajam and Kumar, 2006).

2.1.1 Organogenesis

Plant regeneration via organogenesis has been achieved by using a variety of explants including hypocotyl (Kamat and Rao, 1978; Matsuoka and Hinata, 1979; Alicchio *et al.*, 1982; Hinata, 1986; Sharma and Rajam, 1995a), epicotyl (Magioli *et al.*, 1998), stem nodes (Magioli *et al.*, 1998), leaf (Alicchio *et al.*, 1982; Gleddie *et al.*, 1983; Mukherjee *et al.*, 1991; Sharma and Rajam, 1995a; Magioli *et al.*, 1998), cotyledon (Alicchio *et al.*, 1982; Sharma and Rajam, 1995a; Magioli *et al.*, 1998), root (Franklin and Sita, 2003; Franklin *et al.*, 2004), cell suspensions (Fassuliotis *et al.*, 1981; Gleddie *et al.*, 1983), and protoplast cultures (Sihachakr and Ducreux, 1987a) in various cultivated eggplant varieties. It has been reported that hypocotyl explants are the best for organogenetic response followed by leaf and cotyledon explants (Sharma and Rajam, 1995a). It was also shown that regeneration response varied within single explant and followed a basipetal pattern, which was correlated with the spatial distribution of endogenous polyamines (Sharma and Rajam, 1995a, b). The number of shoots per explant reported by various groups is usually low (~7 shoots/explant), except the reports of Sharma and Rajam (1995a) and Magioli *et al.* (1998, 2000), where they could obtain about 20 shoots/explant. Scoccianti *et al.* (2000) have shown that organogenesis from cotyledon explant is correlated with the hormone-mediated enhancement of biosynthesis and conjugation of polyamines. In addition, several wild species of eggplant were also regenerated using different seedling explants (Rao and Narayanaswami, 1968; Kowlozyk *et al.*, 1983; Gleddie *et al.*,

1985; Kashyap, 2002) and cell (Fassuliotis, 1975) or protoplast cultures (Guri *et al.*, 1987).

In eggplant, shoot regeneration can be achieved by using cytokinins alone (Gleddie *et al.*, 1983; Mukherjee *et al.*, 1991; Sharma and Rajam, 1995a; Magioli *et al.*, 1998) or in combination with auxins. Direct shoot regeneration has been achieved by a combination of benzylaminopurine (BAP) and indole-3-acetic acid (IAA) (Sharma and Rajam, 1995a), kinetin (Mukherjee *et al.*, 1991), or thidiazuron (TDZ) (Magioli *et al.*, 1998, 2000). Indirect shoot regeneration was obtained by using combinations of IAA with BAP, kinetin, or zeatin (Kamat and Rao, 1978; Sharma and Rajam, 1995a), α -naphthalene acetic acid (NAA) and BAP (Matsuoka and Hinata, 1979) and combinations of IAA, 2,4-dichlorophenoxyacetic acid (2,4-D), and kinetin followed by IAA and 2-(isopentyl) adenine (2iP) (Fassuliotis *et al.*, 1981). Organogenic nodules developed in cell suspension culture using IAA and 2iP failed to develop to shoots until transferred to medium containing ascorbic acid or antiauxin *p*-chlorophenoxy isobutyric (PCIB) acid (Fassuliotis *et al.*, 1981).

It has been shown that a variety of factors such as the concentration and combination of phytohormones, genotype, explant source and age, and physical environment can influence the organogenetic response in eggplant (Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003; Kantarajah and Golegaonkar, 2004; Magioli and Mansur, 2005; Rajam and Kumar, 2006). In most cases, the combination of BAP and IAA was found to be the best for shoot regeneration. Further, the ratio between BAP and IAA (the most optimum being 5:1) seems to be critical for better results (Sharma and Rajam, 1995a), which is also observed in other Solanaceae members, including tomato (Madhulatha *et al.*, 2006). However, low concentration of TDZ (100–200 nM) was useful in inducing efficient shoot regeneration (~20 shoots/explant) from leaf and cotyledon explants of five eggplant cultivars (Magioli *et al.*, 1998, 2000).

Tissue or cell cultures of eggplant has been used for the regeneration of plantlets on media supplemented with the pathogen toxins or abiotic stress agents in order to obtain potential lines resistant or tolerant to the above said conditions (Mitra *et al.*, 1981; Mitra and Gupta, 1989;

Sadanandam and Farooqui, 1991; Asao *et al.*, 1992; Ashfaq Farooqui *et al.*, 1997), or to understand gene function and activity (Alicchio *et al.*, 1984). Electrolyte release from regenerating eggplant calli has been used in screening for resistance and susceptibility to *V. dahliae* (Cristinzio *et al.*, 1994). Induction of laterals in root cultures of eggplant in hormone-free liquid medium was also reported and this system was used to demonstrate the role of polyamines in root growth and differentiation of lateral roots (Sharma and Rajam, 1997). They have shown that polyamines, particularly spermidine are intimately involved in root growth and differentiation of lateral roots.

2.1.2 Somatic embryogenesis

Somatic embryogenesis (SE) in eggplant has been very successful (Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003; Kantarajah and Golegaonkar, 2004; Magioli and Mansur, 2005; Rajam and Kumar, 2006) (Figure 2). Auxins alone can induce SE in eggplant and cytokinins have been shown to be inhibitory (Matsuoka and Hinata, 1979; Gleddie *et al.*, 1983). The first induction of SE in eggplant by Yamada *et al.* (1967) was achieved using IAA. Later on NAA (Matsuoka and Hinata, 1979; Alicchio *et al.*, 1982; Gleddie *et al.*, 1983; Matsuoka, 1983; Fillipone and Lurquin, 1989; Rao and Singh, 1991; Mariani, 1992; Sharma and Rajam, 1995a, b; Fobert and Webb, 1988; Hitomi *et al.*, 1998) and 2,4-D (Ali *et al.*, 1991; Saito and Nishimura, 1994; Hitomi *et al.*, 1998) have been widely used. It was reported that 2,4-D could not induce SE in a Brazilian variety F-100, although SE was successfully induced with

NAA. Thus, NAA is very efficient in inducing somatic embryo differentiation. However, its optimal concentration varies with the explant and genotype. Leaf explants required 2–6 mg l⁻¹ of NAA for SE, while hypocotyls required 6–10 mg l⁻¹ (Matsuoka and Hinata, 1979; Gleddie *et al.*, 1983; Sharma and Rajam, 1995a). SE was also affected by nitrogen content with an optimum NO₃⁻/NH₄⁺ ratio of 2:1 and sucrose concentration (0.06 M) (Gleddie *et al.*, 1983). Culturing of seeds in the dark for preparation of seedling explants was found to be helpful in inducing SE (Ali *et al.*, 1991). The use of aseptic ventilative filter has been found to be helpful (Saito and Nishimura, 1994). The occurrence of both SE and shoot primordial has been observed in the same calli after prolonged culture in the presence of 21.6 µM NAA (Fari *et al.*, 1995a). The somatic embryos induced by NAA in eggplant are formed from perivascular parenchyma cells originating indeterminate meristematic masses, which can either give rise to adventitious shoots or proembryogenic masses (Tarre *et al.*, 2004).

The conversion of somatic embryos into plantlets is generally hampered due to the abnormalities like hyperhydricity, lack of apical meristem, fusion of cotyledons, and inefficient maturation (Gleddie *et al.*, 1983; Saito and Nishimura, 1994; Magioli *et al.*, 2001a). However, about 92% conversion rate has been reported by culturing mature embryos on Murashige and Skoog (MS) medium solidified with 1% phytoigel (Saito and Nishimura, 1994; Magioli *et al.*, 2001a).

The most important factor influencing SE in eggplant has been the genotype (Sharma and Rajam, 1995a, b). Genotype-dependant variations in physiology and metabolism in the explants have been decisive in induction of somatic

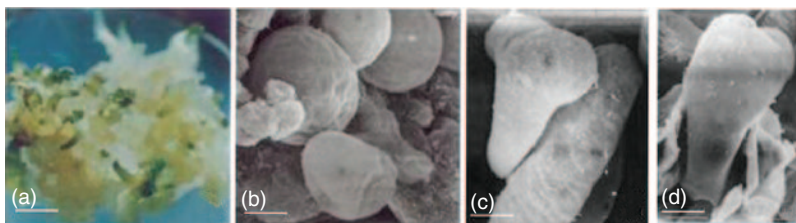


Figure 2 Somatic embryogenesis in eggplant from leaf tissue. (a) Formation of somatic embryos from callus, (b–d) scanning electron micrographs of various stages of somatic embryogenesis—globular, heart shaped, and torpedo somatic embryos

embryos (Sharma and Rajam, 1995a, b). It has been shown that the varietal difference exerted varied growth regulator requirement for optimum SE (Rao, 1992). It has also been validated through hybridization (Ali *et al.*, 1991) and gene expression (Afele *et al.*, 1996) experiments that the somatic embryogenic potential is genetically controlled.

Explant type also has been an important factor determining SE (Ali *et al.*, 1991; Sharma and Rajam, 1995a; Kantarajah and Golegaonkar, 2004). Various explants have been used in different protocols, which include leaves (Gleddie *et al.*, 1983; Rao and Singh, 1991; Sharma and Rajam, 1995a), cotyledons (Fobert and Webb, 1988; Saito and Nishimura, 1994; Sharma and Rajam, 1995a), and hypocotyls (Matsuoka, 1983; Ali *et al.*, 1991; Sharma and Rajam, 1995a). Leaves and cotyledons generally showed higher SE induction compared to hypocotyls (Sharma and Rajam, 1995a). Significant differences for morphogenetic potential were noticed within the different regions of a single explant—hypocotyl (Sharma and Rajam, 1995a, b) and leaf (Yadav and Rajam, 1997), which was attributed to the variation in the spatial distribution of polyamines.

In addition to plant hormones, very little is known about the additional means that may regulate plant morphogenesis *in vitro*. However, recent increasing evidences are showing the participation of polyamines in both SE and organogenesis and also other morphogenetic events from plant cells and tissues *in vitro* (Kumar *et al.*, 2006). Polyamines, which are considered to be the endogenous growth regulators, have been shown to be involved in the regulation of SE in diverse plant species, including eggplant (Kumar *et al.*, 2006). A thorough investigation has shown that the temporal changes in polyamines could be correlated with the variation in SE within the single explant (Sharma and Rajam, 1995a). It has also been demonstrated that the spatial distribution (Yadav and Rajam, 1997) and temporal regulation (Yadav and Rajam, 1998) of polyamines in explant correlates with SE process. Yadav and Rajam (1997) have demonstrated that the explants from different regions of the leaf showed significant variation in SE potential that was correlated with varying polyamine levels especially putrescine. It was also shown that the modulation of polyamine titers in the explants by exogenous addition has

resulted in the improvement of SE, suggesting the positive role of polyamines in the process. The follow-up study has further demonstrated the temporal regulation of SE by precise modulation of the endogenous polyamines during critical stages of SE (Yadav and Rajam, 1998). Data from these and other studies have been instrumental in establishing polyamines as biomarkers for plant regeneration (Shoeb *et al.*, 2001).

Studies on SE in eggplant have been extended recently to understand the molecular mechanisms underlying this important developmental process. A novel group of differentially expressing genes during SE has been identified through differential display (Momiyama *et al.*, 1995). An investigation into the molecular basis of difference among cultivars in SE using differential display has identified unique transcripts specific to the cultivar effectively producing SE in comparison to the one, which does not (Afele *et al.*, 1996). A more recent study has demonstrated the transcriptional expression of an *Arabidopsis thaliana* glycine-rich gene (*Atgrp-5*) during somatic embryo development in transgenic lines of eggplant carrying chimeric constructs using β -glucuronidase (GUS) fusion, where high promoter activity was detected in all cells of proembryogenic cell clusters and early stages in somatic embryo development (Magioli *et al.*, 2001a).

Somaclonal variations have also been reported in eggplant. For example, a salt-resistant line was obtained from cell culture in a medium fortified with 1% sodium chloride (Jain *et al.*, 1988). A somaclonal variant for resistance to little leaf disease was isolated from regenerants from tissue culture of infected plants (Magioli *et al.*, 1998). Synthetic seeds have also been developed by encapsulating somatic embryos with sodium alginate and calcium chloride (Rao and Singh, 1991; Mariani, 1992).

2.2 Protoplast Culture and Somatic Cell Hybridization

2.2.1 Protoplast culture

The control of plant regeneration from culture of organs, tissues, cells, and protoplasts is an essential prerequisite to the application of any techniques of biotechnology to supplement and

complement the conventional techniques aiming at genetic improvement in crops. In eggplant, plants could easily be regenerated through organogenesis and SE, using a variety of explant sources and this has been discussed in the previous section.

As far as the protoplast technology is concerned, plants of *S. melongena*, raised in growth chamber or greenhouse, gave poor yield of protoplasts, because of the presence of thick pubescence at the leaf surface, thus making sterilization difficult. Therefore, *in vitro*-grown plants have become the best source of protoplasts with good ability to high yield and develop, as they were sterilized, juvenile, and homogeneous (Saxena *et al.*, 1981; Sihachakr and Ducreux, 1987a). In most experiments, the primary choice of protoplast sources was concerned with the use of mesophyll tissues, from which high yield of protoplasts could easily be obtained. However, in some species, different steps of protoplast culture may encounter difficulties, needing the use of alternative protoplast sources. Protoplasts isolated from petioles were found to display a very high potential of development, compared to those from leaves and stems (Sihachakr and Ducreux, 1987a). A standard enzyme solution was effective for the isolation of protoplasts from various organs and tissues of eggplant and its wild relatives (Sihachakr and Ducreux, 1987a; Daunay *et al.*, 1993; Collonnier *et al.*, 2003a, b), potato (*Solanum tuberosum*) (Chaput *et al.*, 1990; Fock *et al.*, 2001), and sweet potato (*Ipomoea batatas*) (Sihachakr and Ducreux, 1987b; Sihachakr *et al.*, 1997).

The enzyme solution for eggplant protoplast isolation was composed of 0.5% (w/v) cellulase RS, 0.5% (w/v) macerozyme R-10 (Yakult, Tokyo, Japan), 0.5 M mannitol as osmotic agent and 0.05% (w/v) 2-(N-morpholino) ethane sulfonic acid (MES) buffer (Collonnier *et al.*, 2003a, b). The pH was adjusted to 5.5. About 500 mg of fresh plant material were incubated in 6 ml enzyme solution overnight in dark at 27 °C. The highest yield of eggplant protoplasts was obtained by using mesophyll as protoplast source with 9×10^6 protoplasts/g fresh weight, while petioles and stems gave 3.1 and 1.2×10^6 protoplasts/g fresh weight, respectively (Sihachakr and Ducreux, 1987a; Sihachakr *et al.*, 1993). Isolated protoplasts were highly heterogeneous. Most protoplasts from stems were highly vacuolated and floated on the surface of the culture medium, while those from

mesophyll and petioles, chlorophyllous and denser, dropped to the bottom.

Among numerous media routinely used, liquid basal and modified KM media (Kao and Michayluk, 1975) were found suitable for culture of eggplant protoplasts, particularly when supplemented with 0.2 mg/l 2,4-D, 0.5 mg l⁻¹ NAA, and 1 mg l⁻¹ BAP or zeatin (Sihachakr and Ducreux, 1987a; Collonnier *et al.*, 2003a, b). Glucose used at 0.35 M as osmoticum in the culture medium resulted in high viability and sustained cell division of eggplant protoplasts. Moreover, the addition of 250 mg l⁻¹ polyethylene glycol (PEG-MW 6000) to the culture medium enhanced the protoplast viability, probably due to its antioxidant property (Sihachakr *et al.*, 1993). In culture, protoplasts reformed the cell wall, and underwent cell division within 24 h and 3–4 days, respectively. However, after 7 days of culture, only 50% of cultured protoplasts survived, and some of them sustainably divided, giving rise to microcolonies. The frequency of cell division, observed after 10 days of culture, varied with the protoplast sources. The highest percentage of dividing cells was obtained with protoplasts from petioles, giving 33.4% division, followed by those from stems and lamina with 23.8 and 19.4% division, respectively (Sihachakr and Ducreux, 1987a). The division percentage was reduced to 10–15% by replacing the cytokinin zeatin with BAP in the initial culture medium. Moreover, cell growth was enhanced when diluting 10 times the cultures with fresh KM medium containing 0.1 mg/l 2,4-D and 2 mg l⁻¹ BAP or 0.2 mg l⁻¹ zeatin alone. The presence of zeatin in the diluting medium was shown to be favorable to further plant regeneration from cultured protoplasts of several species, particularly *Solanum nigrum* (Sihachakr and Ducreux, 1987c), *S. khasianum*, and *Solanum laciniatum* (Serraf *et al.*, 1988), and even sweet potato, which was known to be very recalcitrant (Sihachakr and Ducreux, 1987b; Sihachakr *et al.*, 1997). Three weeks after protoplast-derived calli were transferred onto the regeneration medium, containing 2 mg l⁻¹ zeatin and 0.1 mg l⁻¹ IAA, plant regeneration occurred by developing small deep-green spots. Sections of those spots showed embryo-like structures with two large cotyledons and a shoot meristem, but completely lacking the root. Some of them developed into leafy shoots, which can easily be rooted on

hormone-free medium. Interestingly, the callus ability to regenerate shoots was decreased and finally lost after maintaining the callus phase in 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA for more than 10 weeks (Sihachakr and Ducreux, 1987a). When transferred to the greenhouse, rooted shoots grew vigorously and showed similar morphology to the original plants. They intensely flowered and set fruits with viable seeds.

The successful development of the protoplast technology has provided the opportunity to apply biotechnology, such as somatic hybridization and genetic transformation, and gene transfer in view of improvement of eggplant (Collonnier *et al.*, 2001a; Kashyap *et al.*, 2003; Rajam and Kumar, 2006). Moreover, the cellular heterogeneity of the protoplast populations, as a single cell system, combined with an important callus phase during the culture, would result in regenerating somaclonal variants with interesting characteristics, which could further be selected and used in breeding programs. A salt-resistant line of eggplant was isolated from cell culture in a medium containing 1% sodium chloride (Jain *et al.*, 1988). In field trials, potential useful genetic variation for agronomic traits was observed in both embryogenic and androgenic eggplant lines (Rotino, 1996). In sweet potato, plants regenerated from cultured protoplasts showed a very great genetic variability, affecting plant morphology and physiology, particularly development and tuberization (Sihachakr *et al.*, 1997).

2.2.2 Somatic hybridization

The yield of cultivated varieties of eggplant is limited mainly by the susceptibility to diseases and pests, particularly bacterial and fungal wilts, which are the most serious diseases, causing heavy damages to this crop (Sihachakr *et al.*, 1994; Collonnier *et al.*, 2001b). Progress has been made to develop resistant lines by identifying and introgressing traits of disease resistance from wild relatives into cultivated eggplant through sexual crosses (Ano *et al.*, 1991; Bletsos *et al.*, 1998) and somatic hybridization (Sihachakr *et al.*, 1994; Collonnier *et al.*, 2001b; Kashyap *et al.*, 2003; Rajam and Kumar, 2006). Somatic hybridization by protoplast fusion offers the possibility of

overcoming sexual barriers or improving the fertility of hybrids obtained by conventional breeding methods (Daunay *et al.*, 1993; Sihachakr *et al.*, 1994). It provides the means of introgressing desirable agronomic traits without any detailed genetic and molecular knowledge of the genes encoding for such characters from wild into cultivated species (Jones, 1988; Collonnier *et al.*, 2001b). New and unique nucleo-cytoplasmic structures with a very high genetic variability are expected from the mixture of nuclear and cytoplasmic genomes of the fusion partners, occurring during somatic fusion.

The somatic hybridization through protoplast fusion between eggplant and its wild relatives is listed in Table 3. Protoplasts isolated from different sources, particularly leaves, cell suspensions, and calli were used in fusion experiments. Both electrical and chemical procedures, mainly the use of PEG, have successfully been applied for the production of somatic hybrids of eggplant with its wild relatives. In some fusion experiments, somatic hybrids were recovered by using selectable markers based on resistance to antibiotics, such as streptomycin and kanamycin, or chlorophyll-deficient mutant (Toki *et al.*, 1990; Guri *et al.*, 1991). In most experiments, the selection of eggplant hybrids was based on differences in the cultural behavior of hybrids and parental calli (Daunay *et al.*, 1993; Collonnier *et al.*, 2003a, b), and their identification was made upon the vigor and the morphological analysis of *in vitro*- and greenhouse-grown plants, particularly through the plant growth and morphology of leaves, inflorescence, flowers and fruits (Sihachakr *et al.*, 1994; Collonnier *et al.*, 2001b). The hybrid nature of selected plants was further confirmed by biochemical and molecular analysis, and chloroplasts of the parental and hybrid lines were identified by chloroplast DNA (ctDNA) restriction analysis (Daunay *et al.*, 1993; Collonnier *et al.*, 2003a, b). The ploidy level of somatic hybrids was determined by using flow cytometry and confirmed by chromosome count. Moreover, the composition of the hybrid genome was analyzed by using the technique of genomic *in situ* hybridization (GISH) (Collonnier *et al.*, 2003a, b).

The first successful somatic hybridization between cultivated eggplant and its wild species *S. sisymbriifolium* was conducted through PEG-mediated protoplast fusion, resulting in the

Table 3 Somatic hybridization in eggplant (*Solanum melongena* L.)

Fusion partners	Fusion	Culture response	References
Procedure			
<i>S. melongena</i> cv. Imperial Black Beauty + <i>S. sisymbirifolium</i>	PEG	26 hybrids mostly aneuploids with chromosome number close to 48	Gleddie <i>et al.</i> , 1986
<i>S. melongena</i> cv. Dourga + <i>S. khasianums</i>	Electrofusion	83 hybrids mostly tetraploids and (48) a few aneuploids (46–48)	Sihachakr <i>et al.</i> , 1988
<i>S. melongena</i> cv. Black Beauty + <i>S. torvum</i>	PEG	10 hybrids, tetraploids and aneuploids	Guri and Sink, 1988b
<i>S. melongena</i> cv. Black Beauty + <i>S. nigrum</i>	PEG	Two hybrids	Guri and Sink, 1988a
<i>S. melongena</i> cv. Dourga + <i>S. torvum</i>	Electrofusion	19 hybrids mostly tetraploids (46–48)	Sihachakr <i>et al.</i> , 1989b
<i>S. melongena</i> cv. Dourga + <i>S. nigrum</i>	Electrofusion	One hybrid aneuploid close to expected 96	Sihachakr <i>et al.</i> , 1989a
<i>S. melongena</i> cv. Shironasu + <i>N. tabacum</i>	Dextran	Green shoots from two somatic hybrid calli	Toki <i>et al.</i> , 1990
<i>S. melongena</i> cv. Black Beauty + (sexual hybrid Tomato × <i>Lycopersicon Pennellii</i>)	PEG	Two hybrid calli with leaflike primordia	Guri <i>et al.</i> , 1991
<i>S. melongena</i> cv. Dourga + <i>S. aethiopicum</i>	Electrofusion	35 hybrids mostly tetraploids (48)	Daunay <i>et al.</i> , 1993
<i>S. melongena</i> + <i>S. sanitwongsei</i>	PEG	–	Asao <i>et al.</i> , 1994
<i>S. melongena</i> + <i>S. aethiopicum</i> gr <i>aculeatum</i>	Electrofusion	Hybrids resistant to <i>Fusarium</i> wilt	Rotino <i>et al.</i> , 2001
<i>S. melongena</i> cv. Black Beauty + interspecific <i>Lycopersicon</i> hybrid	PEG	Asymmetric hybrids, only four hybrids	Liu <i>et al.</i> , 1995; Samoylov <i>et al.</i> , 1996
<i>S. melongena</i> + <i>S. torvum</i>	PEG	Asymmetric hybrids resistant to <i>Verticillium dahliae</i>	Jarl <i>et al.</i> , 1999
<i>S. melongena</i> cv. Dourga + <i>S. aethiopicum</i>	Electrofusion	30 hybrids, all fertile, mostly resistant to bacterial wilt	Collonnier <i>et al.</i> , 2001b
<i>S. melongena</i> cv. Dourga + <i>S. sisymbirifolium</i>	Electrofusion	Four hybrids resistant to bacterial wilt and <i>Verticillium dahliae</i>	Collonnier <i>et al.</i> , 2003a
<i>S. melongena</i> cv. Dourga + <i>S. torvum</i>	Electrofusion	26 hybrids mostly tetraploids and bacterial and fungal wilts	Collonnier <i>et al.</i> , 2003b

regeneration of 26 aneuploid hybrids. All the hybrids possessed only chloroplast genome of *S. sisymbirifolium*. They were highly resistant to root-knot nematodes and potentially resistant to spider mites, but further exploitation of those hybrids has been limited by strong hybrid sterility (Gleddie *et al.*, 1986). Similarly, four other tetraploid somatic hybrids were obtained by using electrofusion (Collonnier *et al.*, 2003a). They also had chloroplast genome of *S. sisymbirifolium*, and the analysis of the genome composition by using GISH technique clearly demonstrated the

presence of one complete set of chromosomes from both the parents in the hybrid nucleus. Moreover, *in vitro* tests revealed that the somatic hybrids were resistant to two bacterial strains of *R. solanacearum* and the filtrate of culture medium of *V. dahliae*. Since hybrid pollen was weakly fertile, the four somatic hybrids of eggplant with *S. sisymbirifolium* were expected to further be exploited in breeding programs (Collonnier *et al.*, 2003a). Electrofusion has successfully been used for production of 83 somatic hybrids between eggplant and *S. khasianum*, another useful wild

relative displaying resistance to shoot and fruit borer. A sample of six hybrid plants was examined for the chloroplast genome, and all had ctDNA type of eggplant (Sihachakr *et al.*, 1988). The hybrid plants grew vigorously in the greenhouse when grafted on eggplant rootstock because of difficulty in rooting. Despite 12% viable hybrid pollen, only parthenocarpic fruits without any seeds were obtained. Traits of resistance to the herbicide atrazine have been introgressed from *S. nigrum* into somatic hybrids of eggplant through chemical (Guri and Sink, 1988a) and electrical fusion procedures (Sihachakr *et al.*, 1989a). The hybrid plants had *S. nigrum* ctDNA, conferring resistance to 0.1 M atrazine. They developed abnormal morphology, affecting leaves and flowers, and produced only parthenocarpic fruits. The alteration of morphological traits was probably due to the aneuploid status of the hybrids with 90 chromosomes, close to the expected number (*S. melongena*: 24 + *S. nigrum*: 72 = 96) (Sihachakr *et al.*, 1989a). In somatic hybridization between eggplant and *S. torvum*, a very interesting wild relative with resistance to both bacterial and fungal wilts, chemical (Guri and Sink, 1988b) and electrical (Sihachakr *et al.*, 1989b; Collonnier *et al.*, 2003b) procedures have successfully been used, resulting in regeneration of 10 and 35 hybrid plants, respectively. Most of them had the eggplant ctDNA type, except one from electrofusion, displaying a mixture of chloroplasts from both the parents (Collonnier *et al.*, 2003b). The technique of GISH was applied to analyze the composition of the hybrid genome. As expected, the tetraploid hybrid plants contained one complete set of chromosomes from each fusion partner. On spread cytological preparation, the two parental genomes were not spatially separated at any time of the cell cycle. Translocation or recombinant chromosomes could not be demonstrated in the mitotic metaphase. The somatic hybrids were found resistant to nematodes and partially resistant to spider mite (Guri and Sink, 1988b). *In vitro* tests revealed that they had good levels of resistance to *R. solanacearum* and filtrate of culture medium of *V. dahliae*. Moreover, when grown to maturity in greenhouse, the hybrid plants flowered, but the pollen viability was reduced (Collonnier *et al.*, 2003b).

Somatic hybridization between eggplant and *S. aethiopicum* has provided a good example of

successful exploitation of somatic hybrids in breeding programs. Electrofusion was used to produce somatic hybrid plants aiming at introgressing traits of resistance to bacterial and fungal wilts from *S. aethiopicum* into cultivated eggplant (Daunay *et al.*, 1993; Rotino *et al.*, 2001) (Figure 3). Most of the hybrids had the eggplant ctDNA type. Field evaluation showed that the somatic hybrids had better pollen viability, estimated at 30–85%, compared to sexual counterparts with only 20–50%. Some somatic hybrid clones gave a very good yield of fruits up to 9 kg/plant, which was three to four-times as high as that of the parental lines, while the sexual hybrids produced only very few fruits. Tests carried out in a contaminated field in Bogor (Indonesia) revealed that the somatic hybrids were highly resistant to bacterial wilt. Similarly, other fertile somatic hybrids of eggplant with *S. aethiopicum* were shown to also be resistant to *Fusarium* wilt (Rotino *et al.*, 1998, 2001). The fertile tetraploid somatic hybrids have successfully been brought back to diploid status through anther culture (Rizza *et al.*, 2002), and the resulted dihaploid hybrids have been backcrossed to the recurrent eggplant (Rotino *et al.*, 2005). Interestingly, analysis of morphological traits and molecular markers, particularly isozymes and ISSRs (inter-simple sequence repeats), strongly suggested that genetic reassortment/recombination between the genomes of *S. melongena* and *S. aethiopicum* occurred during meiosis and it was expressed in the dihaploid somatic hybrid plants. Moreover, fertility and morphology close to cultivated eggplant were progressively recovered after a series of backcrosses of the dihaploids to cultivated eggplant. Thus, the resulted backcrossed progenies with fungal resistance can easily be incorporated in breeding programs of eggplant (Rotino *et al.*, 2005).

Protoplast fusion was also used to produce intergeneric hybrids of *S. melongena* with a triple mutant, chlorophyll-deficient, streptomycin and kanamycin-resistant *Nicotiana tabacum*, on which was based the selection scheme to recover the hybrids (Toki *et al.*, 1990). Interestingly, somatic hybridization between eggplant and a sexual hybrid of tomato with its wild relative, *Lycopersicon pennellii*, has been performed, resulting in the formation of only hybrid calli with few leaf primordial at first (Guri *et al.*, 1991), and then normal plants were regenerated after

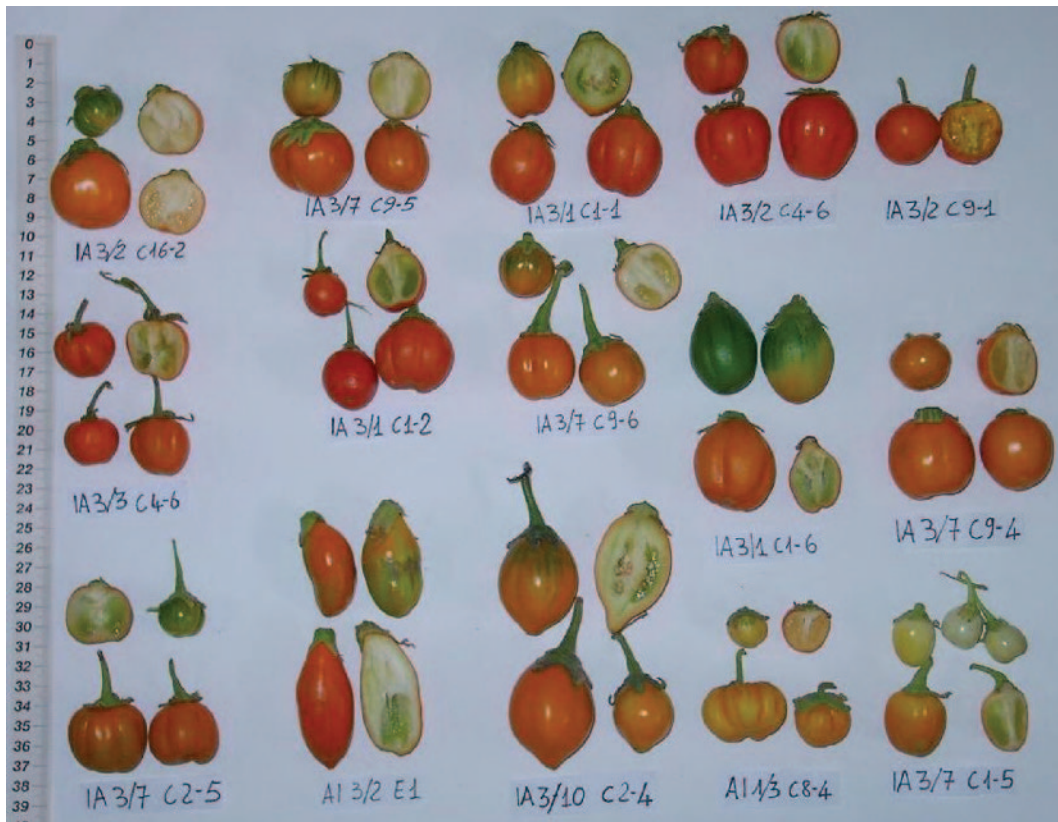


Figure 3 Variability in the shape, size, and color of fruits produced by dihaploid plants derived from anther culture of somatic hybrids between eggplant and *S. aethiopicum*

irradiating one fusion partner with γ -rays (Liu *et al.*, 1995; Samoylov and Sink, 1996; Samoylov *et al.*, 1996).

Although the combination of complete genomes can easily be obtained through somatic fusion, the resulted somatic hybrids, particularly between distant parents, are often sterile, thus limiting their further exploitation in breeding programs (Sihachakr *et al.*, 1994). An additional difficulty is their amphiploid status, and it is needed to bring tetraploid hybrids back to the diploid status, which is more suitable for intensive backcrosses to the diploid recurrent eggplant for introgression of desirable traits. Moreover, when the fusion partners are distantly related, the final product is often an asymmetric combination of the two genomes (Gleba *et al.*, 1984; Pelletier *et al.*, 1985; Pijnacker *et al.*, 1989). Therefore, a potential approach could be the production of highly asymmetric somatic hybrids bearing

few chromosome fragments from one parent associated with a complete set of chromosomes from the other parent (Jones, 1988; Sihachakr *et al.*, 1994). Interestingly, fertile somatic hybrids with tolerance to *Verticillium* wilt and morphology close to the cultivated eggplant have successfully been obtained after fusion between eggplant protoplasts and x-rays irradiated protoplasts of *S. torvum* (Jarl *et al.*, 1999). Furthermore, as plant regeneration is difficult from fusion between remote species, it would be of great interest to increase cytoplasmic variability in eggplant through organelle transfer via asymmetric fusion of protoplasts.

In conclusion, through several successful examples of somatic hybridization in eggplant, it is needed to include the technique of protoplast fusion in breeding programs of eggplant to complement and supplement the conventional breeding techniques for genetic improvement of

this crop. The somatic fusion provides the means for a rapid introgression of useful traits without any detailed knowledge of the genes encoding for the traits from wild into cultivated species.

2.3 Genetic Transformation

The most important prerequisite for genetic manipulation of a plant species is the availability of a standard procedure for the introduction of foreign genes. Members of the family Solanaceae are highly amenable for tissue culture and genetic transformation. Several groups achieved genetic transformation of a number of eggplant varieties via *Agrobacterium* during the last two decades (Table 4). The production of transgenic plants was first reported by Guri and Sink (1988c), using leaf explants and the cointegrate vector pMON 200 carrying the *nptII* gene. In the following year, Fillipone and Lurquin (1989) reported stable transformation of leaf and cotyledon explants using a binary vector. Since then, transgenic eggplants have been obtained using several *Agrobacterium* strains harboring binary vectors carrying *nptII* gene as a selection marker and different reporter genes, including *gus*, *cat* (chloramphenicol acetyl transferase) and *luc* (luciferase).

Transformation efficiency of eggplant is mainly influenced by preconditioning of the explants, regeneration pathway, antibiotics used to eliminate bacteria, bacterial density, and the explant type. Therefore, these factors should be evaluated for different genotypes in order to establish a successful protocol.

Preconditioning of explants before co-cultivation with *Agrobacterium* by culture in regeneration inducing medium has been reported for several species and is a determining factor to produce or improve the production of transgenic eggplant. The use of a preculture period of 48 h on solid medium has been adopted both for cotyledon and leaf explants (Rotino and Gledie, 1990; Arpaia *et al.*, 1997; Iannacone *et al.*, 1997). Fari *et al.* (1995b) demonstrated that a preculture of cotyledon explants for a period of 48 h followed by a 48 h co-culture in liquid regeneration medium period significantly improved the recovery of transformants. No kanamycin-resistant tissues were formed following 24 h co-culture and

transformation efficiency decreased by half when the preculture was not adopted. Magioli *et al.* (2000) also showed that a 48 h preculture of cotyledon explants in solid or 24 h in liquid regeneration medium (MS + 200 nM TDZ) resulted in a significant increase in the frequency of kanamycin-resistant calli formation and shoot production. More recently, a preculture period of 48 h on MS medium supplemented with 2 mg l⁻¹ kinetin or 2.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ IAA was found to be optimum to ensure transformation from leaf explants (Kumar and Rajam, 2005).

The production of transgenic eggplant through SE either fails to occur (Fillipone and Lurquin, 1989) or is achieved with very low efficiency (Fari *et al.*, 1995b) when using cefotaxime as the bactericidal antibiotics. Both co-cultivation with *Agrobacterium* and the presence of bactericidal antibiotics used in transformation protocols cause a reduction of up to 80–99% in the number of embryos/explant (Magioli *et al.*, 2001b). The inhibitory effect on somatic embryos development may result from the interaction between the physiological alterations caused by these treatments and the delicate processes of gene regulation that are induced in early culture stages. Antibiotics used to eliminate *A. tumefaciens* can also influence the efficiency of transformation protocols based on organogenesis. For example, augmentin can cause enhanced shoot regeneration induced by TDZ (Billings *et al.*, 1997).

Bacterial culture densities in the range of A₆₀₀ = 0.2–0.6 usually result in high transformation rates. Although higher number of GUS-expressing foci in transformed explants can be achieved with higher densities, the regeneration potential is greatly reduced, resulting in the production of low number of transgenic shoots (Kumar and Rajam, 2005).

The great majority of transformation protocols of eggplant is based on the use of leaf, cotyledons, and hypocotyl explants of *in vitro*-grown seedlings and generally cotyledons show the highest transformation efficiency. For example, the use of leaf segments resulted in two times lower transformation efficiency as compared to cotyledons (Fari *et al.*, 1995b; Magioli *et al.*, 2000). Interestingly, recent experiments using *Agrobacterium tumefaciens* strain A348 (pSM358) harboring a *VirE::lacZ* fusion construct, which carries hygromycin phosphotransferase gene (*hpt*) as

Table 4 Genetic transformation of eggplant

Explant	Gene	Main result	References
Leaf	<i>nptII</i> ^(a)	Success only with a cointegrate vector	Guri and Sink, 1988c
Cotyledon / Leaf	<i>nptII</i>	Stable transformation with a binary vector	Fillipone and Lurquin, 1989
Leaf	<i>nptII</i> , <i>cat</i> ^(c)	Transformation efficiency of 7.6%	Rotino and Gleddie, 1990
Cotyledon	<i>nptII</i> , <i>gus</i> ^(g)	7.5% transformation through organogenesis 0.8% transformation through embryogenesis	Fari <i>et al.</i> , 1995b
Hypocotyl	<i>Bt (cryIIIB)</i> ^(d)	Resistance to CPB was not observed	Chen <i>et al.</i> , 1995
Hypocotyl	Mutagenized <i>Bt (cryIIIB)</i>	Resistance to CPB	Arpaia <i>et al.</i> , 1997
Cotyledon	Mutagenized <i>Bt (cryIIIB)</i>	Resistance to CPB	Iannacone <i>et al.</i> , 1997
Leaf	<i>Bt (cryIIIB)</i>	Influence of growth regulators and antibiotics on transformation efficiency	Billings <i>et al.</i> , 1997
Leaf	<i>Bt (CryIIIA)</i> ^(e) Synthetic <i>Bt (cryIIIA)</i>	Resistance to fruit borer Resistance to CPB	Hamilton <i>et al.</i> , 1997 Jelenkovic <i>et al.</i> , 1998
Cotyledon	Synthetic <i>Bt (cryIAb)</i> ^(f)	Resistance to <i>Leucinodes orbonalis</i>	Kumar <i>et al.</i> , 1998
Leaf	<i>Luc</i> ^(h)	Evaluation of the stability of luciferase gene expression	Hanyu <i>et al.</i> , 1999
Cotyledon	<i>PAtgrp-5</i> ⁽ⁱ⁾ :: <i>GUS</i>	Optimization of factors which influence transformation efficiency	Magioli <i>et al.</i> , 2000
Cotyledon	<i>DefH9-iaaM</i> ^(j)	Parthenocarpic transgenic plants	Rotino <i>et al.</i> , 1997b; Donzella <i>et al.</i> , 2000; Acciarri <i>et al.</i> , 2002
—	yeast Δ -9 desaturase	Increased resistance to <i>Verticillium</i> wilt	Xing and Chin, 2000
Cotyledon	<i>MtID</i> ^(k)	Tolerance against osmotic stress	Prabhavathi <i>et al.</i> , 2002
Root	<i>nptII</i>	Efficient and stable transformation	Franklin and Sita, 2003
Leaf, cotyledon, hypocotyl	<i>Gfp:gus</i> , <i>hpt</i> ^(b) , <i>VirE::LacZ</i>	Increased transformation rate and <i>vir</i> genes expression by adding 100 μ M acetosyringone	Kumar and Rajam, 2005
Cotyledon	<i>Mi-1.2</i>	Resistance to root knot nematode	Goggin <i>et al.</i> , 2006
Cotyledon	<i>Oryzacystatin</i>	Resistance to aphids	Ribeiro <i>et al.</i> , 2006

^(a)*nptII*, neomycin phosphotransferase II^(b)*hpt*, hygromycin phosphotransferase^(c)*cat*, chloramphenicol acetyltransferase^(d)*Bt (cryIIIB)*, *Bacillus thuringiensis* cryIIIB^(e)*Bt (cryIIIA)*, *Bacillus thuringiensis* cryIIIA^(f)*Bt (CryIAb)*, *Bacillus thuringiensis* cryIAb^(g)*gus*, β -glucuronidase^(h)*Luc*, luciferase⁽ⁱ⁾*pAtgrp-5*, regulatory region of the *Arabidopsis thaliana* glycine-rich protein 5^(j)*DefH9-iaaM*, regulatory region of the DEFICIENS 9 gene from snapdragon and the auxin-synthesizing gene coding region (IaaM) from *Pseudomonas syringae* pv savastanoi^(k)*mtID*, bacterial mannitol-1-phosphodehydrogenase gene

a selection marker and a green fluorescent protein (*gfp*):*gus* fusion gene as a reporter, demonstrated that leaf, cotyledon, and hypocotyl explants are poor inducers of *vir* genes. Modulation of *vir* genes induction by adding 100 μ M acetosyringone during infection and co-cultivation steps resulted in a twofold to threefold increase in transformation frequency. It has also been demonstrated that although hypocotyls explants are superior to leaf and cotyledon explants regarding the percentage of explants showing transient GUS expression, the latter two are significantly more efficient in regenerating transformed shoots (Kumar and Rajam, 2005).

A high transformation efficiency was achieved with an organogenic system from root explants and *A. tumefaciens* strain LBA4404 harboring the binary vector pBAL2 with the reporter gene *gus* containing an intron (GUS-INT) and the marker gene *nptII*. Transgenic calli of the variety MEBH 11 were obtained on medium supplemented with 0.1 mg l^{-1} TDZ and 3.0 mg l^{-1} N-6-benzylaminopurine. Root explants demonstrated a high susceptibility to *Agrobacterium* and quick regeneration capacity on selection media containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime, resulting in 82.5% of transgenic calli induction with a means of 24 transgenic shoots per callus (Franklin and Sita, 2003).

Protocols based on the use of leaf, cotyledon and hypocotyl explants were successfully used to introduce agronomic traits into eggplant (Table 4). Resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say) (CPB), a pest that has developed resistance to synthetic insecticides and became a serious problem for agriculture in Europe and America (Arpaia *et al.*, 1997), has been pursued by a number of groups. Chen *et al.* (1995) have produced transgenic eggplant lines with the introduction of *Bacillus thuringiensis* (*Bt*) genes, but resistance to CPB was not observed. Later, different groups obtained lines resistant to CPB by using mutagenized versions of *cryIIIB* (Rotino *et al.*, 1992a; Arpaia *et al.*, 1997; Iannacone *et al.*, 1997) and a synthetic version of *cryIIIA* (Jelenkovic *et al.*, 1998) *Bt* genes. Field trials demonstrated high levels of resistance in transgenic plants produced after the introduction of a mutagenized *Bt cryIIIB* gene, without detrimental effects on nontarget arthropods (Rotino *et al.*, 1992a; Acciarri *et al.*, 2000; Mennella *et al.*, 2005).

Resistance to *L. orbonalis*, a lepidopteran insect that causes extensive damages to fruits and shoots, was obtained in transgenic plants produced through transformation of cotyledon explants with the strain EHA105 harboring a synthetic *cryIAb* gene modified for rice codon usage and carrying a castorbean catalase intron (Kumar *et al.*, 1998). Eggplant lines expressing *Bt-cryIFal* gene were recently developed and tested to be more effective than *cryIAb*-expressing lines (Kumar, unpublished). Efforts to commercialize eggplant hybrids expressing CryIAc protein are currently underway in India.

Recently, a gene encoding oryzacystatin was introduced in eggplant and the effect on *Myzus persicae* and *Macrosiphum euphorbiae* was examined (Ribeiro *et al.*, 2006). The transgenic eggplant reduced the net reproductive rate, the instantaneous rate of population increase, and the finite rate of population increase of both aphid species compared with a control eggplant line. Age-specific mortality rates of *M. persicae* and *M. euphorbiae* were higher on transgenic plants. These results indicate that expression of oryzacystatin in eggplant has a negative impact on population growth and mortality rates of *M. persicae* and *M. euphorbiae* and could be a source of plant resistance for pest management of these aphids. Expression of *Mi-1* gene isolated from tomato in eggplant cv. HP 83 conferred resistance to root knot nematode *Meloidogyne incognita* (Goggin *et al.*, 2006).

Attempts have also been made to engineer eggplant for fungal resistance. Overexpression of an yeast Δ -9 desaturase gene in eggplant has resulted in higher concentrations of 16:1, 18:1, and 16:3 fatty acids, and such transgenics exhibited increased resistance to *Verticillium* wilt (Xing and Chin, 2000). Transgenic plants challenged by *Verticillium* could also result in a marked increase in the content of 16:1 and 16:3 fatty acids. They have shown that *cis*- Δ 9 16:1 fatty acid was inhibitory to *Verticillium* growth. Transgenic eggplants resistant to *Verticillium* and *Fusarium* wilts by overexpression of pathogenesis-related genes, such as glucanase, chitinase and thaumatin (singly and in combination) were also produced (Singh, 2004).

Tolerance against osmotic stress induced by salt, drought and chilling stress was achieved in eggplants expressing the bacterial mannitol-1-phosphodehydrogenase (*mtlD*) gene, which is

involved in the mannitol synthesis (Prabhavathi *et al.*, 2002). Interestingly, these transgenic plants also showed enhanced resistance to fungal wilts caused by *V. dahliae* and *F. oxysporum*. Further, various transgenic eggplants overexpressing different genes (namely, arginine decarboxylase, ornithine decarboxylase, S-adenosylmethionine decarboxylase, and spermidine synthase) encoding enzymes in the polyamine metabolic pathway have also been generated. These transgenic plants showed increased tolerance to multiple abiotic (salinity, drought, extreme temperature, and heavy metals) as well as biotic (fungal pathogens) stresses. A gene pyramiding approach is also being pursued, in which the genes responsible for various agronomical important traits, including abiotic stress tolerance and fungal disease resistance, are being introduced in combination into eggplant through co-transformation.

Transgenic eggplants with parthenocarpic fruits were also developed by manipulating the auxin levels during fruit development through the introduction of *iaaM* gene from *Pseudomonas syringae* pv. *savastanoi* under the control of the ovule-specific promoter DefH9 from *Antirrhinum majus* (Rotino *et al.*, 1997b). These transgenics produced seedless parthenocarpic fruit in the absence of pollination without the external application of plant hormones, even at low temperature, which normally prohibit fruit production in untransformed lines. Further, these transgenics have exhibited significantly higher winter yields than the untransformed plants and a commercial hybrid under an unheated glasshouse trial (Donzella *et al.*, 2000). Trials in open field for summer production and greenhouse for early spring production confirmed that transgenic parthenocarpic eggplant F₁ hybrids gave a higher production coupled with an improved fruit quality with respect to the untransformed controls (Acciarri *et al.*, 2002). Quality parameters of frozen parthenocarpic fruits did not show any significant changes when compared to their controls (Maestrelli *et al.*, 2003).

3. CONCLUSIONS

Extensive studies have been performed on eggplant regeneration via both organogenesis and SE using tissues, cells, and protoplasts. Efficient regeneration systems have been achieved in a wide

variety of genotypes. Such regeneration protocols were successfully utilized to develop protocols for genetic transformation of eggplant, particularly via *Agrobacterium* and also somatic hybridization through protoplast fusion. Significant progress has been made in genetic improvement of eggplant through the use of conventional as well as biotechnological tools such as androgenesis, somatic hybridization, and genetic transformation. The studies on molecular markers like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) have not been exploited fully in eggplant breeding, and considerable attention is needed for developing comparative phylogenetic maps and molecular markers for pest and disease resistance as well as for various other useful traits. The genetic engineering approach seems to be very promising for the improvement of eggplant as transgenic lines with interesting traits, particularly resistance or tolerance to insects, nematodes, diseases, and abiotic stress, as well as parthenocarpic fruits have successfully been developed. Value addition to eggplant in terms of vitamins and iron will help ameliorate problems of malnutrition and under nourishment in many Asian countries. Tomato genome sequencing is currently underway as part of the larger initiative "International Solanaceae Genome Project" (SOL) (Mueller *et al.*, 2005). This sequencing project would offer exciting new perspectives and opportunities for eggplant improvement as the key genes that are responsible for various agronomically important traits, including plant architecture, disease and pest resistance, and fruit yield and quality would soon be available. A high degree of sequence similarity among the genomes of Solanaceae members facilitates cross utilization of microarray (genomics) tools and resources and will impact the improvement of all the cultivated members. An intensive effort to dissect the functional aspects of eggplant genome will hasten our efforts to improve the crop.

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Peppers

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1. INTRODUCTION

The genus *Capsicum* is one of the most important members of Solanaceae crops that include tomato, potato, eggplant, tobacco, petunia, and others. Almost five billion people in the world utilize peppers not only for food and spices but also for colorants and medicines. Peppers are cultivated in six continents ranking seventh in the harvested area under vegetable crops in the world and the classical breeding program for pepper cultivation has been well established in Asia and Europe with increasing number of elite F₁ hybrid varieties. Traditional pepper breeding has been focusing on traits such as yield, environment adaptation, fruit color and shape, and disease resistance. However, limited genetic resources for breeding and increasing demand for better pepper varieties require new tools for pepper breeding.

Two most important biotechnological tools for crop improvement are plant transformation and molecular marker-assisted breeding. For the last 10 years, farmers have constantly increased their cultivation area under genetically modified (GM) crops every year since GM crops were first commercialized in 1996. However, no GM pepper varieties have been commercialized; no GM peppers have been under field test due to the difficulties in transformation of peppers. One of the most important steps in plant transformation

is plant regeneration (Valera-Montero and Ochoa-Alejo, 1992; Ebida and Hu-C 1993; Harini and Sita, 1993). Despite continuous efforts, successful reports of pepper transformation are very limited. Even among the successful groups in pepper transformation, the transformation efficiencies have been very low and the transformation was neither consistent nor reproducible. Therefore, commercial GM varieties in peppers are still a long way to go. In this review, we will present a general overview of the genus *Capsicum* and current status of pepper transformation. In addition, we will introduce one of the successful protocols for pepper transformation at the end of this review.

1.1 History, Origin, and Distribution

The origin of *Capsicum* species, commonly known as peppers, is believed to be in South America, either central Bolivia along Río Grande (Andrews, 1995) or Brazil along the Amazon (DeWitt and Bosland, 1993). These areas concentrated with large wild species of peppers are also known as the nuclear areas (DeWitt and Bosland, 1993; Andrews, 1995). There are at least two centers of domestication, one in central America and the other in the Andean region of South America. Five species, among approximately 30 in the genus, were independently domesticated and have been

cultivated primarily for use as spice and vegetable for thousands of years (Andrews, 1995). Columbus introduced *Capsicum* into Europe in the 15th century, subsequently it was distributed rapidly around the world. Following its arrival in western Europe, both pungent and nonpungent forms of the species *C. annuum* came into wide cultivation, assuming a particularly important role in the cuisines of some parts of Europe, e.g., Hungary, West Africa, and many regions of Asia.

In western Europe and North America, relatively large, nonpungent bell type (*C. annuum*) became dominant in the 18th and early 19th century (Boswell, 1937). The pungent types remain favored in Latin America, Asia, and Africa. Today *Capsicum* is grown in tropical, subtropical, and temperate regions worldwide and *Capsicum* varieties are utilized for a diverse range of food products as vegetable and spice, fresh, dehydrated or processed, in medicine, in pest and animal control, and even in law enforcement, which make this crop of immense cultural and economic importance (Bosland and Votava, 2000).

1.2 Botanical Features

1.2.1 Taxonomy

The genus *Capsicum* belongs to the tribe Solanae, a family member of the Solanaceae (Hunziker, 2001). *Capsicum* is closely related to *Solanum*, to which two other important vegetable crops, tomato (*S. lycopersicum*) and potato (*S. tuberosum*), belong (Olmstead *et al.*, 1999; Martins and Barkman, 2005). About 30 species are currently recognized under the genus of *Capsicum* (Baral and Bosland, 2002), although the actual number of species is still debatable. In fact, taxonomy of the genus of *Capsicum* has long been confusing and arguable. Botanists traditionally define nomenclature and classification of *Capsicum* species based on morphological and anatomical characteristics. Phylogenetic relationship can also be determined by investigating hybridization performance between the *Capsicum* species (Tong and Bosland, 1999). Recently, molecular phylogenetic study has been carried out with 11 species of *Capsicum* with the aim of clarifying the phylogenetic ambiguity related to *Capsicum* (Walsh and Hoot, 2001).

C. annuum, *C. chinense*, and *C. frutescens* are the three main cultivated *Capsicum* species in agriculture worldwide. Types of cultivated *C. annuum* include bell pepper, jalapeño, New Mexico chile, ancho, Anaheim, and banana pepper, to name just a few. Many local varieties and landraces are also grown widely mostly in Latin America. In addition to these three species, two other domesticated species, *C. pubescens* and *C. baccatum*, are grown primarily in Latin America. *C. pubescens* has distinctive purple flowers and black seeds and was domesticated in the Peruvian and Bolivian Andes. Wild forms of all domesticated species have been identified, except of *C. pubescens* (Pickersgill, 1997).

1.2.2 Germplasm

Germplasm collection is extremely important in pepper breeding. Diversity of genetic resources is utilized for bringing new traits, particularly for resistance to new diseases and alternative resistance sources for existing diseases. Besides crop improvements, germplasm also plays an important role in various scientific researches, such as elucidation of evolution and classification of *Capsicum* species, understanding biology and biochemistry in peppers. Agricultural Research Service of United States Department of Agriculture (USDA-ARS) in Griffin (Georgia, USA) and Asian Vegetable Research and Development Center (AVRDC), the world's vegetable center based in Taiwan, have the world's largest collections of *Capsicum* germplasm and are active in characterization, evaluation and conservation. AVRDC has a total of 7726 accessions of *Capsicum* spp. as of September 2006 (<http://www.avrdc.org>); USDA-ARS has more than 4700 accessions of 16 *Capsicum* taxa in its active collection program in Griffin (Stoner, 2004). These two organizations also maintain databases for their collections and distribute seeds to pepper researchers and breeders all over the world. Other institutions, universities, government or nongovernment organizations, and private seed companies have various scales of *Capsicum* germplasm collections (Berke and Engle, 1997). Germplasm of *Capsicum* of at least 15 countries have been reported in *Capsicum and Eggplant Newsletter* (CENL) in the past 20 years (1983–2002).

1.2.3 Habitat and morphology

Wild *Capsicum* plants are perennial shrubs; commercial pepper cultivars are usually grown as annual crops. As a perennial, the plant turns from herbaceous to woody with age. *Capsicum* species has perfect and complete flowers. As a member of Solanaceae, flowers of *Capsicum* typically have five sepals, petals, stamens, and pistil. Color of petals (or corolla), stamens, and pistil can be white, greenish white, greenish yellow, or purple, combination varies depending on species and varieties. Cultivated *Capsicum* species are primarily self-pollinated and do not display inbreeding depression. Peppers can also be cross-pollinated by insects, outcrossing rate varies depending on the cultivars. Temperature, particularly night-time temperature is critical for fruit set (Andrews, 1995). The optimal day temperature for fruit setting of cultivated pepper is between 20–30 °C in chili pepper and 21–24 °C in sweet pepper, but fruits fail or have difficulty to set when night temperature exceeds 24 °C; growth of the plants is also reduced when air temperature is less than 15 °C or more than 32 °C for chili pepper and less than 18 °C or more than 27 °C for sweet pepper (<http://www.avrdc.org>).

Pepper fruit is the commodity of the pepper plant; therefore, fruit morphology, flavor, and pungency are the most economically important characteristics in *Capsicum*. A tremendous amount of genetic variation is known with respect to fruit traits such as size, shape, color, and flavor, resulting in more than 50 commercially recognized pod types. Bosland (1992) and Andrews (1995) described the major fruit types.

1.2.4 Genome size, karyotype, and ploidy level

The genome size of pepper was estimated to be 7.65 pg/nucleus for *C. annuum* and 9.72 pg/nucleus for *C. pubescens* (Belletti *et al.*, 1998). Genome size in nucleotides is estimated to be about 3000 Mbp (mega base pairs) (Arumuganathan and Earle, 1991). Most *Capsicum* species has 12 pairs of chromosomes; however, *C. ciliatum* has 13 pairs of chromosomes. Polyploidy is very rarely observed in *Capsicum* (Lippert *et al.*, 1966). The development of doubled haploid (DH) lines by

anther culture is common to obtain homozygous genotypes of interest and to develop populations for inheritance studies and the construction of genetic maps (Pochard *et al.*, 1983a; Lefebvre *et al.*, 1995). A set of trisomic lines were produced by Pochard (1977) and used for the chromosomal assignment of several mutations. Various types of chromosomal rearrangements are prevalent in the genus both within and between species (Pochard, 1977; Onus and Pickersgill, 2004).

1.3 Economic Importance and Consumption of Pepper

Peppers are the third-most important vegetable crops worldwide, grown in most countries in the world with production more than 22 million tons annually (FAO, 2002). The world's largest producer of peppers is China (more than 8 million tons in 2001) followed by Mexico and Turkey, which produced 1 900 000 and 1 400 000 tons, respectively in 2001. Spain, Italy, and the Netherlands are the main growers in Europe. Peppers are important source of income for farmers in most parts of the world.

Consumption of pepper is closely associated with cultures and diets. People in different countries or different regions of a country have different demands for fruit type, color, shape, maturity, taste, and pungency of peppers. For fresh- and dried-vegetable market, *C. annuum* is the dominated species of cultivated *Capsicum*, although *C. chinense* and *C. frutescens* are also commonly grown in some regions (Andrews, 1995). *C. pubescens* and *C. baccatum* have minor market in Latin American culture. Pepper fruit is mainly used as fresh and dried vegetable; leaf of pepper plants is also consumed as leafy vegetable in some cultures. Fresh and dried vegetable peppers can also be used in making sauces; the famous “Salsa” sauce is made with serrano and jalapaños (Andrews, 1995).

Peppers are also important crops for processed food industry. Three species of *Capsicum* are used for processed pepper: *C. annuum*, *C. chinense*, and *C. frutescens*. The major uses of peppers in processing industries are pickle, sauce and powder. Thompson (1995) collected and described more than 350 hot sauces from all over the world, including the best-known pepper sauce,

Tabasco (*C. frutescens*). Pepper fruits are dried or dehydrated and processed to produce pepper powder, also known as paprika in Europe. Pepper powder is an important spice particularly in Hungary and India.

In addition to its importance as a food and spice, peppers have been used and continue to be used widely for diverse medicinal applications (Bosland and Votava, 2000). Because capsaicinoids interact specifically with the mammalian pain receptor, VR1, they have been widely used as topical analgesics (Caterina *et al.*, 2000). While pain control is the most familiar application, hundreds of publications each year report various pharmacological applications of these molecules from weight loss to cancer.

1.4 Chemical Composition and Nutrition

1.4.1 Capsaicinoids

Capsicum may be best known for its biosynthesis of capsaicinoids that account for the pungent or “hot” sensation when consumed. Capsaicinoids have only been found in *Capsicum* species, but not all *Capsicum* species are pungent, for instance, nonpungent form can be found in *C. annuum*, *C. ciliatum*, and *C. chacoense*. Capsaicinoids are thought to play an important role in the evolution of *Capsicum* species. Tewksbury *et al.* (2006) studied the geographic variation of pungency within three species of ancestral *Capsicum* in Bolivia and found that production of capsaicinoids shifts across elevations. It was suggested that capsaicinoids entails both costs and benefits in response to selection pressure (Tewksbury *et al.*, 2006). It has been hypothesized that this trait evolved to deter mammalian herbivory, where crushing molars and acidic digestion are detrimental to seed survival (seed predator). The favored agents of dispersal are various species of birds. Avian species do not perceive pain in response to capsaicin and are attracted by the brightly colored fruit of *Capsicum* (Tewksbury and Nabhan, 2001).

Capsaicinoids include a family of up to 25 related alkaloid analogs produced in epidermal cells of the placenta or dissepiments of the fruit. The two major capsaicinoid compounds are capsaicin and dihydrocapsaicin. The gene *AT3*

(acyltransferase 3) at *Pun1* locus was identified for the biosynthesis of capsaicin (Stewart *et al.*, 2005). Nevertheless, the genetic base of biosynthesis of other quantitatively inherited capsaicinoids is still poorly known (Blum *et al.*, 2003). Biosynthesis of capsaicinoids is complicated and accumulation of capsaicinoids varies dramatically within and between *Capsicum* species. A number of analytical techniques have been reported for the determination of pungency in pepper and summarized by Pruthi (2003). Scoville index and high-performance liquid chromatography analysis represent the two generations of the most widely used techniques in pungency measurement (DeWitt, 1999); spectrophotometric method is used as ISO Reference method (Pruthi, 2003).

1.4.2 Pigments and vitamins

The most common colors of pepper fruits are green, red, yellow and orange, and chocolate or purple. Green peppers are usually not mature; the mature color ranges from lemon or yellow through orange, peach, and red. Growing and storage conditions alter color of pepper fruits (Gómez *et al.*, 2003). Pepper color is associated with its pigment content. Carotenoid and anthocyanin pigments are responsible for fruit color and for nutritional value of *Capsicum* fruit. Red color is resulted from the accumulation of different carotenoids in fruit chromoplasts during fruit ripening. The predominant red pigments are capsanthin and capsorubin, the yellow and orange pigments are lutein, β -carotene (provitamin A), zeaxanthin, violaxanthin, and antheraxanthin (Buckenhüskes, 2003). Carotenoid biosynthetic pathway in pepper fruit and putative correspondences with quantitative and qualitative organ-color loci identified in the Solanaceae was proposed by Thorup *et al.* (2000).

Peppers are well known as nutritious crop with very low calories, particularly rich in vitamin A and C. Peppers are ranked first in antioxidant content among vegetables with very high levels of vitamin C (Palevitch and Craker, 1995). Consumption of a single pepper fruit is enough to meet an adult Recommended Dietary Allowances for this vitamin. A list of nutrients of different peppers is provided at www.chilepepperinstitute.org. Vitamin C concentration is high in green fresh fruits, but is

lower in mature fruits and it breakdowns in dried or dehydrated pepper products. As for vitamin A, its accumulation increases as fruits turn matured to red or orange. Unlike vitamin C, high level of vitamin A is retained in dried fruits (DeWitt, 1999).

1.5 Breeding Objectives

1.5.1 Breeding for yield

Yield, like all other crops, is always a major objective in pepper breeding. Yield in pepper often associates with plant vigor, number, and concentration of fruit set, fruit weight and size, harvesting period. In addition, flowering time, fruit set at extreme temperature, growth habit (adaptation to open field or greenhouse production, mechanical or hand harvesting) are also considered. Besides actual increase in yield, stability of pepper production is equally important.

1.5.2 Breeding for disease resistance

Disease is often the major constraint of pepper production worldwide; therefore, disease resistance is one of the major objectives in pepper breeding and genetic studies (Paran *et al.*, 2004). Pernezny *et al.* (2003) has an overview of most of the known pepper diseases. Resistance sources in wild species or domesticated peppers have been reported for tobacco mosaic virus (TMV) (Boukema, 1980), cucumber mosaic virus (CMV) (Pochard, 1982; Shifriss and Cohen, 1990), potato virus Y (PVY) (Pochard *et al.*, 1983b), pepper ventral mosaic virus (PVMV) (Hobbs *et al.*, 1998), tomato spotted wilt virus (TSWV) (Rosello *et al.*, 1996), bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) (Hibberd *et al.*, 1983), bacterial wilt (*Ralstonia solanacearum*) (Perera *et al.*, 1992), *Verticillium* wilt (Gil Ortega *et al.*, 1990), *Fusarium* wilt (Jones and Black, 1992), Anthracnose (*Colletotrichum* spp.) (Voorrips *et al.*, 2004), *Phytophthora* root rot, stem rot, and foliar blight caused by *Phytophthora capsici* (Pochard and Daubeze, 1982), and nematodes, etc.

Disease resistance breeding and genetics have been studied extensively in the past years.

Introgression of disease resistance genes from wild germplasm to elite backgrounds contributes significantly in crop improvement, particularly in terms of yield and quality enhancement and stability in pepper production. Successful examples are as follows: resistance to TMV from *C. chinense* (L^3) and *C. chacoense* (L^4) (Boukema, 1980; Berzal-Herranz *et al.*, 1995; De la Cruz *et al.*, 1997), resistance to TSWV from *C. chinense* (Boiteux *et al.*, 1993), and resistance to bacterial leaf spot disease ($Bs2$) from *C. chacoense* (Cook and Guevara, 1984; Kim and Hartmann, 1985; Hibberd *et al.*, 1987) have been introduced to commercial *Capsicum annuum* cultivars. Additional genes for resistance to potyviruses, CMV, nematodes, *P. capsici*, and powdery mildew have been identified in several *Capsicum* species and are being utilized.

However, introgression of disease resistance genes into elite cultivars is particularly difficult when those resistance traits are inherited by complex quantitative mechanism and linked to undesirable horticultural and economic traits, such as low yield and small fruit size. In case of CMV and many other virus diseases, due to recombination and wide diversity of the virus, breeding for sustainable resistance remains a difficult task. With these challenges, breeding for disease resistance remains a prioritized breeding objective in pepper breeding in the future. Identification of new resistance sources, breeding for multiple disease resistances, and pyramiding different sources of resistance will be important in breeding for sustainable agriculture.

1.5.3 Breeding for quality

There are many types of peppers that are being utilized for different purposes, each with different quality requirements and traits required for successful production. A unique aspect of pepper breeding is of course the degree of pungency. Understanding people's diet and favors in terms of pungency level is particularly important in pepper breeding. Pungency level is particularly important for breeding for processed food industry. The industry also emphasizes on color, stability and uniformity of color, and color intensity (Bosland, 1993). For quality control reason, analytical

techniques for both color and pungency are often applied to assist breeding (Pruthi, 2003). For fresh vegetable pepper, major traits under selection are always related to fruit quality and taste: fruit color and color intensity, size, shape, pericarp thickness, taste, and degree of pungency. Furthermore, given the important role of long-distance transportation of vegetable produces now a days, shelf life also becomes an important breeding objective for fresh vegetable pepper. The major market types and their important fruit quality traits were summarized by Poulos (1994). These include fresh consumption; fresh for processing into sauce, paste, canned or pickled product; dried for spice (whole fruits and powder); oleoresin extraction; and ornamental types.

1.6 Tools and Strategies of Pepper Breeding

1.6.1 Classical genetics and conventional breeding

Classical inheritance studies and quantitative genetics have been used to study pepper genetics; conventional breeding methods and strategies have been used for crop improvement in pepper, for instance, single plant selection by pedigree method, population improvement by recurrent selection, backcrossing, single-seed descent (SSD), etc. A set of recombinant inbred lines derived from PSP-11 \times PI201234, produced by SSD has been generated and utilized for breeding and genetic studies of *P. capsici* resistance in pepper (Ogundiwin *et al.*, 2005). For commercial peppers, both open-pollinated and F₁ hybrid varieties are available.

1.6.2 Hybrids

F₁ hybrids (or single-cross hybrids), which are obtained from crossing between two homozygous inbred lines, were firstly used in corn. Now a days, F₁ hybrid cultivars are produced in many other field and vegetable crops. Hybrid cultivars are exploited on the basis of heterosis or hybrid vigor. F₁ hybrid peppers generally show significant improvements in yield, plant vigor, number of fruit set, disease resistance, etc. and have been produced by seed companies worldwide. The drawback of F₁

hybrids is the cost of seed production, as it involves hand emasculation of each flower. The use of male-sterility can, therefore, dramatically reduce the cost of production of F₁ hybrid peppers by eliminating emasculation in hybridization. Cytoplasmic-genic male-sterility, also known as cytoplasmic male-sterility (CMS) was discovered in pepper (*C. annuum* L.) by Peterson in 1958. Pepper CMS system is similar to that found in rice, petunia, and radish. Generally, in CMS system, male-sterility is caused by a cytoplasmic factor (S) and inherited maternally; normal cytoplasm is termed as (N). Recently, an additional abnormal mitochondrial gene *orf456* and a dysfunctioning mitochondrial gene *atp6* have been found in association with male-sterility in pepper (Kim and Kim, 2005, 2006); a dominant nuclear gene, *Rf*, carries out male-fertility restoration. In hybrid seed production, a CMS line (A line), (S) *rfrf*, serves as female and pollinated with a male-fertile restorer line (R line), (N) *RfRf*. A CMS line is maintained by an isogenic male-fertile maintainer line (B line) (N) *rfrf*. CMS system can give rise to 100% male-sterility and has been utilized particularly in hybrid seed production. However, this system tends to be more stable in certain types of hot pepper, for instance, hot dry-type pepper in Korea (Shifriss, 1997; Lee, 2001), but shows instability in other hot/sweet types, especially under low temperatures (Shifriss and Guri, 1979; Shifriss, 1997). R lines are quite common in hot peppers, but rare in sweet peppers (Zhang *et al.*, 2000; Kumar *et al.*, 2001; Yazawa *et al.*, 2002). Pepper types, therefore, restrict the utilization of CMS in hybrid seed production. An alternative male-sterility system, genic male-sterility (GMS), is available and has been used for sweet pepper production. In GMS system, male-sterility is conferred by a pair of homozygous recessive gene (*ms/ms*), homozygous dominant or heterozygous (*Ms/Ms* or *Ms/ms*) plants are male-fertile. To maintain and produce a male-sterile line, two isogenic lines with only difference at the *Ms* locus (*Ms/Ms* and *ms/ms*) are crossed, that gives rise to a progeny mixture of 50% male-fertile (*Ms/ms*) and 50% male-sterile (*ms/ms*) plants. In hybrid seed production field, the male-fertile plants are manually identified and removed, the remaining male-sterile (*ms/ms*) plants are used as female to pollinate with the second parental line (*Ms/Ms*) for hybrid seed production.

1.6.3 Tissue culture in pepper breeding

In vitro haploid plant production is exploited by plant breeders to facilitate the development of inbred lines particularly for hybrid breeding and widely adaptation in various field and vegetable crops. Nitsch and Nitsch produced haploid plants from pollen grains in 1969. A comprehensive protocol for anther culture in pepper was published by Dumas de Valuix *et al.* (1981). Various modifications or improvements of the original protocol have been reported (Qin and Rotino, 1993; Mityko *et al.*, 1995; Mityko and Juhasz, 2006). Other approaches for generating haploid plants have also been studied, including microspore culture and shed-microspore culture (Mityko and Fari, 1994; Supena *et al.*, 2006). In principle of haploid culture, plants are generated directly from haploid gamete ($n = x = 12$ in *C. annuum*), chromosome number of haploid plants is doubled by treating with colchicine and homozygous DH lines are produced (Sopory and Munshi, 1996). Once a protocol is established, robust tissue culture can be carried out to generate a number of DH lines. In conventional breeding, it takes 6–8 generations to produce homozygous inbred lines. In comparison, with haploid and DH technology, number of homozygous DH lines with different recombinant traits can be generated by culturing recombined haploid gametes from hybrid plants. The resulting DH lines are ready for testcross trials for hybrid breeding (Khush and Virmani, 1996). Tremendous time and cost could be saved by this strategy. Furthermore, DH can also be used in other aspects of genetic research, such as in genetic mapping and evaluation of complex quantitative traits (Thabuis *et al.*, 2003, 2004b).

Embryo culture is an important *in vitro* culture technique for plant breeding. One of the most significant applications is to overcome hybridization barrier between distant crosses (Brown and Thorpe, 1995). In the genus of *Capsicum*, only a few species are utilized for cultivation. However, tremendous genetic resources, for instance disease resistance genes, are hidden in other wild species. Most of these species belong to the secondary or tertiary gene pools, where crosses between different species may fail or may give rise to weak or sterile F_1 or backcross plants because of hybridization barriers. Even within the primary gene pool

barriers may occur between *C. annuum*, *C. chinense*, and *C. frutescens*. Hybridization barriers can occur in different stages. Embryo culture is particularly useful to overcome postzygotic or poor endosperm development, where endosperm fails to properly nourish the embryo and seed development is aborted (Brown and Thorpe, 1995). This technique allows researchers and breeders to transfer desirable genes/traits from a wild-related species. In practice, immature embryos are collected after hybridization and transferred to grow on basal growth medium and plants are regenerated from the rescued embryos. In addition, embryo culture can also be utilized in overcoming seed dormancy, slow seed germination, and immaturity in seeds. Besides embryo culture, other methods used to overcome hybridization barrier include using bridge cross through related species, mixing of pollens, and protoplast fusion, etc.

1.6.4 Molecular approaches in breeding

The use of molecular markers and genetic maps in pepper breeding is currently in developmental stage. The aim of molecular breeding is to supplement conventional breeding, to achieve faster and more efficient breeding through marker-assisted selection (MAS) and/or marker-assisted backcrossing (MAB). Molecular markers that are closely linked to the trait of interest are to be identified and applied in gene pyramiding, facilitating introgression of desirable traits into cultivars, early selection, etc. For more complex traits conferred by polygenes, quantitative trait loci (QTL) analysis is carried out. Furthermore, molecular markers and genetic maps also have significant contributions in other plant science research, particularly in map-based cloning.

Molecular markers and genetic maps are important resources for breeding and genetic studies, molecular resources have been building up in the past 20 years. The first generation of molecular markers are protein based isozymes. The first study in which isozymes were used for linkage mapping in *Capsicum* was reported by Tanksley (1984) who mapped 14 isozyme markers in an interspecific cross of *C. annuum* and *C. chinense*, of which nine were arranged in four linkage groups. From the 1990s, different types of DNA markers: AFLP (amplified fragment

length polymorphism), RAPD (random amplified polymorphic DNA), and RFLP (restriction fragment length polymorphism) were applied in pepper. Livingstone *et al.* (1999) used RFLP markers derived from tomato complementary-DNA (cDNA) and genomic DNA of pepper, which also allowed comparative genetic studies between tomato and pepper. Kang *et al.* (2001) developed sets of pepper-specific DNA probes for RFLP system. Toward the 21st century, molecular marker development has preceded to user-friendly polymerase chain reaction (PCR)-based DNA markers. Simple sequence repeat (SSR) markers in pepper are available in both private and public sectors (Lee *et al.*, 2004a; SOL Genomic Networks, <http://www.sgn.cornell.edu>). The future development is expected to move toward development of single-nucleotide polymorphism (SNP) and single-feature polymorphism (SFP) markers. SNP/SFP markers are believed to have the most potential in application of markers in crop improvement.

Several pepper genetic maps have been published in the past, most of these maps were produced in F₂ interspecific crosses: AC99, (*Capsicum annuum* × *C. chinense*) (SOL Genomic Networks); FA03, (*C. frutescens* × *C. chinense*) (Sol Genomic Networks); SNU, (*C. annuum* × *C. chinense*) (Kang *et al.*, 2001); and SNU2, (*C. annuum* × *C. chinense*) (Lee *et al.*, 2004a). SNU2, FA03, and AC99 are SSR-based genetic maps containing 46, 489, and 359 SSR markers, respectively. An integrated map has been developed recently by pooling data of six genetic maps, including two interspecific genetic maps (*C. annuum* × *C. chinense*) and four intraspecific genetic maps (*C. annuum* × *C. annuum*) (Paran *et al.*, 2004). The integrated map covering 1832 cM (centi-Morgans) contains 2262 markers including 1528 AFLP, 440 RFLP, 228 RAPD, 3 isozyme, and 3 morphological markers. This integrated map significantly reduced the length of gaps between markers to 0.8 cM and is expected to serve as an important platform for locating markers and aligning with other pepper genetic maps.

A large number of dominant and recessive genes have been identified and mapped in pepper through both classical and molecular approaches, including other potyviruses (*Pvr4*, *pvr6*) (Caranta *et al.*, 1996; Caranta *et al.*, 1999; Arnedo-Andres *et al.*, 2002) and bacterial spot resistance (*Bs3*) (Pierre *et al.*, 2000); resistance to TSWV (*Tsw*)

(Jahn *et al.*, 2000; Moury *et al.*, 2000), nematodes (*Me3*, *Me4*) (Djian-Caporalino *et al.*, 2001) and TMV (*L*) (Lefebvre *et al.*, 1995). As for more complex disease resistance traits, QTL studies have been carried out for resistance to CMV (Caranta *et al.*, 1997; Ben Chaim *et al.*, 2001; Caranta *et al.*, 2002), potyvirus (Caranta *et al.*, 1996), powdery mildew (Lefebvre *et al.*, 2003), anthracnose (Voorrips *et al.*, 2004) and *Phytophthora* (Nahm, 2001; Thabuis *et al.*, 2003; Thabuis *et al.*, 2004a; Ogundiwin *et al.*, 2005).

To date, two genes have been cloned in pepper conferring disease resistance by candidate gene and map-based cloning approaches respectively: *pvr1* (= *pvr2*), a recessive gene for potyvirus resistance (Ruffel *et al.*, 2002; Kang *et al.*, 2005); *Bs2*, a dominant, disease resistance gene (R gene) for bacterial spot resistance against *Xanthomonas campestris* pv. *vesicatoria* (Tai *et al.*, 1999; Tai and Staskawicz, 2000). Although the progress of gene cloning is relatively slow, with successful gene cloning, PCR-based molecular markers can be developed and applied in plant breeding program (Yeam *et al.*, 2005).

1.7 Limitations of Genetic and Molecular Resources

The current status of genetic and molecular resources limits their use in applied pepper breeding. Genetic resource is extremely important, however, the progress of characterization of collected germplasm is relatively low, for instance, 800 accessions have been characterized for USDA-ARS's *Capsicum* germplasm collection. Furthermore, not many valuable genetic populations, such as nearly isogenic lines, are available in pepper.

As for molecular resources, although the recent publication of the integrated map (Paran *et al.*, 2004) has improved the situation, there is still lack of correspondence between different pepper maps and QTL analyses in pepper (Varshney *et al.*, 2005). Most of these QTL studies relied on AFLP markers, which have relatively low reproducibility and do not include overlapping sets of markers. These studies also suffer to varying degrees from common limitations of QTL analyses: QTL from individual studies may be either overestimated or underestimated because of environmental factors and QTL intervals are

too often very large (Yano and Sasaki, 1997; Carlborg and Haley, 2004). The differences in types of populations, inoculation and scoring methods, and QTL assessment between different studies of *P. capsici* resistance in pepper, may affect estimation of QTL. In addition, resolution of QTL was further constrained by limited polymorphism often due to lack of polymorphism in intraspecific populations. Considering the limitations of QTL analysis and genomic resources in pepper, the diversity of the pathogens, and the complexity of genetic resources for resistance, molecular markers have important potential for MAS in pepper. Particularly, breeding for complex disease resistance traits in pepper remains a challenge for plant breeders.

With the limitations of genetic and molecular resources, genetic transformation provides an alternative approach for crop improvement in *Capsicum*. Transformation further overcomes the barriers between different species and allows us to introduce “useful” genes or novel traits into pepper.

2. DEVELOPMENT OF TRANSGENIC PEPPERS

2.1 Donor Genes: Sources, Isolation, Cloning and Designing Transgenes

The first pepper transformation was reported in 1990 (Liu *et al.*, 1990). However, Liu and his colleagues failed to regenerate the transgenic pepper plants. In fact, whole plant regeneration of pepper has been known as difficult with poor reproducibility that has been a key limiting factor for pepper transformation. Several studies on establishing and optimizing protocols for reliable regeneration have been reported in the past 15 years (Lee *et al.*, 1993; Manoharan *et al.*, 1998; Mihalka *et al.*, 2000; Kim *et al.*, 2002; Li *et al.*, 2003). Recently, Lee *et al.* (2004b) have reported an improved protocol by callus-mediated shoot formation. Despite these efforts, pepper transformation and regeneration efficiency are relatively poor comparing with that of other solanaceous crops, such as tomato and tobacco, where transformation protocols are well developed and have been routinely used for both biological studies and crop improvement.

Summaries of pepper transformation, regeneration methods and medium used for pepper transformation are presented in Tables 1 and 2, respectively. Besides establishing transformation and regeneration protocol, researchers are mostly interested in pepper transformation for disease resistance, particularly, virus resistance (Table 1). It has been found that by introducing viral gene(s) to the host plant, transgenic plants displayed resistance to the target and related virus. This disease resistance mechanism was first demonstrated by Abel *et al.* (1986) by transforming coat protein (CP) encoding gene of TMV into tobacco; the transgenic plants showed delay of disease development when infected with TMV and related tobamoviruses (Abel *et al.*, 1986; Beachy, 1999). Based on this resistance mechanism, pepper transformation has been studied by different research groups for resistance to TMV by introducing *TMV-CP* gene (Lee *et al.*, 2004b), pepper mild mottle virus (PMMV) by Pepper-PMMV interaction 1 transcription gene (Lee *et al.*, 2004b), tomato mosaic virus (ToMV) by *ToMV-CP* gene (Shin *et al.*, 2002b), CMV by *CMV-CP* gene (Shin *et al.*, 2002a) and CMV-satellite RNA (Kim *et al.*, 1997). Such transgenic virus resistance mechanism was later known as RNA silencing (Voinnet, 2001). Production of transgenic peppers by RNA silencing approach is considered to be specific and effective against targeted virus and/or viral groups.

Other donor genes might be used for developing broad-spectrum resistance in pepper (Table 1). Shin *et al.* (2002b) demonstrated transformation into and overexpression in pepper of *Tsi1*, a tobacco pathogenesis-related (PR) gene and the resulting transgenic plants displayed resistance or improved resistance to a broad spectrum of pathogen types including PMMV, CMV, a bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* and an oomycete pathogen *P. capsici*.

Besides transformation for disease resistance, studies on other aspects of transformation in pepper are limited. In other plants like *Arabidopsis*, tobacco, and tomato, transformation technology is also widely applied for functional studies of genes. Due to preliminary transformation technology in pepper, study on gene expression in transgenic pepper is extremely rare. Characterization of isolated pepper genes has not been carried out by transformation in foreign plants. With

Table 1 Summary of pepper transformation

Pepper cultivars/ genotypes	<i>Agrobacterium</i> strains	Pepper tissue for transformation	Selection marker/ resistance	Gene-of-interest/ promoter	Plasmid/vector	Reference
Yolo Wonder, Early California Wonder, NVH3051, Jupiter, Liberty Bell, Guatemalan wild accession Golden tower	A281, C58 (GV3850) LBA 4404	Young true leaves, cotyledons, hypocotyls	<i>nptII</i> /kanamycin	GUS/CaMV 35S	p3-1-GUS	Liu <i>et al.</i> , 1990
Zhong Hua No. 2	GV3111-SE	Cotyledons	Kanamycin, carbenicillin	Cucumber mosaic virus I ₁₇ N-satellite RNA/CaMV 35S <i>CMV-CP</i> /CaMV 35S	pRok1/105 pHCM40	Lee <i>et al.</i> , 1993 Zhu <i>et al.</i> , 1996
Pusa jwala No. 40017	EHA 105 C58C1Rif ^R , LBA 4404, EHA 101, A281	Young true leaves, cotyledons, hypocotyls Cotyledons Cotyledons	Kanamycin <i>nptII</i> /kanamycin <i>nptII</i> /kanamycin and geneticin, <i>hpt</i> /hygromycin, <i>dhfr</i> /methotrexate, <i>bar</i> /phosphinothricin <i>nptII</i> /kanamycin <i>surB</i> /chlorsulfuron	GUS/CaMV 35S GUS/CaMV 35S	pBI 121 pRGG plasmid set (contracted in this study)	Manoharan <i>et al.</i> , 1998 Mihalka <i>et al.</i> , 2000
Nockkwang VS300-1	LBA 4404 LBA 4404	Hypocotyls Young embryonic tissue, cotyledons	 <i>nptII</i> /kanamycin	<i>OsMADS1</i> /CaMV 35S <i>CaCell</i> /CaMV 35S	pGA1209 pWTT2132	Kim <i>et al.</i> , 2001 Harpster <i>et al.</i> , 2002
Golden Tower	LBA 4404	Cotyledons, hypocotyls	<i>nptII</i> /kanamycin	<i>CMV-CP</i> , <i>ToMV-CP</i> / CaMV 35S <i>Tsi</i> /CaMV 35S	pMBP2 pMBP2	Shin <i>et al.</i> , 2002a Shin <i>et al.</i> , 2002b
Nockkwang	–	Cotyledons, hypocotyls	<i>nptII</i> /kanamycin			
F ₁ Xiangyan 10, Zhongjiao, Zhongjiao 5, Zhongjiao 6	LBA 4404	Cotyledons	<i>nptII</i> /kanamycin	GUS/CaMV 35S	pBI121	Li <i>et al.</i> , 2003
Fehérozón	ShooterGRif ^R GV3170Rif ^R	Cotyledons	<i>hpt</i> /hygromycin or <i>nptII</i> /kanamycin	GUS/CaMV 35S	pRGG <i>hpt</i> , pRGG <i>neo</i>	Mihalka <i>et al.</i> , 2003
P915, P4090, P410, P101	EHA105, LBA4404	Cotyledons, hypocotyls	<i>nptII</i> /kanamycin	<i>TMV-CP</i> , <i>PP1II</i> /CaMV 35S	pCambia	Lee <i>et al.</i> , 2004b

Table 2 Summary of regeneration methods and medium used for pepper transformation

Regeneration method	Shoot inducing medium/selection medium	Elongation medium	Root inducing medium	Regeneration rate	Transformation rate	Reference
Direct regeneration	MSL2 medium: MS salts, L2 medium, BA (10 mg l ⁻¹), IAA (1 mg l ⁻¹) (whole plant regeneration failed)	–	–	–	–	Liu <i>et al.</i> , 1990
Direct regeneration	MS salts, B5 vitamins, 2% sucrose, kanamycin sulfate (100 mg l ⁻¹), carbenicillin (250 mg l ⁻¹), NAA (0.05 mg l ⁻¹), zeatin (2.0 mg l ⁻¹)	MS salts, B5 vitamins, 2% sucrose, kanamycin sulfate (100 mg l ⁻¹), carbenicillin (250 mg l ⁻¹), NAA (0.01 mg l ⁻¹), zeatin (2.0 mg l ⁻¹)	MS salts and vitamins, 3% sucrose, kanamycin sulfate (50 mg l ⁻¹)	57.5%	4%	Lee <i>et al.</i> , 1993
Direct regeneration	MS basal medium, kanamycin (50 mg l ⁻¹), carbenicillin (30 mg l ⁻¹), sucrose (30 g l ⁻¹), BA (8 mg l ⁻¹), IAA (2 mg l ⁻¹)	EM1: MS basal medium, BA (2 mg l ⁻¹), GA ₃ (2 mg l ⁻¹), ABA (2 mg l ⁻¹); EM2: MS basal medium, BA (2 mg l ⁻¹), GA ₃ (2 mg l ⁻¹), ABA (0.5 mg l ⁻¹)	MS basal medium with kanamycin (50 mg l ⁻¹), carbenicillin (30 mg l ⁻¹), NAA (0.1 mg l ⁻¹)	–	–	Zhu <i>et al.</i> , 1996
Direct regeneration	MS medium, 2% sucrose, kanamycin (50 mg l ⁻¹), cefotaxime (400 mg l ⁻¹), thidiazuron (TDZ) (0.5 mg l ⁻¹)	SER medium: Half strength MS medium, kanamycin (25 mg l ⁻¹), cefotaxime (200 mg l ⁻¹), IAA (0.5 mg l ⁻¹)	21.2–84.3% (independent form transformation)	–	–	Manoharan <i>et al.</i> , 1998
Direct regeneration	MSB5gl: MS salts, B5 vitamins, 2% glucose, cefotaxime (500 mg l ⁻¹) or augmentin (400 mg l ⁻¹), benzyladenine (BAP) (4 mg l ⁻¹), indol-3-acetic acid (IAA) (0.5 mg l ⁻¹) (optimized regeneration protocol)	–	–	–	–	Mihalka <i>et al.</i> , 2000

(continued)

Table 2 Summary of regeneration methods and medium used for pepper transformation (*continued*)

Regeneration method	Shoot inducing medium/selection medium	Elongation medium	Root inducing medium	Regeneration rate	Transformation rate	Reference
Direct regeneration	MS medium, cefotaxime (500 mg l ⁻¹), kanamycin (150 mg l ⁻¹), zeatin (3 mg l ⁻¹), IAA (0.3 mg l ⁻¹)	MS medium, kanamycin (50 mg l ⁻¹), NAA (0.3 mg l ⁻¹)	–	0.8%	–	Kim <i>et al.</i> , 2001
Direct regeneration	MS basal medium, kanamycin (100 mg l ⁻¹), sucrose (30 g l ⁻¹), zeatin (2 mg l ⁻¹), NAA (0.1 mg l ⁻¹)	–	–	–	–	Shin <i>et al.</i> , 2002a
Direct regeneration	MS basal medium, kanamycin (200 mg l ⁻¹), sucrose (30 g l ⁻¹), zeatin (2 mg l ⁻¹), NAA (0.1 mg l ⁻¹)	–	–	–	–	Shin <i>et al.</i> , 2002b
Direct regeneration	MS salts, B5 vitamins, 2% sucrose, kanamycin (500 mg l ⁻¹), IAA (1.0 mg l ⁻¹), BA (3.0 mg l ⁻¹), AgNO ₃ (5.0 mg l ⁻¹), AgNO ₃ (10 mg l ⁻¹), DJ nutrients (5000 mg l ⁻¹ ; prepared in this study)	MS salts, B5 vitamins, 2% sucrose, kanamycin (500 mg l ⁻¹), IAA (1.0 mg l ⁻¹), BA (3.0 mg l ⁻¹), AgNO ₃ (10 mg l ⁻¹), DJ nutrients (5000 mg l ⁻¹ ; prepared in this study)	MS salts, B5 vitamins, 2% sucrose, NAA (0.2 mg l ⁻¹), IAA (0.1 mg l ⁻¹)	81.3% (differentiation); 61.5% (elongation); 89.5% (rooting)	40.8% of regenerated plants	Li <i>et al.</i> , 2003
Direct regeneration	MSB5gl medium, cefotaxime (500 mg l ⁻¹)	MSB5gl medium, cefotaxime (300 mg l ⁻¹)	MS basal medium, glucose (15 mg l ⁻¹), maltose (15 mg l ⁻¹), IAA (0.5 mg l ⁻¹); MS basal medium, glucose (15 mg l ⁻¹), maltose (15 mg l ⁻¹), kanamycin (150 mg l ⁻¹)	–	2.4%	Mihalka <i>et al.</i> , 2003
Indirect callus-mediated shoot regeneration	MS medium, kanamycin (80–100 mg l ⁻¹), cefotaxime (300 mg l ⁻¹) or lincillin, zeatin (2.0 mg l ⁻¹) + NAA (0.5 mg l ⁻¹) or zeatin (2.0 mg l ⁻¹) + IAA (0.1 mg l ⁻¹)	MS medium, kanamycin (60–100 mg l ⁻¹), cefotaxime (300 mg l ⁻¹), zeatin (2.0 mg l ⁻¹) + NAA (0.01 mg l ⁻¹) or zeatin (2.0 mg l ⁻¹) + IAA (0.01 mg l ⁻¹)	MS medium, kanamycin (20–30 mg l ⁻¹), cefotaxime (200 mg l ⁻¹)	28/37 500 (number of shoots with roots/ number of explants)	0–0.19%	Lee <i>et al.</i> , 2004b

an exception, Harpster *et al.* (2002), who demonstrated suppression of a ripening-related endo-1,4- β -glucanase in transgenic pepper. On the other hand, transformation of foreign genes identified in other plant species into pepper is also uncommon. Nonetheless, by transformation and constitutive expression of a rice gene, *OsMADS1*, a dwarf transgenic pepper was successfully produced (Kim *et al.*, 2001).

Classical transgene designs have been used in pepper transformation (Table 1). The transgene construct, which contains β -glucuronidase (GUS) gene as reporter gene, driven by cauliflower mosaic virus (CaMV) 35S promoter and with nopaline synthase (NOS) as terminator, is dominated in pepper transformation studies, particularly for the studies aiming at establishing and optimizing pepper transformation protocol (Liu *et al.*, 1990; Manoharan *et al.*, 1998; Li *et al.*, 2003; Mihalka *et al.*, 2003). CaMV 35S promoter is commonly used for overexpression of genes of interest (Zhu *et al.*, 1996; Kim *et al.*, 2002; Shin *et al.*, 2002b; Lee *et al.*, 2004b).

2.2 Methods Employed

2.2.1 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation system is commonly employed for pepper transformation. Other transformation systems, for instance gene gun, have not been reported. Wild type *A. tumefaciens* generally is not considered as a pathogen of *Capsicum* spp. under field conditions, although certain strains of *Agrobacterium* showed virulence to pepper (De Cleene and De Lay, 1976). Capability of *Agrobacterium* strains to induce gall formation under laboratory conditions is required for successful genetic transformation. Use of strains also determines the efficacy of transformation (Liu *et al.*, 1990; Mihalka *et al.*, 2000). Liu *et al.* (1990) compared the effect of two different *Agrobacterium* strains in pepper transformation. The strain C58 consistently showed distinctively higher average percentage of explants forming tumors than the strain A281.

Cotyledons and/or hypocotyls are the most commonly used explants (Manoharan *et al.*, 1998; Pozueta-Romero *et al.*, 2001; Kim *et al.*, 2002;

Lee *et al.*, 2004b). In addition, use of young leaf for pepper transformation has also been demonstrated (Liu *et al.*, 1990; Zhu *et al.*, 1996). For transformation, *Agrobacterium* is co-cultured with the pepper explants in Murashige and Skoog (MS) medium (Pozueta-Romero *et al.*, 2001; Lee *et al.*, 2004b) or MSB5gl medium (MS salts with B5 vitamins and glucose) (Mihalka *et al.*, 2000, 2003).

Different pepper cultivars/genotypes also show variation in response to *Agrobacterium*-mediated transformation (Liu *et al.*, 1990; Lee *et al.*, 2004b). Transformation has been carried out mainly in *C. annuum*, both chili pepper and sweet pepper (Zhu *et al.*, 1996; Manoharan *et al.*, 1998); transformation of other *Capsicum* spp. is rarely tested except for *C. frutescens*.

2.2.2 Selection of transformed tissue

Antibiotic resistance has been the most commonly used method for the selection of transformed pepper tissue (Table 1). To screen for positive transformation, explants are transferred to antibiotic-containing medium after transformation or gall formation for selection, positive transformants with antibiotic resistance would survive. Similar to other protocols for selection of transformants in other plants, kanamycin resistance is commonly used for selection in pepper transformation. Kanamycin resistance is contributed by the neomycin phosphotransferase II gene (*nptII*) in the vector construct (Liu *et al.*, 1990; Kim *et al.*, 1997; Manoharan *et al.*, 1998; Mihalka *et al.*, 2000; Pozueta-Romero *et al.*, 2001; Shin *et al.*, 2002a, b; Li *et al.*, 2003). *NptII* is usually driven by a NOS gene promoter (Manoharan *et al.*, 1998; Kim *et al.*, 2002; Li *et al.*, 2003). *NptII* confers resistance to kanamycin as well as geneticin. Alternative selection markers for pepper transformation have been demonstrated to be effective by using hygromycin resistance gene (*hpt*) for hygromycin resistance, methorexate resistance gene (*dhfr*) for methorexate resistance and phosphinothricin acetyltransferase gene (*bar*) for phosphinothricin resistance (Mihalka *et al.*, 2000).

However, use of antibiotic or herbicide resistance genes as selection markers for transformation has risen environmental and health concerns.

Alternative selection methods have been investigated. Shin *et al.* (2002a) discussed the potential use of ToMV-CP gene as a selection marker. In this screening system, TMV is inoculated to the transgenic plants and positive transformants are identified by a distinctive hypersensitive response. However, further experiment is necessary to evaluate the efficiency and effectiveness of this selection system.

Another alternative selection system was demonstrated by transformation and expression of isopentenyl transferase gene (*ipt*) identified from “shooter” mutants of *Agrobacterium* (Mihalka *et al.*, 2003). With the presences of *ipt*, positive transformants were shown to produce direct shooting on growth regulator-free medium. The significant morphological characteristic allows visual selection of positive transgenic plants. This system has also been demonstrated in transformation of other crops like tomato, tobacco, and muskmelon. The use of binary vector in pepper transformation has also been reported (Mihalka *et al.*, 2003). Selection markers, which might carry undesirable genes, and the genes of interest are integrated in different chromosomal positions in the transgenic pepper under the binary vector system. Therefore, selection marker genes could easily be removed subsequently.

2.2.3 Regeneration of whole plants

Regeneration of pepper plants has long been a challenging task for pepper transformation studies. Several studies attempted to improve, modify, and optimize protocols for regeneration of transgenic pepper plants. However, these experiments are often hardly reproducible.

Direct regeneration from shoot is the most common practice for transgenic pepper regeneration. In this system, explants transformed with *Agrobacterium* are grown on shoot inducing medium or selection medium for 3–4 weeks, shoots or multishoots are formed directly at the sites of wounded explants. Then induced shoot buds are transferred to the elongation medium and grown for 20 days to 8 weeks. The shoots formed are excised and grown on root-inducing medium. Recently, an alternative regeneration method, named callus-mediated shoot formation, has been proposed (Lee *et al.*, 2004b). In this study,

six different types of callus have been identified from transformed tissue and two of which were able to form shoots. Desirable type of callus were isolated and propagated (instead of direct shooting); propagated transformed callus was then subcultured for shoot formation. Although the transformation rate remains low (<0.19%), callus-mediated shoot formation method is believed to be more reproducible and reliable. More detailed protocol of this approach is presented in the next section.

MS medium is used as basal medium for transgenic plant regeneration, depending on the research group; different concentrations and compositions of supplements were applied (Table 2). Particularly, effects of hormones have been studied by several research groups with the aim of optimizing regeneration conditions. Nevertheless, there was lack of consensus between different research groups. Different compositions and ratios of cytokinins (benzyladenine, BAP), thidiazuron (TDZ), naphthalene acetic acid (NAA), zeatin, (2-isopentyl adenine (2IP)) to auxin (indol-3-acetic acid, IAA) have been tested (Lee *et al.*, 1993; Zhu *et al.*, 1996; Manoharan *et al.* 1998; Mihalka *et al.*, 2000; Li *et al.*, 2003). Furthermore, kanamycin in media is commonly used for selection of positive transformants. Concentration and effects of other antibiotics (geneticin, hygromycin, methorexate, phosphinothricin) in the regeneration media have also been studied (Mihalka *et al.*, 2000). In addition, effects of other important factors on regeneration have been studied, for instance, effect on explant tissue for shoot bud initiation (Zhu *et al.*, 1996), genotypes/cultivars used for plant transformation and regeneration (Liu *et al.*, 1990; Kim *et al.*, 2002; Lee *et al.*, 2004b).

2.3 Case Study: Transformation Using Callus-Mediated Indirect Regeneration Method

Most of the previously described transformation procedures for pepper transformation are not very useful for routine transformation due to the lack of reproducibility. The low rate of pepper transformation indicates that DNA transfer via *Agrobacterium* infection into cut-injured cotyledon or hypocotyl tissues hardly occurs. This also suggests that *Agrobacterium* may not

penetrate and/or infect cotyledon or hypocotyl tissues of pepper. Lee *et al.* (2004b) demonstrated that selection of the right callus type is most critical for successful pepper transformation. Indeed, the transformation via callus-mediated shoot formation proved to be reproducible and this selection method provided a reliable system for pepper transformation. The protocol for successful pepper transformation and the results obtained using the protocol are described below.

2.3.1 Transformation protocol

Seeds from commercial inbred lines (*C. annuum* inbred line P915, P409, P410, P101: Nongwoo Bio Co. proprietary) are commonly used for the experiment. These lines showed a very high rate of regeneration (90%) among the 30 inbred lines tested (Kim *et al.*, 2002).

2.3.1.1 Germination and explant

Seeds are surface-disinfested in 95% ethanol for 30 s, 50% bleach for 10 min and rinsed three times with sterilized water. Sterilized seeds are then placed in 1/2 MS medium (Murashige and Skoog, 1962) and allowed to germinate in light or dark at 25°C. Germination under light or dark conditions does not influence the transformation rate. Cotyledons and hypocotyls from 8- to 10-day-old plants are excised and used as explants.

2.3.1.2 Preculture and *Agrobacterium* inoculation

Explants are transferred to a pre-culture medium consisting of MS medium supplemented with zeatin 2 mg l⁻¹ and NAA 0.05 mg l⁻¹ or IAA 0.1 mg l⁻¹, and placed in light room at 25°C for 2–36 h and this preculture step is necessary. For transformation, *Agrobacterium* strain EHA105 is the best choice for the pepper transformation. *Agrobacterium* is grown in YEP (2% bacto peptone; 1% yeast extract) media supplemented with kanamycin 50 mg l⁻¹, rifampicin 50 mg l⁻¹ and 100 µM acetosyringone. The *Agrobacterium* culture is centrifuged and then diluted with MS to optical density 0.3–0.8. This bacterial suspension

is then mixed with MS liquid containing 100 µM acetosyringone and inoculated to explants for 10–20 min, co-cultured in dark for 38–96 h and then washed with cefotaxime 500–800 mg l⁻¹ or lilacilline 500–800 mg l⁻¹ three times.

2.3.1.3 Callus and shoot formation

In order to obtain callus formation and development, explants are transferred to a selection medium consisting of 80–100 mg l⁻¹ kanamycin, 300 mg l⁻¹ cefotaxime or lilacilline with zeatin 2.0 mg l⁻¹ + NAA 0.05 mg l⁻¹ or IAA 0.1 mg l⁻¹ for 6–8 weeks. For shoot formation and elongation, the calli are transferred to a regeneration medium consisting of 60–100 mg l⁻¹ kanamycin, 300 mg l⁻¹ cefotaxime with zeatin 2.0 mg l⁻¹ + NAA 0.01 mg l⁻¹ or IAA 0.01 mg l⁻¹ for 7–10 weeks.

2.3.2 Transformation experiment using the protocol

Two general patterns of pepper shoot formation were identified. Firstly, a shoot or multishoots formed directly from the wounding or cut region of explants (direct regeneration) (Figure 1). This pattern was frequently observed in many cases of pepper transformation and the stages of shoot development observed were direct shoot formation, multishoot elongation, shoot elongation, and root formation. Secondly, shoots were selected from callus tissues that had formed around cut after 4–5 weeks after culture on the shoot selection medium (callus-mediated indirect regeneration) (Figure 2) These were unusual because calli are not easily formed from the wounded region of cotyledons. Five stages of shoot development were distinct: callus formation, callus development, shoot formation, shoot elongation, and root formation. Six different types of callus developed from the explants have been identified. Shoot regeneration capability was dependent on the callus types. Those that were able to form shoots were designated type A (white hard surface and green tissue inside) and type B (dark green callus with hard surface, but a little bit moist) (Figure 3). The regeneration rate of type A was much higher than that of type B (ca. 90%, data

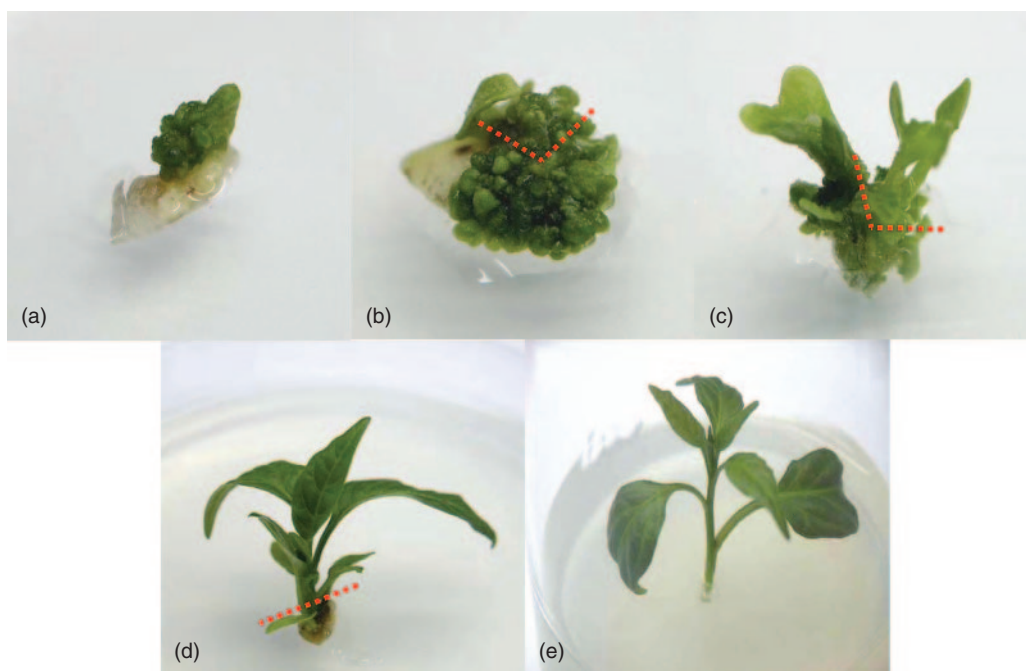


Figure 1 Development of direct shoot formation after co-culture: (a) shoot formation (5 weeks old on selection medium); (b) multishoot formation (7 weeks old); (c) multishoot elongation (9 weeks old); (d) a single shoot elongation from multishoot; (e) root formation (14 weeks old) (the red dot line indicates a cut to move to the next culture)

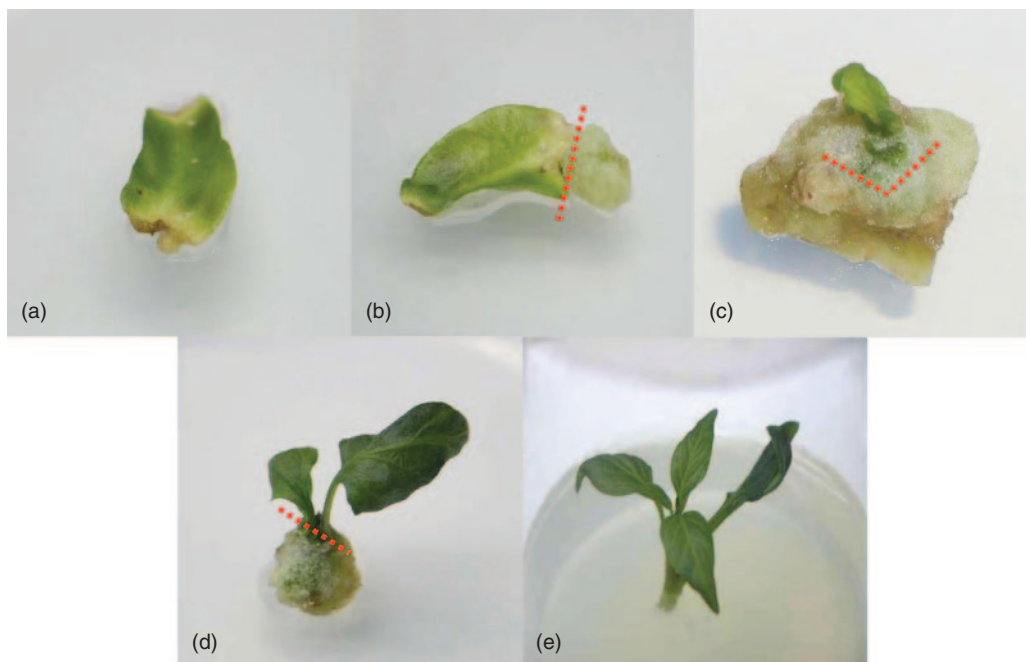


Figure 2 Development of indirect shoot formation after co-culture: (a) callus formation (5 weeks old on selection medium); (b) callus development (7 weeks old); (c) shoot formation (9 weeks old); (d) shoot elongation (11 weeks old); (e) root formation (14 weeks old) (the red dot line indicates a cut to move to the next culture)

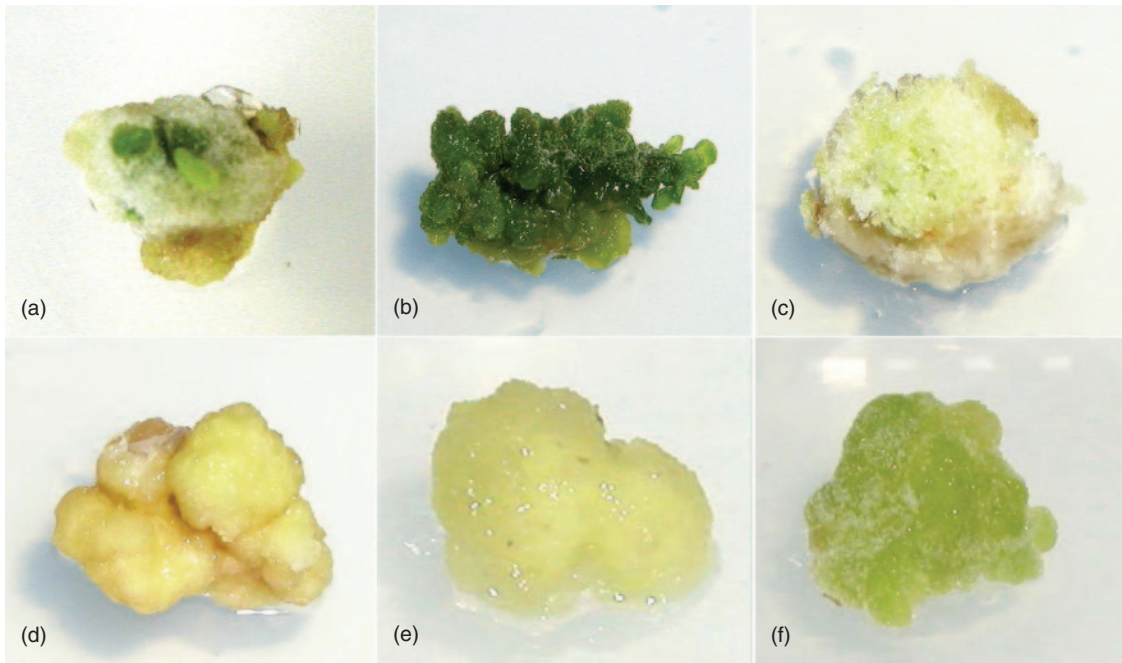


Figure 3 Callus types for shoot formation (a and b) and for nonshoot formation (c, d, e, f): (a) white hard surface and green tissue inside; (b) dark green callus with hard surface, but a little bit moist; (c) yellow and easily brittle; (d) yellow and hard surface; (e) moist and a little bit transparent; (f) green, moist, and easily brittle

not shown). The callus types that were not able to produce shoots were designated type C (yellow and brittle) and also included type D (yellow and hard surface), type E (moist and a little transparent), and type F (green, moist, and brittle).

In order to investigate the direct shoot formation rate after transformation, 151 700 explants from four different lines were transformed with the *TMV-CP* and *PPII* genes. The rate of developing a direct shoot in the shoot medium after co-culture was 5.3% (8089/151 700) (Table 3). The rate of shoot survival in the rooting medium was 0.93% (1407/151 700). Among four lines, P915 line

showed the highest rate of shoot development. There was no significant shoot formation rate between transformation experiments of two genes. To find the indirect shoot formation rate, 37 500 explants from four different lines were transformed with the *TMV-CP* and *PPII* genes. The frequency of generating calli from explants was 1.2% (459/37 500) (Table 4). However, all calli did not produce the shoot. The frequency of shoot development from callus was 11.6% (53/459). The frequency of root formation from the callus-mediated shoots was 52.8% (28/53). Out of four lines, 68% (19/28) of the total shoots

Table 3 Frequency of direct shoot formation

Gene	Number of explant				Number of shoot				Number of shoot with root			
	P915	P409	P410	P101	P915	P409	P410	P101	P915	P409	P401	P101
TMV-CP	30 312	26 039	21 983	21 983	2106	1017	1697	628	392	156	311	49
PPII	14 413	14 080	7 488	13 078	1024	587	720	310	186	103	176	34
Subtotal	44 925	40 110	31 595	35 060	3130	1604	2417	938	578	259	487	83
Total	151 700				8089				1407			

Table 4 Frequency of callus development and shoot formation from the callus

Gene	Number of shoot				Number of shoot callus				Number of shoot from callus				Number of shoot with root			
	P915	P409	P410	P101	P915	P409	P410	P101	P915	P409	P410	P101	P915	P409	P410	P101
TMV-CP	5 188	6917	5	1	7	5491	7 210	81	30	25	94	14	12	3	0	2
PP11	2 903	3056	4	2	5	3798	2 937	107	46	17	59	15	7	2	1	1
Subtotal	8 091	9973	9	3	12	9289	10 147	188	76	42	153	29	19	5	1	3
Total	37 500				459				53				28			

in the rooting medium were obtained from P915 line, suggesting a genotype preference of shoot formation.

A total of 1407 direct shoots grown in the rooting media were tested by PCR to identify transformed pepper plants and none of the shoots contained inserts (Tables 3 and 5). This indicates that the direct shoots grown from explants are not transformed. To test the transformation rate of indirect callus-mediated shoots, the final 28 shoots in the rooting medium were analyzed by PCR (Tables 4 and 5; Figure 4). The transformation rate was 0.19% (15/37, 500) for P915 line and 0.03% (3/37, 500) for P409 line. However, the transformation rate as determined by the number of PCR positive versus the number of callus-mediated shoot was 34% (18/53), indicating that shoots grown from the callus could be transformed with high probability. Therefore, the selection of a callus-mediated shoots from among a large number of shoots growing on selection medium tended to discriminate transformed plants from nontransformed plants.

DNA samples from transformed peppers (T_0), randomly chosen from the PCR positive peppers, were isolated and 30 μ g DNA was digested to completion with *Xba*I for *TMV-CP* and *Bgl*II for the *PP11* gene. Figure 5a shows a Southern

blot analysis of *TMV-CP* transformed peppers digested by *Xba*I. Transformed peppers showed different *TMV-CP* insertion sites and those had one copy of *CP* gene inserted. The *PP11* insert digested with *Bgl*II was also localized at different sites in *PP11* transformed peppers (Figure 5b). The 5-kb band on the Southern blot was present in all T_0 plants, and represented the pepper *PP11* gene internally embedded in the genome, whereas the other inserts were newly transformed. Interestingly, all of the transformed peppers had only one copy of the *PP11* gene inserted.

In summary, the protocol described here provides a more reliable pepper transformation platform. However, several key points should be taken into account for successful transformation. First, plant material for pepper transformation is critical. For maximum transformation efficiency, callus-mediated indirect shoot should be used. Most shoots seem to grow well directly from the wounded surface of explants on selection medium, however, no transformants have been obtained from shoots grown directly. In contrast, callus-mediated indirect shoots showed very high-transformation efficiency. Another important factor is to select a right callus type, which can produce transgenic shoot. Among the six different callus types, the type A callus generated most

Table 5 Transformation efficiency of direct and indirect shoot formation

Gene	Direct shoot				Indirect shoot			
	P915	P409	P410	P101	P915	P409	P410	P101
TMV-CP	0	0	0	0	10	5491	7210	81
PP11	0	0	0	0	5	3798	2937	107
Total	0	0	0	0	15	3	0	0
Transformation rate ^(a) (%)	0	0	0	0	0.19	0.03	0	0%

^(a) Percentages were obtained by dividing the number of PCR positive with the number of explant used

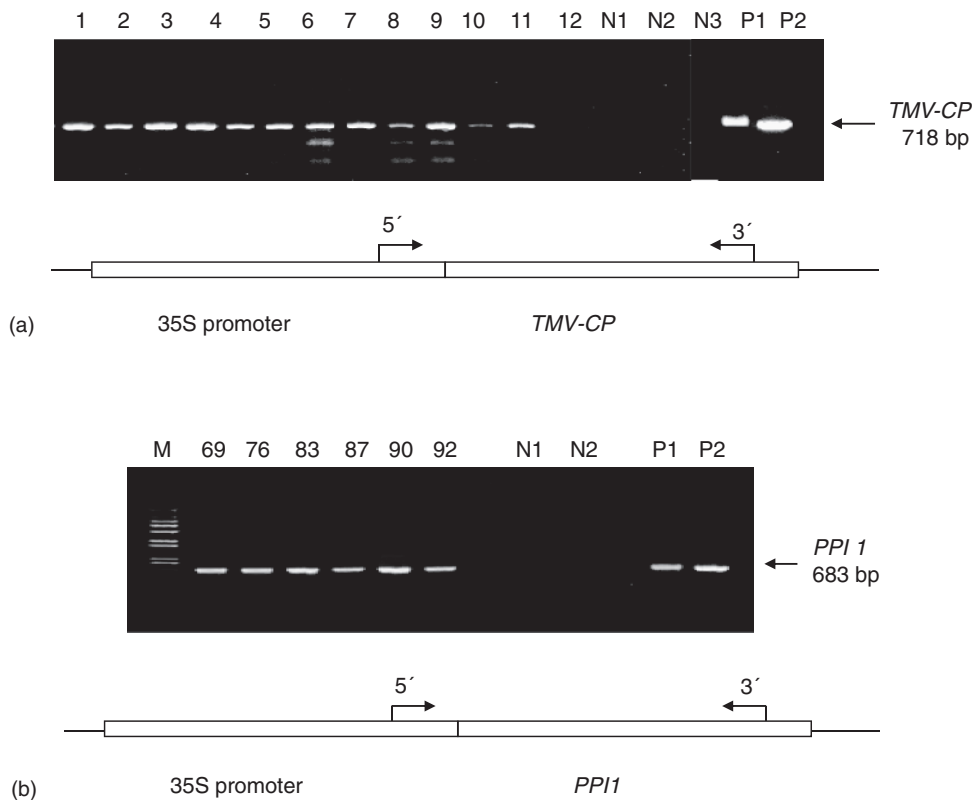


Figure 4 (a) PCR analysis of transformed peppers with *TMV-CP* gene (T_0): 1–12, transformed; N1–N3, nontransformed; P1–P2, positive control (cloned bacterial cell and p*TMV-CP*, respectively) (b) PCR analysis of transformed peppers with *PPI1* gene (T_0): 69, 76, 83, 87, 90, and 92, transformed; N1–N2, nontransformed; P1–P2, positive control (cloned bacterial cell and p*PPI1*, respectively)

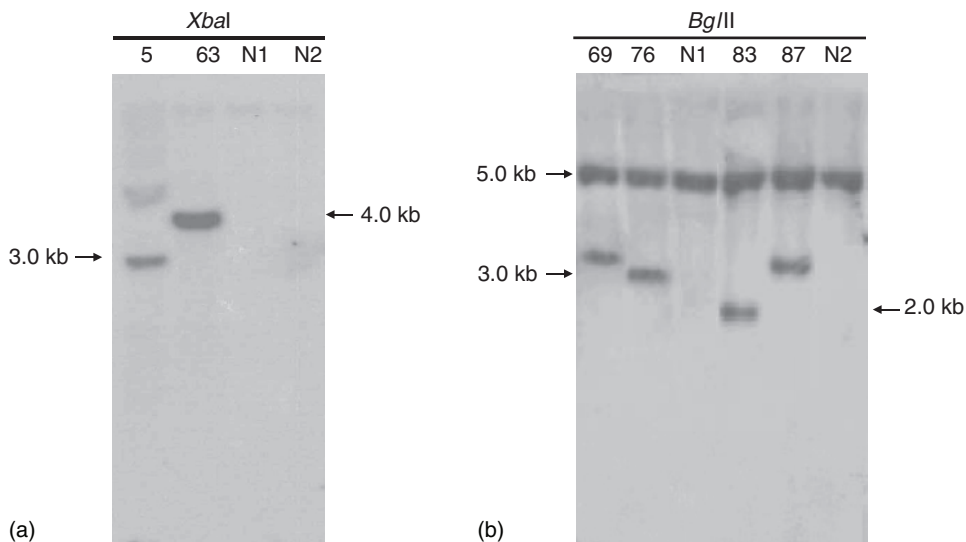


Figure 5 (a) Southern blot analysis of transformed peppers with *TMV-CP* gene (T_0): 5 and 63, transformed; N1–N2, nontransformed (b) Southern blot analysis of transformed peppers with *PPI1* gene (T_0): 69, 76, 83, and 87, transformed; N1–N2, nontransformed (the 5.0-kb band belongs to the endogenous gene of *PPI1*)

transgenic shoots. Therefore, the best way to screen putative transformed shoots during the pepper transformation procedure is to identify shoots regenerated from type A callus. Thirdly, plant genotypes and *Agrobacterium* strains for the transformation are very important factors. Therefore, regeneration rate of each pepper line should be tested. The higher regeneration rate produces the better transformation rate. According to our experimental results *Agrobacterium* strain, EHA105, is best strain for pepper transformation.

3. FUTURE ROAD MAP

Capsicum transformation is still at its infant stage comparing with that of other well-studied solanaceous crops, such as tomato and tobacco. Therefore, no transgenic pepper lines are under field test; no GM pepper varieties have been commercialized. In spite of continuous efforts worldwide, successful reports of pepper transformation are very rare. Even among the successful groups, the transformation efficiencies have been extremely low and inconsistent. We presented a callus-mediated indirect shoot transformation method and expect that this will provide a better platform for pepper transformation.

Nevertheless, further improvement in transformation efficiency is required. Since both transformation efficiency and plant regeneration is genotype dependant. In order to obtain maximum transformation rate and for routine transformation, the most virulent/effective *Agrobacterium* strain and the most susceptible pepper genotypes with the highest regeneration rate should be identified. Genotypes with the highest transformation rate do not necessarily regenerate well (Liu *et al.*, 1990). However, screening large numbers of pepper inbred lines may require joint efforts of laboratories worldwide.

Since pepper transformation efficiency is extremely low at present, introducing transgenes to elite lines can also be very slow even if any useful transgenic lines may be available. In order to expedite transgene introgression, conventional backcrossing and molecular marker technique should be utilized. Molecular linkage maps and various types of molecular markers are currently available for genome scanning and marker-assisted selection. Hence, as was shown in soybean and

other well-studied field crops, useful transgenes are transferred to the genotype, which can be easily transformed, and introgressed by backcrossing to elite lines those are recalcitrant to be transformed.

Virus resistant peppers are expected to be the first possible commercialized transgenic peppers because these traits have been most extensively studied and are relatively simple to manipulate. For virus resistance, virus CPs are easiest target genes to be transformed into pepper. For instance, CMV resistant transgenic peppers harboring CMV-CP gene are very promising future product. In addition to viral genes, engineering host factor can be a target for virus resistance. Since disease resistance breeding by conventional method relies on the availability of disease resistance gene(s) in existing *Capsicum* germplasm and confined by sexual barriers between domesticated and wild *Capsicum* species. Transformation technique is, therefore, extremely useful against potential pathogens when there is lack of genetic resources among available *Capsicum* species.

Although insect resistance has not been a dominated research target in the past pepper transformation studies, *Bacillus thuringiensis* (*Bt*)-transgenic pepper also has potential to be commercialized (if it is available), particularly when political and regulatory issues are considered. Transformation of *Bt*-gene, mainly *Cry1Ac*, for insect resistance has been demonstrated in many other crops and the related products have been tested extensively and released for commercial production for long time; health and environmental assessments and regulations; administrative and political processes for the release of *Bt*-transformed products are well established. In fact, release of *Bt*-transgenic eggplant (another closely related relative) in several targeted Asian countries is under progress. These experiences in *Bt*-transformed products and *Bt*-GM eggplant will serve as an example for future release of *Bt*-pepper.

Since for breeding pungency level is particularly important and difficult object, development of transgenic plant with different pungency level will be very useful approach. The gene, which controls presence and absence of capsaicinoid, was recently identified as acyltransferase 3 (*AT3*) and expression level of this gene controls pungency level. Therefore, it is theoretically feasible to control pungency level by controlling expression level of *AT3*. Other possibility of transgenic pepper

development is biofortification for micronutrient enhancement. Although peppers are rich in vitamin A and C, transformation approach for further increase or stable vitamins' production will benefit consumers and growers/manufacturers as well particularly in the areas with malnutrition problems.

Finally, once transgenic pepper plants are developed, potential risks should be addressed. For example, damage to human health and environment, disruption of current cropping schemes and food production in developed countries, and of traditional practices and economies in less developed countries, the regulatory measures adopted by Institutional Biosafety Committee (IBC), Animal and Plant Health Inspection Service (APHIS), Food and Drug Administration (FDA), Environmental Protection Agency (EPA), and international agreements for new transgenic products are also to be considered.

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Vegetable Brassicas

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1. INTRODUCTION

The Brassicaceae family contains about 3000 species in 350 genera including many important vegetable species such as water cress (*Nasturtium officinale*), garden cress (*Lepidium* spp.), radish (*Raphanus sativus*), horse radish (*Armoracia lapathifolia*), roquette (*Eruca sativa*), sea kale (*Crambe maritima*), and many ornamental species such as *Matthiola* and *Malcolmia* (stocks), *Iberis* (candytuft), and *Alyssum*. In addition, the Brassicaceae family includes the economically important *Brassica* genus, which consists of about 85 species including several important vegetables. In addition to many important vegetable species, the Brassicaceae family includes condiment producing plants such as the common horseradish (*Armoracia*), Japanese horseradish (*Wasabi japonica*), and mustard (*Brassica*); forage crops such as turnip, swede, rape, kale (all *Brassica* spp.); and important oilseed plants such as rape and turnip (*Brassica*) and *Crambe* (Bailey and Bailey, 1976). Turnips and swedes provide winter fodder for sheep and cattle. In New Zealand, arable Brassica crops have been important for animal production since 1870. They are used to supplement pasture for sheep and cattle when pasture growth is not sufficient (Palmer, 1983). *B. oleracea* originated in southern Europe, where it grows along the coasts of the Mediterranean Sea (Nieuwhof, 1993).

The six cultivated *Brassica* species are interrelated with the three amphidiploids (*B. napus*, *B. juncea*, and *B. carinata*) arising from interspecific

hybridization between the three diploid species (*B. oleracea*, *B. nigra*, and *B. rapa* (syn. *B. campestris*) (U, 1935). Genome size, as determined by flow cytometry by Arumuganathan and Earle (1991), ranges from 600 to 662 Mbp (mega base pair) for the haploid C genome of *B. oleracea*. The A genome of *B. rapa* Pak choi is smaller (507 Mbp).

Vegetable Brassicas are economically important and grown worldwide for consumption as both fresh and frozen produce. The major group of Brassica vegetables are members of *B. oleracea* ($n = 9$), which is a polymorphic species consisting of 14 varieties, distinguished by variations in internode length, stem swelling, axillary bud development, foliage curling, and inflorescence development. *B. oleracea* is an important source of vegetables in the Brassicaceae with practically every part of the plant utilized including the leaves (cabbage, kale, collards), terminal buds (early cauliflower), axillary buds (Brussels sprouts), stems (kohlrabi), floral primordia (late cauliflower), and flower buds (broccoli). Other vegetable members are present in *B. rapa* where it is the leaves (Chinese cabbage) and swollen hypocotyl (turnip) that are utilized.

Vegetable Brassicas are highly nutritious, particularly broccoli, which contains high amounts of calcium, carotene, vitamin C, and vitamin A. In addition, vegetable Brassicas are gaining in popularity as they contain compounds with anticancer properties. Worldwide, cabbage is the most commonly grown vegetable Brassica with over 3 million hectares grown in 2005. The main area of vegetable Brassica production is in China where 1.5 Mha was grown in 2005. India, Russia,

and Asia in general are also important areas of vegetable production. Cauliflower is also an important vegetable Brassica with approximately 857 000 ha grown worldwide. In 2003, 16 million tons of cauliflower were produced worldwide with China and India accounting for 75% of this figure (<http://www.FAO.org>). In developing countries, Brassica crops, such as cabbage, cauliflower, kale, mustard, are important food plants. In India, for example, cabbage and cauliflower are grown by more than 20 million small-holding farmers, over 250 000 ha of land, or over more than 10% of the total area devoted to vegetable production (<http://www.CIMBAA.org>). India is the world's largest cauliflower grower and second largest cabbage grower (after China). Cabbage and cauliflower are important in the diets in this country where vegetarianism is widespread. The one and a half million cabbage and cauliflower fields occupy 440 000 ha in India with a production of 6.4 million tons annually (<http://www.CIMBAA.org>).

In recent years, the development of plant transformation procedures has enabled the genetic modification of many crop plants, including *Brassica* species. Transformation protocols have been developed for a wide range of members of the Brassicaceae family, with the major emphasis on the economically important oilseed rape *B. napus*. Development in this crop, particularly in Canada, has progressed to the stage where herbicide resistant genetically modified (GM) *B. napus* are grown over wide areas. Use of *Agrobacterium tumefaciens*-based transformation methods are by far the most efficient for use with most vegetable Brassicas and have been successfully used to produce transgenic plants of several other vegetables. In this chapter, we summarize the results obtained with the introduction of traits covering a range of characteristics including introduction of pest and disease resistance, altered postharvest attributes and alteration of flowering and breeding systems.

2. DEVELOPMENT OF TRANSGENIC VEGETABLE BRASSICAS

2.1 Transformation Methods

Vegetable Brassicas are susceptible to attack from several insect pests and to infection by a range

of bacterial and fungal diseases. In addition, postharvest deterioration can limit shelf life. Genetic engineering (GE) technology offers the opportunity to produce pest- and disease-resistant varieties by enabling transfer of genes that could not be possible by conventional breeding methods. An advantage of GE is that a small amount of well-characterized DNA can be quickly added into the genetic background of the elite lines. This DNA can be from any source; plant, animal, bacterial, viral, or artificially synthesized. Since the first reports of transgenic vegetable Brassicas in the late 1980s, GE of vegetable Brassicas has now progressed to the stage where agronomically useful traits have been introduced and numerous plants have been field tested. However, no commercial release of a transgenic vegetable Brassica cultivar has yet occurred.

2.1.1 *Agrobacterium*

Agrobacterium-based methods are the most widely used approach for producing transgenic vegetable Brassicas and have been used to produce transgenic plants in most vegetable types (Table 1). Binary vectors based on the nopaline strain LBA4404 are the most commonly used *Agrobacterium* strain followed by EHA101 (reviewed in Christey and Braun, 2004). The prerequisites for efficient production of transgenic plants are susceptibility to *Agrobacterium* and an efficient shoot regeneration system. Previously, we have reviewed ways to increase transformation rates by consideration of the culture conditions and genetic factors (Christey and Braun, 2004).

In addition to susceptibility to *Agrobacterium*, an efficient shoot regeneration system is required for the successful application of *Agrobacterium*-mediated transformation for the introduction of agronomically important genes. High rates of shoot regeneration have been reported from many vegetable Brassicas from many explant sources. *B. oleracea* vegetables are particularly amenable to *in vitro* manipulation as shoot regeneration has been demonstrated for 13 types from a number of different vegetative explants and also from reproductive or floral organs (reviewed in Christey and Braun, 2004). In addition to explant source, choice of cultivar is important for achieving high rates of shoot regeneration as demonstrated by

Table 1 Summary of traits transformed into vegetable *Brassicas*^(a)

Trait	Vegetable	Gene of interest ^(b)	Marker or reporter gene used ^(c)	Reference
Caterpillar resistance	Rutabaga	<i>cry1A</i>	<i>npII</i>	Li <i>et al.</i> , 1995
	Chinese flowering cabbage	<i>cry1Ab</i> , <i>cry1Ac</i>	<i>npII</i>	Xiang <i>et al.</i> , 2000
	Cauliflower	<i>cry1A(b)</i>	<i>npII</i>	Chakrabarty <i>et al.</i> , 2002
	Chinese cabbage	<i>cry1C</i>	<i>hpt</i>	Cho <i>et al.</i> , 2001
	Chinese cabbage	<i>CpTI</i>	<i>npII</i>	Zhao <i>et al.</i> , 2006 ^(d)
	Cabbage	<i>cry1Ac</i>	<i>npII</i>	Metz <i>et al.</i> , 1995
	Cabbage	<i>cry1A(b)</i>	<i>npII</i>	Bhattacharya <i>et al.</i> , 2002
	Cabbage	<i>cry1Ba1</i> , <i>cry1C</i>	<i>npII</i>	Christey <i>et al.</i> , 2006a
	Cabbage	<i>cry1Ab3</i> , <i>cry1Ia3</i>	<i>npII</i>	Jin <i>et al.</i> , 2000
	Collards	<i>cry1Ac</i> , <i>cry1C</i>	<i>npII</i> or <i>hpt</i>	Cao <i>et al.</i> , 2005
	Cauliflower	<i>cry9Aa</i>	<i>hpt</i>	Kuvshinov <i>et al.</i> , 2001
	Cauliflower	<i>cry1Ba1</i> , <i>cry1C</i>	<i>npII</i>	Christey <i>et al.</i> , 2006a
	Cauliflower	<i>CpTI</i>	<i>npII</i>	Iingling <i>et al.</i> , 2005
	Broccoli	<i>cry1Ba1</i> , <i>cry1C</i>	<i>npII</i>	Christey <i>et al.</i> , 2006a
	Broccoli	<i>cry1C</i>	<i>hpt</i>	Cao <i>et al.</i> , 1999
	Broccoli	<i>cry1Ab</i>	<i>npII</i>	Cao <i>et al.</i> , 2001
Virus resistance	Chinese cabbage	TMV	<i>npII</i>	Jun <i>et al.</i> , 1995
	Chinese cabbage	Antisense <i>NiB</i>	none	Zhandong <i>et al.</i> , 2007 ^(e)
	Cauliflower	CaMV capsid gene, antisense gene VI	<i>npII</i>	Passelègue and Kerlan, 1996
Altered SI	Broccoli × Chinese kale	SLG or SRK mutants	<i>hpt</i>	Conner <i>et al.</i> , 1997
	Broccoli	SLG	<i>hpt</i>	Toriyama <i>et al.</i> , 1991
Bacterial resistance	Chinese kale	SLG	<i>hpt</i>	Toriyama <i>et al.</i> , 1991
	Cauliflower	Shiva	<i>npII</i>	Braun <i>et al.</i> , 2000
	Cauliflower	Magainin	<i>npII</i>	Braun <i>et al.</i> , 2000
	Chinese cabbage	Shiva, cecropin	<i>npII</i>	Zhao <i>et al.</i> , 2006 ^(g)
Male sterility	Cauliflower	Trypsin inhibitor	<i>npII</i>	Ding <i>et al.</i> , 1998
	Cauliflower	Antisense pollen specific gene	<i>npII</i>	Bhalla and Smith, 1998a
	Cabbage	DTx-A	<i>hpt</i>	Lee <i>et al.</i> , 2003
Herbicide resistance	Chinese cabbage	CYP86MF	<i>npII</i>	Yu <i>et al.</i> , 2004 ^(d)
	cabbage	<i>bar</i>	<i>bar</i>	Lee <i>et al.</i> , 2000
	Cauliflower	<i>bar</i>	<i>bar</i>	de Block <i>et al.</i> , 1989
		<i>bar</i>	<i>bar</i>	Rajicic <i>et al.</i> , 2002
Altered postharvest attributes	Broccoli	<i>pat</i>	<i>pat</i>	Waterer <i>et al.</i> , 2000
	Broccoli	EAS	<i>npII</i>	Henzi <i>et al.</i> , 1999a, b ^(f)
	Broccoli	ACOII	<i>npII</i>	Gapper <i>et al.</i> , 2002
	Broccoli	<i>ipt</i>	<i>hpt</i>	Gapper <i>et al.</i> , 2002
	Broccoli	<i>ers</i>	<i>npII</i> or <i>hpt</i>	Chen <i>et al.</i> , 2004a
	Broccoli	BoCP5	<i>npII</i>	Eason <i>et al.</i> , 2005
	Broccoli	<i>BoINV2</i>	<i>npII</i>	Eason <i>et al.</i> , 2007
	Broccoli	ACC syn., ACC ox.	<i>gfp</i>	Higgins <i>et al.</i> , 2006 ^(g)
	Broccoli	<i>ipt</i>	<i>npII</i>	Chen <i>et al.</i> , 2001
Fungal resistance	Broccoli	Endochitinase	<i>npII</i>	Mora and Earle, 2001
	Cabbage	Glucose oxidase	<i>hpt</i>	Lee <i>et al.</i> , 2002
Increased anthocyanin production	Rapid cycling	Lc	<i>npII</i>	Braun <i>et al.</i> , 2006
Enhanced salt tolerance	Cabbage	<i>betA</i>	<i>npII</i>	Bhattacharya <i>et al.</i> , 2004
Flooding tolerance	Chinese cabbage	<i>LEA</i>	<i>npII</i>	Park <i>et al.</i> , 2005
	Cabbage	<i>vhb</i>	<i>npII</i>	Li <i>et al.</i> , 2005
T-DNA tagging/plasmid rescue	Chinese cabbage	<i>ori</i>	<i>hpt</i> and GUS	Lee <i>et al.</i> , 2004

^(a) All reports use *Agrobacterium tumefaciens*-mediated explant transformation unless noted; this is not a comprehensive list but provides key references

^(b) ACOII, antisense version of broccoli ACC oxidase II; *bar*, *pat*, phosphinothricin acetyltransferase; *betA*, glycinebetaine biosynthesis; BoCP5, antisense cysteine protease; *BoINV2*, antisense acid invertase; *CpTI*, cowpea trypsin inhibitor; *cry*, *Bacillus thuringiensis* toxin gene; DTx-A, diphtheria toxin A chain; EAS, antisense version of tomato ACC oxidase; *ers*, ethylene response sensor; *ipt*, isopentenyl transferase; *LEA*, late embryogenesis abundant gene; Lc, leaf color; *NiB*, turnip mosaic virus replicase gene; SLG, S-locus glycoprotein; SRK, S-locus receptor kinase; TMV, tobacco mosaic virus L coat protein; *vhb*, *Vitreoscilla* hemoglobin

^(c) *hpt*, hygromycin phosphotransferase; *npII*, neomycin phosphotransferase II; GUS, β -glucuronidase

^(d) Pollen tube transformation

^(e) Floral dipping

^(f) *Agrobacterium rhizogenes*-mediated transformation

^(g) *Agrobacterium tumefaciens*-mediated transformation of protoplasts

the detailed studies on the effect of cultivar on regeneration ability in cauliflower (Bhalla and Smith, 1998b) and cabbage (Kuginuki and Tsukazaki, 2001).

B. campestris explants are more recalcitrant to shoot regeneration than *B. oleracea*, but the use of ethylene inhibitors has been beneficial. Chi and Pua (1989) improved shoot regeneration rates from cotyledons of *B. campestris* ssp. *chinensis* (Chinese cabbage) through the addition of the ethylene inhibitors AgNO₃, Ag₂SO₄, or aminoethoxyvinylglycine (AVG). Explant source and age were also important factors influencing shoot regeneration as 3-days-old cotyledons showed higher shoot regeneration than 5-days-old explants and hypocotyl explants showed rare shoot regeneration. Shoot regeneration from cotyledons and hypocotyls of recalcitrant *B. campestris* comprising ssp. *chinensis*, ssp. *parachinensis*, and ssp. *pekinensis* was also markedly enhanced by the presence of either AgNO₃ or AVG, though in some cases AVG was inhibitory at high concentration (Chi *et al.*, 1990). Palmer (1992) also found that AgNO₃ enhanced both the percentage shoot regeneration and the number of shoots per explant from *B. campestris* cotyledon explants.

Ethylene inhibitors are also beneficial for regeneration from *B. oleracea* explants. Sethi *et al.* (1990) studied the effect of ethylene inhibitors on ethylene level and on shoot regeneration from cauliflower hypocotyl explants. They found that AVG, CoCl₂, and AgNO₃ induced higher rates of shoot regeneration, with reduced ethylene level, compared to controls. de Block *et al.* (1989) found that AgNO₃ was important for efficient shoot regeneration in cauliflower. Ding *et al.* (1998) obtained a significant improvement in shoot regeneration from cauliflower hypocotyl explants when the explants were pretreated with callus-inducing medium for 3 days, followed by supplementing the regeneration medium with silver ions.

In addition, Gapper *et al.* (2002) noted that transformation of broccoli with an anti-sense 1-aminocyclopropane-1-carboxylate synthase (ACC) oxidase gene significantly increased transformation rates with cotyledon petioles, but not with hypocotyl explants. They attributed the increase in transformation rate to an ethylene biosynthesis blocking effect.

2.1.1.1 *A. tumefaciens*-mediated transformation

The most common method used for vegetable Brassica transformation involves *A. tumefaciens* with all major vegetable types being successfully transformed by this method. However, transformation efficiencies are low, usually less than 5% (reviewed in Christey and Braun, 2004). Seedling explants, such as hypocotyls or cotyledonary petioles, are the most common explants used. Recent developments in *A. tumefaciens*-mediated transformation are summarized in Table 1 and indicate that a range of traits of agronomic importance have been introduced into vegetable Brassicas.

2.1.1.2 *A. rhizogenes*-mediated transformation

A. rhizogenes is a soil bacterium responsible for the development of hairy root (HR) disease on a range of dicotyledonous plants. Transgenic plants have been obtained after *A. rhizogenes*-mediated transformation in 89 different taxa, representing 79 species from 55 genera and 27 families (reviewed in Christey, 2001). Co-cultivation of explants with *A. rhizogenes* results in the production of HRs that are easily distinguished by their rapid, highly branching growth on hormone-free medium and plagiotropic root development. Root tips are then transferred to callus induction media for shoot regeneration, though regeneration may also occur spontaneously.

A. rhizogenes-mediated transformation has been used to obtain several transgenic vegetable Brassicas including broccoli, cabbage, cauliflower, Chinese cabbage, and Brussels sprouts (reviewed in Christey and Braun, 2004). Overall, rates of transformation are low, but in some cases, transgenic vegetable Brassicas were more efficiently obtained via *A. rhizogenes*-mediated transformation than *A. tumefaciens* (Christey *et al.*, 1997). In most cases, gene transfer involves marker and/or reporter genes, but genes of agronomic use that have been introduced include reduced ethylene production and resistance to the herbicide Basta (reviewed in Christey and Braun, 2004).

One disadvantage of *A. rhizogenes*-mediated transformation is that once transgenic root

cultures are obtained, shoots still need to be regenerated. In addition, plants regenerated from HRs often exhibit an altered phenotype characterized by several morphological changes including wrinkled leaves, shortened internodes, reduced apical dominance, reduced fertility, and altered flowering time and plagiotropic roots (Christey, 2001). These characteristic phenotypic changes are due to the transfer and expression of four loci (*rolA*, B, C, D) located on the TL-DNA. However, several studies have shown segregation of root inducing (Ri) and tumor inducing (Ti) transfer-DNA (T-DNA) and therefore the recovery of phenotypically normal transgenic plants (Christey *et al.*, 1999; Puddephat *et al.*, 2001).

2.1.2 Direct uptake into protoplasts

While *Agrobacterium*-mediated transformation is the most common method to obtain transgenic vegetable Brassicas, other methods have also successfully been used. Vegetable Brassicas are amenable to regeneration from protoplasts (Chen *et al.*, 2004b; reviewed by Christey, 2004), thus direct DNA uptake into protoplasts is a feasible alternative for the production of transgenic plants. However, despite the fact that shoot regeneration has been established from protoplasts of numerous vegetable Brassicas at high efficiencies for several years, reports of protoplast-mediated transformation are rare. Eimert and Siegemund (1992) co-cultivated cauliflower protoplasts with *A. tumefaciens* and obtained kanamycin resistant calli but only rarely shoots did develop further.

Mukhopadhyay *et al.* (1991) reported an efficient system using polyethylene glycol (PEG)-mediated DNA uptake into cauliflower hypocotyl protoplasts. Large numbers of fertile transgenic plants were produced. Eimert and Siegemund (1992) used PEG-mediated and/or electroporation of cauliflower mesophyll protoplasts but only a few shoots were obtained. Radchuk *et al.* (2002) studied the effect of a range of factors on PEG-mediated direct DNA uptake into cauliflower mesophyll protoplasts. Fertile transgenic plants were obtained with either kanamycin or hygromycin resistance. More recently, Nugent *et al.* (2006) have used PEG-mediated DNA uptake into cauliflower mesophyll protoplasts to obtain

transgenic plants expressing β -glucuronidase (GUS) and hygromycin resistance.

2.1.3 Microprojectile acceleration

Particle gun transformation involves firing small DNA-coated microprojectiles at high velocity into plant cells. It had been widely used for the production of transgenic plants that are not susceptible to *Agrobacterium*. However, use of particle gun transformation is decreasing as monocot plants, such as cereals, grasses, and *Alliums* that were once regarded as not susceptible to *Agrobacterium* have now been transformed by *Agrobacterium*.

Given the excellent rates of shoot regeneration obtained from some vegetable Brassica explants and the problems with *Agrobacterium*-mediated transformation, it is surprising that particle gun transformation has not been used for the production of transgenic vegetable Brassicas. Puddephat *et al.* (1999) have demonstrated transient GUS expression in broccoli cotyledon discs by particle gun transformation. They made a detailed study of the factors that influence several particle gun delivery parameters. However, they only studied transient expression 24 hours after bombardment and did not produce stable transformants. Subsequently, Nugent *et al.* (2006) have also attempted particle gun transformation of cauliflower explants but with no transgenic shoots obtained.

2.1.4 Floral dipping

In *Arabidopsis thaliana*, several simple *in planta* nontissue culture transformation methods have been developed including seed imbibition and floral dipping (reviewed by Bent, 2000). The floral dip method is now widely used for *A. thaliana* transformation and involves dipping flowering plants briefly in a solution of *Agrobacterium* and surfactant and, subsequently, harvesting and screening seed for transgenic progeny. The advantage of this method is that it is simple and convenient with no tissue culture system required, thus decreasing costs and labor input. In addition, possible somaclonal variation from the regeneration process can be avoided. This method

may be applicable to other Brassicaceae that are recalcitrant to regeneration from tissue culture. However, in *A. thaliana* thousands of seeds are easily obtained from one plant and though the overall transformation rates are low, 10–15 transformants may be recovered per plant. Qing *et al.* (2000) adopted this method for transformation of Pak choi and obtained Basta-resistant plants. Whole plants were immersed in *Agrobacterium* under vacuum. Selfed seed were collected and sprayed with Basta. Two Basta-resistant seedlings out of approximately 20 000 were obtained and molecular and progeny analysis confirmed gene insertion and transmission to progeny. While the transformation rate was extremely low, this study demonstrates one of only a few reports of the successful production of transgenic Pak choi. Yu *et al.* (2004) unsuccessfully attempted floral dipping for production of Chinese cabbage but were able to obtain transformants by pollen tube transformation. More recently, Zhandong *et al.* (2007) successfully obtained 43 transgenic Chinese cabbage plants from 1831 seeds (2.35%) using *A. tumefaciens* mediated floral dipping.

2.1.5 Plastid transformation

Plastid transformation offers several advantages over nuclear transformation including the potential for high levels of protein production, the potential for transgene containment in species with maternal inheritance of plastid DNA, absence of gene silencing, and the ability to express multiple genes. Introduced traits include herbicide, insect and disease resistance, abiotic stress tolerances, and vaccines. Plastid transformants have been produced in a number of plants including potato, tomato, carrot, soybean, cotton, petunia, *Arabidopsis*, and *Brassica napus* (reviewed in Maliga, 2004). However, in vegetable Brassicas only a single plastid transformant has been obtained to date in cauliflower using PEG-mediated DNA uptake into mesophyll protoplasts (Nugent *et al.*, 2006).

2.1.6 Selection of transformed tissue

Kanamycin has been widely used as a selection agent for the production of vegetable Brassicas

(see Table 1). However, hygromycin and herbicide resistance have also been used in addition to visual markers such as GUS and green fluorescent protein (GFP) (see Table 1). Cao *et al.* (1999) and Lee *et al.* (2004) found hygromycin to be a more effective selection agent than kanamycin. Zhandong *et al.* (2007) used marker-free selection by selecting transgenic plants on the basis of the trait of interest, i.e., virus resistance.

2.2 Enhancing Transformation Rates

Transgenic plants have been obtained from most vegetable Brassicas, but transformation rates are low. In an effort to increase transformation rates many factors have been studied concentrating on cultural conditions, i.e., both explant and bacterium, and also genetic factors.

2.2.1 Culture conditions

The frequency of production of transgenic vegetable Brassica plants varies widely between species and cultivar. Due to the low efficiency of transformation, several authors have used transient GUS expression as a simple assay for establishing the optimal conditions for transformation. They have found several factors influence transformation efficiency including explant age, duration of co-cultivation with *Agrobacterium*, strain, cultivar, use of feeder cells, acetosyringone, and micropore tape. The GFP reporter gene has also been used to monitor transformation and has the advantage that transformation can be monitored *in situ* nondestructively as shown by Cogan *et al.* (2001) with broccoli.

The explant source, most commonly used for *Agrobacterium* co-cultivation, includes seedling-derived cotyledonary petioles and hypocotyls with a few reports using peduncles or petioles (reviewed in Christey and Braun, 2004). Seedling age is important as Chen *et al.* (2001) reported that hypocotyl and cotyledon explants from 3-days-old broccoli seedlings gave higher putative transformation frequencies than 9-days-old seedlings. Bhalla and Smith (1998a) also found seedling age was important as explants from 4-days-old cauliflower seedlings gave higher rates of transformation than 7–14-days-old seedlings. Chen *et al.* (2001)

compared transformation of broccoli cotyledon, hypocotyl, and peduncle explants using both co-culture and vacuum infiltration for *Agrobacterium*-mediated transformation. However, vacuum infiltration adversely affected regeneration from cotyledon and hypocotyl explants. Regeneration rates on kanamycin selection were slightly higher for peduncle explants after vacuum infiltration (23%) compared with direct soaking (17%). The overall putative transformation rates varied from 0.6% to 14.7% depending on explant source and cultural conditions.

Tsukazaki *et al.* (2002) used transient GUS expression to determine the best conditions for *Agrobacterium*-mediated transformation of cabbage doubled haploid (DH) lines. The best conditions were hypocotyl explants precultured on medium containing 50 μ M acetosyringone for 3 days, followed by co-cultivation with strain LBA4404 for 3 days. Under these conditions, the stable transformation efficiency was 3%.

Manipulation of co-cultivation conditions can also increase transformation rates. Henzi *et al.* (2000a) improved the rate of HR production from leaf explants of Shogun broccoli from 8% to 33% by the use of acetosyringone in the bacterial culture medium, addition of a *B. campestris* feeder layer and use of acetosyringone and mannopine in the co-cultivation medium.

Bhalla and Smith (1998a) found that several factors were important in the successful production of transgenic cauliflower plants including explant age, co-cultivation time, and delayed introduction of selection. Shoots could not be regenerated from cauliflower explants when selection was applied immediately after *Agrobacterium* inoculation. A 1-week delay in selection resulted in the recovery of transgenic plants. Chen *et al.* (2001) found a 7–10-day recovery was needed with broccoli peduncle explants before kanamycin selection was used.

In a detailed study with cauliflower, Chakrabarty *et al.* (2002) used transient GUS expression to evaluate the optimum conditions for *Agrobacterium*-mediated transformation of hypocotyl explants. Explant age, preculture period, bacterial strain, and density were found to be critical factors in the transformation efficiency. The use of the *Agrobacterium* strain GV2260 considerably improved expression rates compared with LAB4404, A208, or EHA105. They also noted that a 7–10-day delay was required to enable

the production of transgenic cauliflower. Optimal conditions for transformation of hypocotyl explants were 2 days of preculture, 2 days of co-cultivation with a 7–10-day delay prior to transfer to 20 mg l⁻¹ kanamycin.

Jin *et al.* (2000) were unsuccessful in applying the seedling explant method developed by Metz *et al.* (1995) for cabbage to their cabbage lines. However, they successfully produced *Bacillus thuringiensis* (Bt)-resistant cabbage with a modified protocol after testing several factors that affect the virulence of *Agrobacterium* and regeneration of transgenic cells. They found that the wounded ends of explants incurred severe necrosis from *Agrobacterium* infection, and the amount of kanamycin used for selection was too high. These problems are commonly reported not only in cabbage but have been also noted in cauliflower and broccoli (M.C. Christey, unpublished observations). Jin *et al.* (2000) found that reducing the *Agrobacterium* concentration used for infection, excluding tobacco cells from culture, increasing the agar concentration to 1%, and decreasing the level of kanamycin used for selection enabled the production of transgenic cabbage plants.

2.2.2 Genetic factors

In vegetable Brassicas, there are clear effects of species and cultivar on *A. tumefaciens*- and *A. rhizogenes*-mediated transformation rates. Sparrow *et al.* (2004b, c, 2007) investigated the genetic basis of two key variables of *A. tumefaciens*-mediated transformation in broccoli. They showed genetic control of shoot regeneration and susceptibility to *Agrobacterium* is controlled by additive and dominant gene effects. They mapped quantitative trait loci (QTL) associated with *Agrobacterium* susceptibility. They demonstrated the ability to introduce or increase shoot regeneration potential and susceptibility to *Agrobacterium* into otherwise recalcitrant lines. Their studies resulted in the identification and use of phenotype markers to select potential candidates for successful transformation. Sparrow *et al.* (2004a) have selected a rapid cycling (RC) *B. oleracea* line, DH1012, for high regeneration and transformation ability. The use of this RC DH1012 line by Christey *et al.* (2006a) enabled the production of large numbers of fertile transgenic

plants quickly. Plants were flowering three months after transfer to the greenhouse. The use of DH1012 enabled the rapid production and analysis of transgenic plants to ensure that the gene constructs were functional (Christey *et al.*, 2006a). The use of this line has been particularly invaluable in our other research where it is important to analyze large numbers of transgenic plants to ensure that the phenotypic changes noted are actually due to the inserted gene and not an effect of somaclonal variation. In addition, the rapid generation of progeny enables progeny analysis to be conducted.

Cogan *et al.* (2001) studied the production of HRs transgenic for GFP production from eight *B. oleracea* cultivars including broccoli, cabbage, cauliflower, and kale. Significant differences in transformation rates were noted between broccoli cultivars ranging from 1% to 58%. Three of the broccoli cultivars were then subjected to anther culture and the transformation rates of the DH lines studied. These lines showed considerable variation for transgenic root production with two populations including lines showing significantly increased transformation rates compared to the parental cultivar. Anther culture derived DH lines of the cabbage cultivar Hawke also showed considerable variation for transgenic root production ranging from 6% to 85%.

Cogan *et al.* (2002, 2004) studied GFP expression after *A. rhizogenes*-mediated transformation on 73 lines of a Chinese kale \times broccoli DH mapping population. They identified genotypes that showed an increase in transformation efficiency from 14% and 20% (parental lines) to over 83%. They identified three QTLs for transgenic root production, two of these were the same as those associated with adventitious root production. They identified lines with high rates of transgenic root production that may be useful in the development of high-throughput transformation systems. The identification of QTL regions in these studies associated with control of *Agrobacterium*-mediated transformation will assist in the identification of the most amenable genotypes and aid in the development of genotype-independent transformation systems. In addition, marker-assisted selection may be used to transfer these alleles to recalcitrant genotypes, enabling the transformation of previously nonamenable genotypes.

2.2.3 Other factors to consider

In addition to the choice of cultivar, *Agrobacterium* strain, selectable marker and other culture conditions, choice of promoter needs to be also considered. The cauliflower mosaic virus (CaMV) 35S promoter is widely used in the production of transgenic plants. However, Al-Kaff *et al.* (2000) have noted that infection of plants with CaMV leads to silencing of genes controlled by the 35S promoter. They analyzed the response to CaMV infection of a transgenic oilseed rape line containing the phosphinothricin acetyltransferase (*bar*) gene regulated by the 35S promoter. CaMV infection altered the expression of the herbicide tolerance gene such that plants became susceptible to the herbicide. Susceptibility to the herbicide was most likely a result of transcriptional gene silencing of the transgene. This is important as it means that CaMV infection of a crop could have a major impact on transgenic expression in a cruciferous crop and destabilize a commercially important trait.

2.3 Agronomic Traits Introduced

2.3.1 Insect resistance

Insect-resistant crops expressing *Bacillus thuringiensis* toxin (*cry*) genes from *Bt* were first grown commercially in 1996, and since then the acreage of *Bt*-transgenic crops has increased dramatically with over 25 Mha planted worldwide in 2005, mainly in maize and cotton (James, 2005). Various *cry* genes from *Bt* have been introduced into vegetable Brassicas including broccoli, cabbage, cauliflower, collards, and swede (Table 1) and been shown to successfully control important insect pests such as diamondback moth (*Plutella xylostella*) and cabbage white butterfly (*Pieris rapae*).

In addition to *cry* genes, the cowpea trypsin inhibitor has also been shown to confer insect resistance (Table 1). Trypsin inhibitors are a type of protease inhibitor that is capable of controlling a wide spectrum of insect pests. Ding *et al.* (1998) produced insect-resistant transgenic cauliflower expressing a sweet potato trypsin inhibitor gene. Transgenic cauliflower plants were obtained with a high degree of protection against

the Brassica Lepidopteran pests *Spodoptera litura* (cut worm) and *Plutella xylostella* (diamondback moth).

As with all traits, the use of genes used in *B. napus* is likely to be of value for use in transformation of vegetable Brassicas as often the same insects and disease are a problem. There are no reports in vegetable Brassicas of the introduction of lectin genes for insect control. However, in *B. napus* good control of the pollen beetle (*Meligethes aeneus*) has been achieved in plants transformed with a construct containing the pea lectin gene under the control of a pollen specific promoter (Ahman *et al.*, 2006).

2.3.2 Disease resistance

Important fungal, bacterial, and viral diseases affecting vegetable Brassicas include black rot, soft rot, downy mildew, clubroot, *Alternaria* blight, and CaMV. Protoplast fusion has been investigated as a means to introgress pest and disease resistance from other Brassicaceae species (Christey, 2004) but transformation technology offers an alternative approach.

2.3.2.1 Bacterial

Braun *et al.* (2000) introduced antibacterial genes from nonplant sources into cauliflower in an attempt to produce black rot-resistant cauliflower. They introduced the Shiva protein, which is a synthetic analogue of cecropin B from the giant silkworm moth, and the magainin II peptide derived from the African clawed frog. *In vitro* bacterial assays using crude leaf extracts confirmed increased resistance to black rot but greenhouse screening failed to show any increased resistance compared to controls. Zhao *et al.* (2006) have also introduced Shiva and cecropin B into Chinese cabbage but while transgenic plants were obtained and gene expression confirmed by Northern analysis, no bacterial assays were conducted.

Glucose oxidase catalyzes the oxidation of glucose generating H_2O_2 as a by-product. H_2O_2 effectively inhibits bacterial and fungal growth and plants transgenic for glucose oxidase show enhanced disease resistance. However, some deleterious phytotoxic effects of glucose oxidase

have been noted in transgenic plants. Cabbage expressing a glucose oxidase gene from *Aspergillus niger* showed enhanced disease resistance to black rot caused by *Xanthomonas campestris* but also showed phytotoxic effects (Lee *et al.*, 2002). Plants showed significant growth retardation, and seed set was dramatically reduced, with only a few seed produced.

2.3.2.2 Fungal

Chitin is an important component of fungal cell walls. Chitinase genes cloned from plants and fungi have been transferred into a number of plant species and resistance to a broad range of fungal pathogens obtained. Mora and Earle (2001) produced transgenic broccoli plants expressing an endochitinase gene from the biocontrol fungus *Trichoderma harzianum*. Transgenic plants were obtained with 14–37 times higher endochitinase levels than controls. Transgenic plants inoculated with *Alternaria brassicicola* showed significantly less severe disease symptoms than controls. Interestingly, polyploid plants were highly susceptible regardless of their endochitinase activity. In contrast, lesion size of plants inoculated with *Sclerotinia sclerotiorum* was not statistically different from controls.

2.3.2.3 Viral

Passelègue and Kerlan (1996) transformed cauliflower with two CaMV-derived genes in an attempt to produce CaMV-resistant cauliflower. They used the capsid gene and the antisense gene VI of CaMV. While reverse transcriptase-polymerase chain reaction demonstrated the presence of CaMV gene transcripts in all plants, the amount of RNA transcribed was very low, in contrast to the hygromycin resistance (*hpt*) gene transcript. In plants transformed with the capsid gene, the capsid protein could not be detected. The response to CaMV infection was not tested. In contrast, Zhandong *et al.* (2007) successfully obtained high levels of resistance to turnip mosaic virus (TuMV) in Chinese cabbage plants transformed with the antisense *TuMV* replicase (*NIb*) gene. Plants were transformed

using marker-free *A. tumefaciens* mediated floral dipping.

2.3.3 Herbicide resistance

The incorporation of herbicide resistance into vegetable Brassicas would enable growers to more efficiently control weeds. Basta resistant broccoli has been produced and field tested by Christey *et al.* (1997) and Waterer *et al.* (2000). Waterer *et al.* (2000) field tested six transgenic lines and noted that herbicide application had little effect on head quality and marketable yield of most lines. Christey *et al.* (1997) field tested four transgenic lines and also noted phenotype was normal though plants were not sprayed in the field. Greenhouse application of Basta to seedlings demonstrated they were resistant.

2.3.4 Flowering control

Hybrid seed production is an important breeding goal in vegetable Brassicas as hybrid seed offers many benefits including increased vigor and greater uniformity. The introduction of male sterility into vegetable Brassicas would aid production of hybrid seed. Transformation approaches have been used in cabbage, cauliflower, and Chinese cabbage to induce male sterility (Table 1). In cauliflower, Bhalla and Smith (1998a) introduced an antisense pollen-specific gene linked to a pollen-specific promoter into cauliflower and obtained the expected sterility of 50% of pollen. In Chinese cabbage, introduction of an antisense version of the *CYP86MF* gene linked to a tapetum-specific promoter induced male sterility (Yu *et al.*, 2004). Lee *et al.* (2003) also used a tapetum-specific promoter to induce male sterility in cabbage through the introduction of the cytotoxic diphtheria toxin A chain.

Self-incompatibility prevents self-fertilization and promotes outcrossing. In vegetable Brassicas self-incompatibility is used for hybrid seed production but has a number of drawbacks, including breakdown of incompatibility, labor intensiveness, and genetic complexity of the system. In Brassicas self-incompatibility is sporophytically controlled by multiallelic genes at the *S*-locus. Two genes

have been identified at the *S*-locus, *S*-locus glycoprotein (*SLG*) and *S*-locus receptor kinase (*SRK*). *SLG* encodes a secreted glycoprotein in the wall of the stigma papillary cells and *SRK* encodes a transmembrane receptor kinase. A self-incompatible response occurs when the same *S*-allele is expressed in pollen and stigma. Toriyama *et al.* (1991) introduced an *SLG* gene from *B. campestris* S8 homozygote and were able to alter the self-incompatibility phenotype of pollen and stigma. The self-incompatible Chinese kale and partial compatible broccoli plants were fully compatible upon self-fertilization.

2.3.5 Postharvest attributes

Broccoli is harvested when the flowering head is immature and growing rapidly. Postharvest senescence is rapid with loss of chlorophyll resulting in yellowing of the head. Ethylene plays an important role in the yellowing of broccoli as chlorophyll loss is associated with an increase in floret ethylene synthesis and chlorophyll loss can be delayed through the use of inhibitors of ethylene action and biosynthesis. Several groups are conducting transgenic research aimed at increasing the shelf life of the ethylene-sensitive broccoli. Antisense versions of two key regulatory genes in the ethylene biosynthesis pathway, i.e., ACC oxidase and ACC synthase have been used to produce transgenic broccoli plants with reduced ethylene synthesis. However, the reduction in ethylene production has only resulted in increases in shelf life of 1–2 days.

In order to delay postharvest senescence in broccoli, Henzi *et al.* (1999a, b) used *A. rhizogenes*-mediated transformation to express an antisense ACC oxidase gene from tomato into broccoli. Plants showed reduced ethylene production but little effect on postharvest senescence (Henzi *et al.*, 2000b). Gapper *et al.* (2002) used *A. tumefaciens*-mediated transformation to introduce an antisense ACC oxidase gene from broccoli into broccoli. This gene was driven by the asparagine synthetase promoter from asparagus that is expressed at harvest as shown by fusion of this promoter to the GUS gene and analysis of expression patterns (Kenel, 1999). Several lines were obtained with reduced ethylene production

and delayed postharvest senescence (Gapper *et al.*, 2002). Higgins *et al.* (2006) have also shown that broccoli transgenic for antisense versions of ACC synthase and ACC oxidase have reduced ethylene production, which correlates with delayed chlorophyll loss.

Cytokinin has also been shown to be involved with broccoli senescence as application of cytokinin to broccoli heads can delay postharvest yellowing. Gapper *et al.* (2002) reported the introduction of an isopentenyl phosphotransferase (*IPT*) gene into broccoli but results on the effect of postharvest senescence were not reported. Some plants had an abnormal phenotype typical of constitutive *IPT* expression. Chen *et al.* (2001) also introduced an *IPT* gene into broccoli under the control of senescence-associated promoters and demonstrated retardation in postharvest yellowing in broccoli heads and leaves. They reported that 31% of transformants exhibited delayed yellowing in detached leaves, 16% in floret branchlets, and 7% in both leaves and floret branchlets.

Eason *et al.* (2005) and Chen *et al.* (2004a) have also delayed senescence in broccoli through transformation with either an antisense cysteine protease or mutant ethylene response sensor gene. In both cases, senescence was only delayed by 1–2 days.

As there are several other genes whose expression is increased at broccoli harvest, it is likely that down-regulation of these genes through antisense or RNA interference will also lead to the production of broccoli with delayed senescence. It is likely that future research will involve the introduction of these genes into green leafy vegetables such as Chinese cabbage and Pak choi that also show postharvest deterioration due to ethylene.

2.3.6 Altered stress tolerance

Due to concerns regarding the increasing demand for water and the need to produce plants with increased tolerance to salt stress, transgenic plants are being produced with drought tolerance and salt stress particularly in rice and maize. Similar research is also being conducted with vegetable Brassicas. For example, Bhattacharya *et al.* (2004) have produced cabbage plants with

enhanced salt tolerance through the introduction of the bacterial glycinebetaine biosynthesis (*BetA*) gene for biosynthesis of glycinebetaine. Detailed analysis of three independent transgenic lines showed improved growth and development under salt stress compared with the control. Park *et al.* (2005) produced Chinese cabbage transgenic for the *B. napus* late embryogenesis abundant (*LEA*) gene and obtained plants with enhanced drought tolerance and salt tolerance.

2.3.7 Altered health benefits

2.3.7.1 Anthocyanins

Flavonoids, such as anthocyanins, are known as antioxidants *in vitro*. Their presence in many fruits and vegetables has prompted researchers to validate epidemiological links between a diet rich in fruit and vegetables and the reduced risk of many diseases related to aging. However, some vegetable Brassicas, such as cauliflower, are low in anthocyanins. In an attempt to manipulate pigment biosynthesis to increase the health benefits of vegetables, the effect of a regulatory locus of flavonoid content was assessed. *Agrobacterium tumefaciens*-mediated transformation of a *Brassica oleracea* line, selected for high transformation ability by Sparrow *et al.* (2004a), was used to produce plants transgenic for the maize *lc* (leaf color) locus. *Lc* is a regulatory gene in the anthocyanin pathway and it is expected that its presence will increase the flavonoid content. Seedling explants were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 containing a binary vector with a neomycin phosphotransferase II (*NPTII*) gene. Under tissue culture conditions *lc*-containing plants were green with no visible increase in anthocyanin production. However, on transfer to greenhouse conditions the higher light resulted in visible signs of pigmentation within 1 week. Increased pigmentation was apparent in stems, petioles, main leaf veins, and sepals. *Lc*-containing lines had 10–20 times higher levels of total anthocyanins compared with controls. In addition, antioxidant activity of *lc*-containing lines was 1.5 times higher than controls (Braun *et al.*, 2006).

2.3.7.2 Sulfur

Sulfur metabolites impart key nutritional and sensory attributes to *Allium* and crucifer crops. S-methyl cysteine sulfoxide (MCSO) occurs in all *Allium* and *Brassica* spp. and is associated with “cabbage” flavors, human health benefits but also kale anemia of ruminants. To investigate biochemical and molecular aspects of plant sulfur metabolism the effect of down-regulating genes involved in thiol precursor biosynthesis and MCSO production was investigated with an RNA interference vector constructed for the enzyme γ -glutamyl cysteine synthetase (GGCS).

Agrobacterium tumefaciens-mediated transformation of a rapid-cycling *Brassica oleracea* line selected for high transformation ability was used to produce transgenic plants. Seedling explants were co-cultivated with *A. tumefaciens* strain LBA4404 containing a binary vector with an *NPTII* gene for selection of transgenic plants. Preliminary results on MCSO levels using high-performance liquid chromatography (HPLC) analysis has shown a wide range of results with some lines showing a large decrease in MCSO levels (Christey *et al.*, 2006b). Over 25 independent T₀ transgenic lines have now been screened for MCSO or precursor compounds using HPLC. A range of results have been obtained with some lines showing a large decrease in MCSO levels. T₁ seed from selected lines segregating for the construct has been collected. Analysis on T₁ seed for a range of metabolites upstream and downstream of GGCS in the sulfur assimilation pathway have been conducted to determine if knockdown of this gene affects sulfur flux. In three transgenic lines there was a glutathione decrease with no apparent effect on growth under normal nutrient conditions, while levels of γ -glutamyl cysteine and cysteine appeared relatively stable. The relationship between this glutathione decrease and MCSOs will be determined in future work.

2.3.7.3 Vitamins and amino acids

Current advances in genetic engineering have enabled the production of plants with alterations in a range of vitamins or amino acids for either improved human or animal nutrition. For

example, Lu *et al.* (2006) have produced transgenic cauliflower with β -carotene accumulation and Wahlroos *et al.* (2004) have produced oilseed *B. rapa* with increased histidine content. It is likely in the future further transgenic vegetable Brassicas with altered vitamin or amino acid content will also be developed.

2.4 Field Testing

The first field trial of transgenic plants was conducted in 1986 with the first commercial release of a transgenic crop in 1994. Since then there has been a rapid expansion in the commercial release of transgenic crops. However, while oilseed forms of *B. napus* and *B. campestris* are no longer regulated in the United States and transgenic canola is widely grown (Christey and Woodfield, 2001), there are only rare reports of vegetable Brassica field trials and there are no nonregulated or commercial use of vegetable Brassica products. Both *A. tumefaciens*- and *A. rhizogenes*-derived plants have been field tested. *A. tumefaciens*-derived Basta resistant broccoli was field tested by Waterer *et al.* (2000) and Christey *et al.* (1997). The Collaboration on Insect Management for Brassicas in Asia and Africa (CIMBAA) aims to bring to Asia (initially India) and Africa a safe and sustainable control strategy for controlling damaging insect pests of cabbages and cauliflowers. *Bt*-containing vegetable Brassicas (cabbage and cauliflower) are being developed and field trials are planned. In New Zealand, *A. rhizogenes*-derived vegetable Brassicas have been field tested by Christey *et al.* (1999). In addition, a field test of a range of *Bt*-containing vegetable Brassicas is in progress in New Zealand (Christey, 2006).

2.5 Environmental Concerns with GM Vegetable Brassica Plants

The field testing and potential commercial release of transgenic vegetable Brassicas has raised concerns about the environmental impact of these crops. In particular, concerns relate to the possible movement of transgenes through pollen transfer from vegetable Brassicas to weedy relatives. However, seed certification schemes

contain internationally recognized guidelines for sowing distances to minimize seed contamination by pollen transfer. If these guidelines and strict management conditions are followed then pollen transfer will be less of a problem (reviewed in Christey and Woodfield, 2001). In addition, genetic strategies are available to prevent pollen transfer. Use of male sterile lines would prevent pollen transfer. Chloroplast transformation has recently been reported in vegetable Brassicas by Nugent *et al.* (2006) and its adoption could alleviate some of the environmental concerns associated with pollen due to maternal inheritance of the cytoplasm in Brassicas. Another method to decrease concerns or to provide more targeted gene expression is the use of tissue or developmental specific promoters. For example, Baranski *et al.* (2004) determined the expression patterns of four tissue-specific promoters fused to GUS for their expression in cauliflower tissues in comparison with the constitutive 35S promoter. This research identified promoters with low expression in curds.

Other concerns relate to nontarget impacts on beneficial organisms and the soil biota. Current field trials of vegetable Brassicas in New Zealand are ensuring these concerns are addressed by undertaking extensive nontarget impacts research on nontarget beneficial organisms (Christey, 2006).

Biosafety concerns are also raised due to the presence of marker genes in the GM plants. A potential advantage of *A. rhizogenes* is its use for the production of marker-free transgenic plants. High rates of co-transformation of Ri and T-DNA occur and therefore the hairy root phenotype can be used to select transgenic roots. The genes may be integrated into different chromosomes and therefore independent segregation in the next generation will produce transgenic plants without the marker gene. This has been demonstrated in cauliflower by Christey *et al.* (1999) and in broccoli by Higgins *et al.* (2006). In addition, the use of the *GFP* gene as a marker gene will overcome concerns raised by the use of antibiotic marker genes. Recently, Zhandong *et al.* (2007) successfully demonstrated the production of transgenic Chinese cabbage plants transformed using marker-free *A. tumefaciens*-mediated floral dipping. Seeds produced after floral dipping were screened for the trait of interest, i.e., virus resistance. However, polymerase chain reaction

analysis was required to confirm gene presence as a large number of TuMV-resistant plants, identified by TuMV inoculation, did not contain the transgene.

3. FUTURE PRODUCTS

Transformation systems have been developed for several vegetable *B. oleracea* and *B. rapa* types, but transformation remains difficult with rates low and genotype dependent. A range of genes of agronomic importance have been introduced including pest and disease resistance. Research is already being conducted to produce plants with altered adaptation to water and salt stress. It is likely that a range of products with altered health benefits are likely to be the next generation of transgenic vegetable Brassicas. While no transgenic vegetable Brassicas have yet been released commercially, this is likely to occur soon if environmental and public concerns are adequately addressed.

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Radish

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Radishes are grown in all continents of the world. One of the first indications on the cultivation of radish were the inscriptions on walls of the Pyramids dating 2000 BC, but Herodotus (c. 484–424 BC) believed radish was a major crop in Egypt some 5000 years ago (Becker, 1962). During such ancient times, it is believed that radishes were cultivated as an oilseed crop. Over 2000 years ago, the cultivation of radishes spread to eastern China, where the crop was domesticated into producing a wide diversity of roots. By the time of Christianity, the cultivation of several forms and colors of radish had been described in Roman writings. However, the introduction of radishes to the New World was not until Columbus discovered the Americas. Indeed, at that time, only the large, late-winter types were grown but by the turn of the 16th century the short-season radishes were introduced.

Radishes are generally categorized into two groups based on the size of their edible swollen hypocotyl and tap root. The small-rooted, short-season (or 1-month) radishes are commonly cultivated in the temperate regions of Europe, the United States (especially coastal regions of California and Florida), and also in Arctic regions under protection. Large-rooted (or 3-month) radishes are mainly grown in Asia but are suited to both temperate and tropical conditions.

There are two other forms of radish that fail to produce an edible root. Fodder radish, is grown in Southeast Asia and Europe as leaf fodder for animal feed and also as a green manure. Mougridish or rat-tail, is grown for its leaves and long immature seed pods (80 cm length) in Southeast Asia.

The small-rooted radishes are grown for salads and consumed as a raw vegetable. These roots can vary in shape (elongated to flattened spheres) and in skin color (white or red; Figures 1a and b). This vegetable is popular in gardens as an intercrop between slower growing crops and also with specialist growers in market gardens.

The large roots are more diverse than the European types especially in terms of their skin color (green, yellow, purple, and black) and flesh color (white, red, purple, or green; Figure 1c). Remarkably, the large, spherical Asian varieties grown in China and Japan can weigh up to 40 kg. In Tibet and Northeast China, where food is in short supply during winter months, the roots are stored in the ground. In Korea, the roots are commonly pickled with other vegetables in large pots underground and sometimes consumed as *kimchi*, which is the country's national dish. This radish is also culturally extremely important in Japan, with the building of temples at sites, where radish has been cultivated for centuries (e.g., Asakusa), and restaurants specializing in an array of dishes using both root and leaves for the making of bread, soup, jelly, and ice cream!

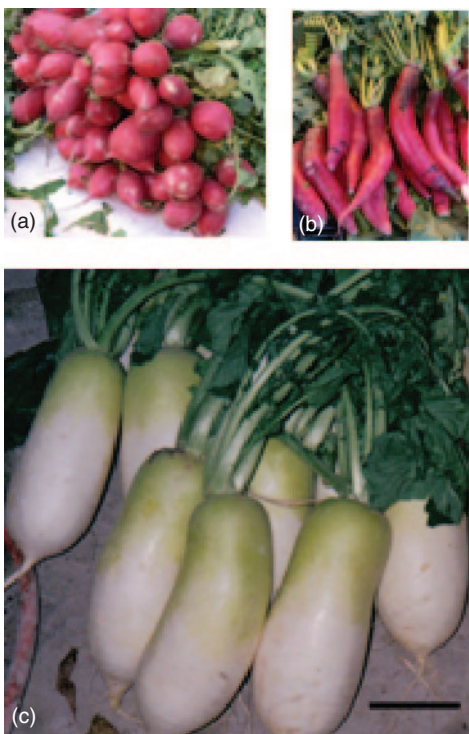


Figure 1 The diversity of radish roots: (a) globe and (b) elongated small-rooted radishes from Mexico with red skins (bar = 6 cm); (c) the popular white-fleshed 3-month-old radish cultivated in Korea (bar = 10 cm)

1.2 Botanical Description

For many years the taxonomy of radishes has been based on inadequate knowledge of the variation within the species. To date, the most reliable classification scheme combines the main cultivar form *Raphanus sativus* with its wild relatives *R. raphanistrum*, *R. landra*, and *R. maritimus* (Pistrick, 1987). These taxa have $2n = 2x = 18$ chromosomes and cross to form fertile hybrids (Lewis-Jones *et al.*, 1982). As these wild taxa occur as agricultural weeds, gene flow between cultivated and wild species seems inevitable. The cultivated forms of radish can be divided into three groups:

- Convar. *oleifera* – oilseed and fodder radishes
- Convar. *caudatus* – rattail radish (or var. *mougri*)
- Convar. *sativus* – all edible root forms, which can be further divided into geographic types, in-

cluding European, Chinese (including Japanese types as they are derived from the same ancestor), and Indian.

At present there is no division between small-rooted (also referred to as var. *radicula*) and long-rooted types (var. *nigra*, *niger*, *sinensis*, *acanthiformis*, or *longipinnatus*) but the phenotypic differences between the two groups appear to be a result of commercial development.

During the early part of the 20th century, crosses were made between *R. sativus* and *Brassica oleracea* ($2n = 18$), *B. rapa* ($2n = 20$), and *Sinapis arvensis* ($2n = 18$) to understand the affinities between their genomes (Karpechenko, 1927). The *Raphanus*–*B. oleracea* hybrids, including fertile allopolyploids, revealed that both genomes were similar. However, in the wild, there is no evidence for such hybrids to exist.

Radish belongs to the family Brassicaceae and exists as an annual and biennial. The 1-month radishes are annuals and are cultivated mainly in spring and summer; the large-rooted radishes are harvested later in the year and are classified as both annual and biennial. The DNA amount in the unreplicated haploid nuclear genome, or 1C value (Swift, 1950) of *R. sativus* L. is 0.5 pg (picogram) (Bennett and Leitch, 1997). In comparison, the radish genome is more than double the size of *Arabidopsis thaliana* (L.) Heynh. (0.2 pg) but less than half the size of the allotetraploid *Brassica napus* (1.4 pg).

During early stages of growth the radish forms a rosette of leaves attached by a short stem. The short-season radishes develop a shorter top growth (20–30 cm height) and the root swells within 2–3 weeks, larger rooted radishes produce an extensive rosette (90–120 cm), and some cultivars can form edible roots more than 150 cm in length.

A cold period (4–6 °C) for approximately 7 days under long-day conditions (16-h daylength) can cause the bolting of biennials. The annuals form bolts once the root reaches edible size, especially when the plants are exposed to warm day temperatures (above 18 °C). The bolting of a radish plant results in the nutrients from the root being transferred into the developing stem resulting in a radish unfit for the market.

In the open field, radishes are not aggressive competitors. The crop can tolerate most well-drained soils, which are free from excessive debris

and stones. Radishes are very hardy, in that, they can be sown before the end of frosty weather. However, the large-rooted Korean ecotypes will form bolts when exposed to 4–6°C for 7 days. The 1-month-old radishes are grown in rows 20 cm apart and 2 cm between plants; large-rooted types require greater spacing. Uniform soil moisture is critical for the formation of healthy roots. Periods of drought can cause surface cracking of the enlarged root and the flesh to become tough and fibrous. The number of crops per year depends on the length of the growing season. As a guide, the market gardens in the United Kingdom can produce more than four crops of the small-rooted radishes per year and those in Asakusa (Japan) can produce 2–3 crops of the long-rooted radishes.

1.3 Economic Importance

Although radishes are grown globally, in the Far East, its cultivation is far more widespread. Japan alone produces 30 times more radishes by weight compared to the whole of Europe (Crisp, 1995). Recent governmental figures for Korea and Japan revealed that radish cultivation is ranked fifth and fourth, respectively, in terms of its popularity as a vegetable crop (Table 1). In China, the large-rooted radishes are grown in over 1.2 million ha of arable land (Curtis, 2004) making radish the most widely grown root crop in the Far East.

Table 1 Ten most popular crops grown in Korea and Japan (area = 1000 × ha, yield = 1000 t)

Korea ^(a)			Japan ^(b)		
	Area	Yield		Area	Yield
Soybean	85	139	Soybean	150	236
Red pepper	68	410	Potato	87	2885
Potato	48	988	Red bean	43	91
Chinese cabbage	45	2865	<i>Radish</i>	40	1620
<i>Radish</i>	36	1710	Sweet potato	40	1009
Sesame	32	21	Cabbage	33	1279
Garlic	30	358	Spinach	24	289
Green onion	28	700	Leek	24	485
Perilla seed	24	18	Onion	23	1125
Watermelon	22	824	Lettuce	22	509

Source: ^(a)Ministry of Agriculture & Forestry, Republic of Korea, 2004, ^(b)Abstract of Statistics on Agriculture Forestry & Fisheries in Japan, 2004

Table 2 Nutritional composition of 100 g (f. wt.) of large-rooted radish roots^(a)

Component	Quantity (mg)
Water	94 000
Crude protein	800
Crude oil	200
Carbohydrate	4500
Crude fiber	600
Dietary fiber	1300
Ash	500
Vitamin A	0.0
Vitamin B1	0.01
Vitamin B2	0.02
Niacin	0.4
Vitamin B6	0.03
Vitamin C	18
Sodium	23
Potassium	200
Calcium	27
Magnesium	10
Phosphorus	13
Iron	0.2
Zinc	0.2

^(a)Source: R. Yang (Asian Vegetable Research and Development Center, AVRDC, Taiwan)

Despite the popularity of radish cultivation worldwide, the crop has been regarded as adding little to nutrition (Pearce, 1987). However, studies in the 1950s reported that radish is an excellent source of vitamins B and C and a good source of calcium (Quisumbing, 1951). To understand the nutritional composition of radish roots, a recent study was performed on the large-rooted types of radish (R. Yang, personal communication). This study performed at the Asian Vegetable Research and Development Center (AVRDC) in Taiwan confirmed that the root is an important source of nutrients (Table 2).

Since the 1920s, the radish has been used for medicinal purposes in India. The seeds were used as an emmenagogue for the treatment of gonorrhoea and the root was found to be useful for treating haemorrhoids, syphilis, and bladder problems (Nadkarni, 1927). At that time, there was no indication which chemicals in the plant were involved in treating such medical problems. In a recent review, two chemicals have been identified in radish that could have potential as being important medicinally (Curtis, 2007).

In summary, peroxidase is present in rich amounts in the roots of radish and acts as an

oxidoreductase enabling it to scavenge dangerous-free radicals. Such an enzyme could be useful in reducing the occurrence of coronary heart diseases and atherosclerosis. To determine the value of peroxidase on reducing excess fat in the blood, a clinical trial was conducted using hyperlipidemic mice fed with different purities of peroxide from radish and horseradish (Wang *et al.*, 2002). Their results revealed that the levels of total serum cholesterol, triglyceride, blood glucose, and lipid peroxidation in the small intestine and liver were significantly reduced compared to mice not fed with peroxidase. This preliminary trial suggests that peroxidase from radish may help in reducing the chance of hyperlipidemia in mice, but such trials are necessary on people to be able to truly evaluate its value to human medicine.

The wounding of crucifers can result in a large production of organic isothiocyanates. These highly reactive chemicals are valuable to the medical world as they have antimicrobial, antimutagenic, and anticarcinogenic properties. A study was made to see whether the main isothiocyanate in radish, 4-(methylthio)-3-butenyl isothiocyanate (MTBITC) had a positive effect as an antimutagen on UV-induced *Escherichia coli* b/r WP2 (Nakamura *et al.*, 2001). Although there were varietal differences in the amount of MTBITC being produced from wounded roots, this bacterial mutagenic assay revealed a positive response as an antimutagen. If such a response can be repeated in human cells, then a medically important chemical would have been identified in radish.

1.4 Traditional Breeding

For many centuries, radish breeding has been achieved by mass selection or combined mass pedigree selection. The radish plant has a sporophytic incompatibility system, where the papillae on the stigma surface forms an elusive barrier to pollen tube penetration. Breeders have overcome this problem by disrupting the stigma surface or by applying pollen to the conducting tissue of the style to allow normal seed set after selfing. However, more recently, breeders have adapted the bud pollination procedure to produce seed (Roggen and Van Dijk, 1973).

The 1960s saw the production of hybrid seed utilizing the incompatibility system and the Ogura (Ogura, 1968) cytoplasmic male-sterility (CMS) in Japan. Although the production of F₁ hybrid seed is expensive, the higher economic value of the large-rooted Asian radishes made such a product counter productive. Japanese seed companies exploited the incompatibility system in radish for the marketing of the F₁ long white “mouli” type to western growers. Recent molecular studies have revealed that the Ogura CMS is under the control of *orf138* (Bonhomme *et al.*, 1992) from the wild relative *R. raphanistrum* (Yamagishi and Terachi, 1997). The Ogura CMS has recently been transferred into the small-rooted radishes to aid the production of F₁ hybrid seeds.

The breeding objectives for small-rooted radishes are quite different from those of the large-rooted types. For the small-rooted types, the most important trait is early bulbing. Such a trait prevents the swollen hypocotyl and root becoming pithy or “wooden” in texture, which makes the crop inedible for human consumption. Hence, breeders have focused on selecting for early growth, fast growth rate, and an annual cycle, which can be found in both *radicula* and early *niger* types of radish. These types of radishes do not require vernalizing temperatures to bolt but will flower especially under long days. For this reason, such types of radishes would not be suitable for summer cultivation. However, through selection, summer varieties, which exhibit decreased sensitivity to photoperiod, are on the market (Tsukamoto and Konishi, 1959). The small-rooted radishes are also commonly grown under glass during the winter season. Under conditions of poor light and warm temperatures, the net assimilation of the radish plant may be too low to allow thickening of the developing root and so the plant will bolt prematurely without the formation of a harvestable root. However, breeders have been able to overcome the problems of early bolting by conventional breeding (Banga and Van Bennekon, 1962).

In terms of the breeding of large-rooted radishes, farmers in China and Japan have mainly focused on the introduction of disease resistance genes. During the early 1950s, most of the radish crop in Japan was destroyed by radish yellows (Katano, 1952). This disease is caused by the casual organism *Fusarium oxysporum* f.

sp. *conglutinans* race 2, which infects a wide range of cruciferous vegetables causing vascular browning and premature leaf fall. Through intensive breeding programs, it was discovered that both the Chinese and Japanese radishes had a rich source of resistance genes against *Fusarium* (Hida and Ashikawa, 1985), but also toward *Albugo candida* (Williams and Pound, 1963) and some viruses (Shimizu *et al.*, 1963).

In Korea, the main breeding objective is to produce vegetables out-of-season. Korean varieties of radish are sensitive to low temperatures and bolt before reaching maturity, if grown during the autumn season. Breeders in Korea have attempted to transfer the late-flowering trait of the Japanese variety “Tokinashi” into Korean varieties to produce winter cropping varieties. During the late 1960s, a late-flowering hybrid was released to the market but its root was found to be too slender and soft textured (Lee, 1987). Although breeders were able to produce autumn cropping varieties in later trials, such varieties often bolted when cultivated during the summer.

1.5 Rationale for Transgenic Breeding

The conventional breeding of radish has been a major success. Over a course of several thousand years, the radish has emerged from being an oilseed crop to a plant producing a large diversity of root shapes and sizes with different flesh and skin colors. Despite the plant having an incompatibility system, breeders have been able to utilize this in the production of F₁ hybrid seeds and also through CMS. Over the last 40 years, breeders have been able to introduce disease resistance genes into the crop and been able to generate new varieties capable of growing under a wide range of climates and different seasons. However, one of the major drawbacks of the conventional breeding of radish is that such methods remain time consuming and labor intensive (8–12 years to produce a new variety). Over the last 30 years, a new “tool” has emerged that could potentially help to accelerate the output of new varieties, this being plant genetic engineering. The progress made toward the generation of new germplasms of radish by tissue culture and other gene transfer methods will be described.

2. DEVELOPMENT OF TRANSGENIC RADISHES

2.1 Tissue Culture Approach in Radish

A prerequisite for the production of transgenic plants in culture is to establish an efficient shoot regeneration system from cultured cells or plant organs. In the case of radish, early studies have shown that the crop has a poor responsiveness toward various culture media in terms of shoot production. Such studies revealed that plant regeneration via organogenesis from hypocotyls (Matsubara and Hegazi, 1990), embryogenic calli (Jeong *et al.*, 1995), and microspores (Lichter, 1989; Takahata *et al.*, 1996) showed a poor regeneration frequency. During the mid 1990s, studies focused on the use of ethylene inhibitors and polyamines in culture media to improve the shoot production of seedling explants of radish, as previous reports highlighted their benefits toward shoot production in related members of the Brassicaceae family. For example, the ethylene released by cultured explants of mustard appeared to impair shoot regeneration (Pua and Lee, 1995) and the inclusion of polyamines in culture media benefited the regeneration of shoots of Chinese cabbage cotyledon explants (Chi *et al.*, 1994). In the case of radish hypocotyls explants, the addition of silver nitrate and L- α -2-aminoethoxyvinylglycine (AVG) to the shoot regeneration medium N1B2 enhanced the shoot regeneration frequency to 40% (Pua *et al.*, 1996). In addition, the supplementation of the polyamine and putrescine at 10–25 mM in the presence of silver nitrate or AVG also greatly benefited the regeneration of hypocotyl explants on N1B2 medium. In a more recent study, the value of ethylene indirectly, polyamines, and gelling agent on the regeneration of hypocotyl and cotyledon explants of the radish Korean ecotype “Jin Ju Dae Pyong” were investigated (Curtis *et al.*, 2004). Using established regeneration media for hypocotyls (N1B2 medium) and cotyledons explants (CR medium; initially used for *B. napus* by Moloney *et al.*, 1989), a shoot regeneration system was established for this commercially important Korean ecotype. In terms of gelling agent, the use of 0.8% (w/v) agar was superior in promoting shoot regeneration in both explants compared to the 0.4% (w/v) agarose treatments (Figures 2a–d).

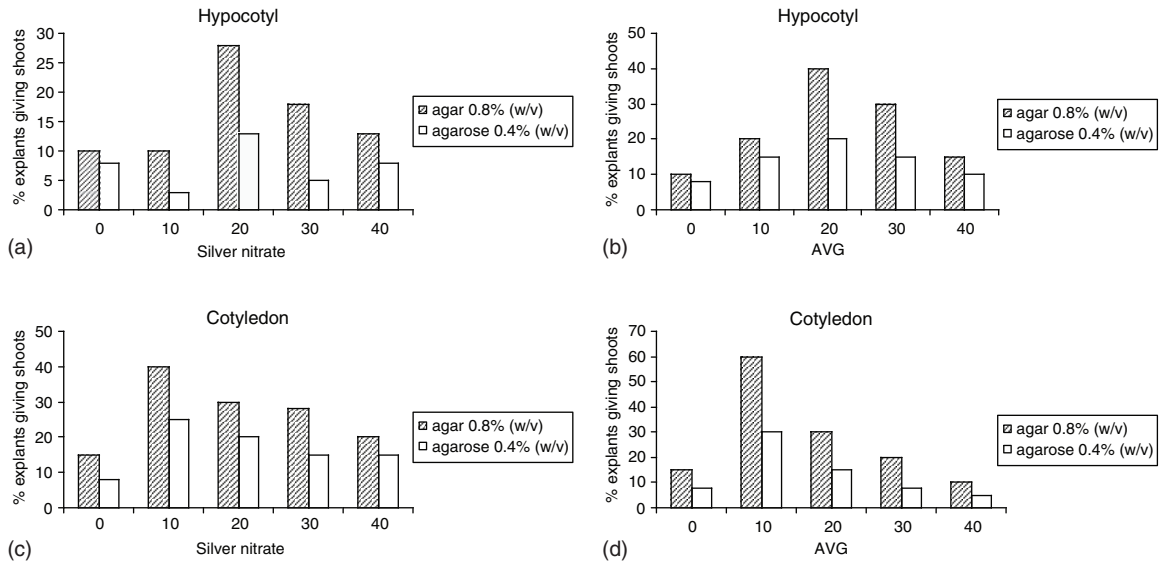


Figure 2 The effects of gelling agent, silver nitrate, and AVG on the regeneration of shoots from (a, b) hypocotyls and (c, d) cotyledon explants of the Korean ecotype “Jin Ju Dae Pyong”, 40 days after culture

This contradicted a previous study by Pua *et al.* (1996), who demonstrated that Chinese radish hypocotyl explants produced more shoots when cultured on agarose medium compared to agar medium. A possible reason why agar treatments were superior compared to agarose treatments for the study with Korean radish was that a highly purified agar was used and that any inhibitory cell growth components may have been removed. The supplementation of either silver nitrate or AVG was significantly better in promoting shoot regeneration compared to treatments without these agents for both hypocotyls and cotyledon explants. However, in terms of shoot production, the hypocotyl (20 μM, 40% regeneration frequency) and cotyledon (10 μM, 60% regeneration) explants required a different concentration of AVG for the optimal regeneration of shoots. Such improved regeneration frequencies for radish explants in culture should in the near future aid the possible production of transgenic radish plants in tissue culture.

In order to assess the usefulness of tissue culture as a means of producing phenotypically normal radish plants, tissue culture-derived plants, and seed-derived plants were compared (Curtis *et al.*, 2004). In this study, a population of 144

regenerated plants (74 from 1-month-old cultures, 70 from 3-month-old cultures) derived from hypocotyl and cotyledon explants from various treatments were compared phenotypically with 62 seed-derived plants under glasshouse conditions. Both populations of regenerated plants bolted and flowered earlier compared to seed-derived plants. In terms of pollen viability, wild type plants revealed more fluorescein diacetate-stained pollen grains (69.0 ± 10.0) compared to tissue culture-derived plants (1 month old, 53.8 ± 7.8 ; 3 months old, 38.9 ± 6.9). Regenerants from 3-month-old cultures (1772 ± 219 mg) yielded larger seed weights compared to plants from 1-month-old cultures (1301 ± 120 mg) and wild type (1490 ± 179 mg).

To be able to study the likelihood of alterations in chromosome number in regenerated plants, root tip squash preparations from 10 randomly selected R_1 lines (10 plants from each line) were hydrolyzed and stained according to the method by Dyer (1963). From 100 cell counts per plant from each line, between 85% and 90% of cells from seed-derived plants and 78–88% from 1-month-old shoot cultures had the same chromosome complement from the diploid number ($2n = 2x = 18$) (Figure 3a). However, plants derived from

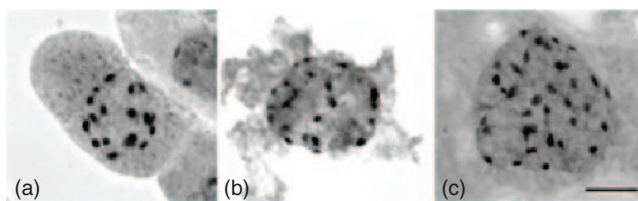


Figure 3 Chromosome preparations from squashed root tips: (a) seed-derived plant exhibiting the normal 18 chromosome complement; (b, c) aberrant chromosome number from a R_1 plant derived from a 3-month-old culture with (b) 22 and (c) 36 mitotic chromosomes (bar = 10 μ m)

3-month-old shoot cultures were less synchronized in terms of number of chromosomes per cell compared to the other treatments. Between 50% and 80% of the cells from the 3-month-old shoot cultures exhibited between 20 and 40 chromosomes per cell (Figure 3b–c). To confirm whether root tip squashes were a good indicator for detecting ploidy in radish, leaves from selected 3-week-old plants were excised and analyzed by flow cytometry. Nuclei from such leaves were stained with 4'6-diamidino-2-phenylindole (DAPI) then passed through a particle analysing system (PAS)-type flow cytometer. These stained nuclei were analyzed in terms of the relative DNA content per nuclei. As a standard, leaves from seed-derived plants were set to 100 fluorescence units corresponding to the chromosome complement of diploid plants. Both wild type and plants derived from 1-month-old cultures produced a major peak at 100 fluorescence units (Figures 4a–b). In terms of the lines derived from 3-month-old cultures, two lines formed a major peak at 120 units (Figure 4c) and another independent line gave two major peaks at 100 and 120 units (Figure 4d). The seven remaining 3-month-old culture lines formed a peak at 100 units.

Further studies were performed on these R_1 lines to see whether plants with deviations in DNA content per nuclei also showed unusual phenotypes. The three R_1 lines with a relative DNA content per nuclei greater than wild type (Figures 4c–d) produced larger leaf areas compared to lines derived from 1-month-old cultures and wild type. However, the numbers of seeds produced by these three R_1 lines were significantly lower compared to wild type and the 1-month-old culture lines. This study demonstrated that prolonged culture of radish

explants can cause a higher turnover in the number of genetically variable plants. This observation supports a previous study in tobacco, where plants regenerated from protoplasts exhibited a higher frequency of ploidy following prolonged culture (Huang and Chen, 1988). Overall, this study in radish emphasized the need for a short culture period of explants to be able to produce phenotypically normal plants in culture and for the future of producing genetically stable transformants through *Agrobacterium*-mediated systems in tissue culture.

2.2 Somatic Hybridization

Plant protoplasts, a wall-less cell, can be used for several techniques directed at plant genetic manipulation. Such cells can be used as recipients for plasmid DNA and organelles including chloroplasts and mitochondria. One of the most important uses of protoplasts is that they can be induced to fuse, either chemically or through the use of electrical currents, with protoplasts of other species to produce a novel hybrid or somatic hybrid. This method allows sexually incompatible species to be combined at the genome level but also allow hybrid organelle combinations.

In terms of radish, the use of somatic hybridization has been mainly focused on transferring useful characters from radish into *Brassica* species (Sakai and Imamura, 1990; Hagimori *et al.*, 1992). This technology is useful as sexual hybrids between *R. sativus* and *Brassica oleracea* (*Raphanobrassica*) have limitations. Firstly, such sexual crosses can only be achieved, if radish is used as the female parent and so the cytoplasm of *B. oleracea* cannot be introduced into the hybrids. In addition, the

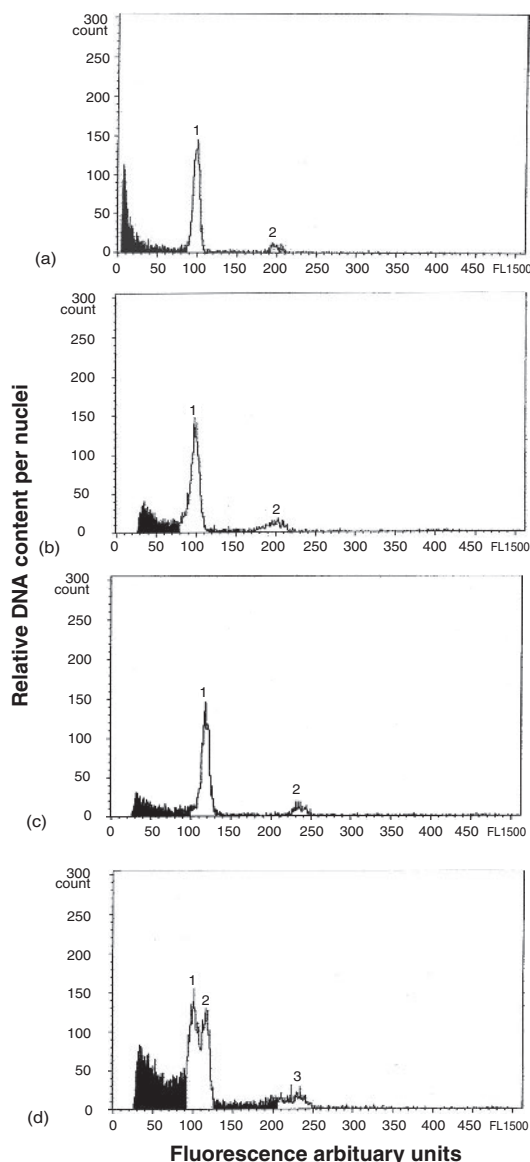


Figure 4 Relative fluorescence of nuclei stained with DAPI of (a) seed-derived plant and R_1 plants derived from (b) 1-month-old culture and from (c, d) 3-month-old cultures

fertility of *Raphanobrassica* is poor (McNaughton, 1973) and backcrossing to *B. oleracea* remains difficult.

One successful application of somatic hybridization using radish as a parent was the transfer of clubroot resistance into cauliflower (Hagimori *et al.*, 1992). Japanese radishes carry resistance to this root infection caused by the fungus

Plasmodiophora brassicae (Yoshikawa *et al.*, 1979). Somatic hybrids were produced after the electrofusion of iodoacetamide-treated cauliflower protoplasts with untreated radish protoplasts. From 40 regenerated plants, 37 were true hybrids based on phenotype, isozyme, and restriction fragment length polymorphism analysis. All hybrids exhibited resistance to clubroot disease equal to that of the Japanese radish.

Recently, somatic hybrids between *A. thaliana* ecotype Columbia and a male-sterile radish line MS-Gensuke were produced by fusing protoplasts of the two species with polyethylene glycol (Yamagishi and Glimelius, 2003). By using an established protoplast culture system for *Arabidopsis* (Yamagishi *et al.*, 2002), 11 shoots/rooted plants from a population of 41 shoots showed hybrid DNA patterns when analyzed at the molecular level. The chloroplast genomes of these hybrids were exclusively of radish origin but the mitochondrial DNA revealed a combination of both parental genomes. Those somatic hybrids, which flowered, were male-sterile regardless of the presence of the Ogura CMS gene *orf138*. This report offers the opportunity for the gene pool of radish to be expanded if such hybrids are backcrossed to radish. Such material would also be useful for studying nuclear–organellar genetic interactions as the *Arabidopsis* genome has been completely sequenced.

2.3 Production of Transgenic Plants by *Agrobacterium*-Mediated Gene Transfer

There is now a wealth of protocols that describe the production of transgenic crops. Such methods often rely on the culture of plant cells, tissues, or organs on complex media and then foreign DNA is transferred by *Agrobacterium*-mediated transformation or by biolistics. In the case of radish, the production of transgenic plants has been severely delayed due to the poor responsiveness of cultured explants to regenerate shoots. For this reason, researchers looked at alternative procedures of trying to produce genetically modified radish to widen the gene pool of this important crop. The production of transgenic radish was finally achieved by a nontissue culture method, which is classified as *in planta* transformation. Today, there are two *in*

planta transformation systems available for the production of transgenic radishes.

2.3.1 Floral dipping

The first report on the production of transgenic radish by *Agrobacterium*-mediated transformation was achieved by a method commonly known as “floral dipping” (Curtis and Nam, 2001). This procedure involves the dipping of a recently flowering plant into a container containing a surfactant, energy source, and the biological gene transfer agent *Agrobacterium tumefaciens*. The method itself is relatively simple and an experienced plant cell culturist is not required. One of the first reports on the use of *in planta* transformation involved *A. thaliana* L. (Bechtold *et al.*, 1993). In this report, the inflorescences of the plants were subjected to a vacuum during inoculation to aid transformation. A later investigation using *Arabidopsis* identified that the use of a vacuum was not necessary and the term “floral dipping” was employed (Clough and Bent, 1998). Subsequently, this technique has been used successfully in other plant systems including *B. rapa* L. ssp. *chinensis* (Qing *et al.*, 2000) and *Medicago truncatula* (Trieu *et al.*, 2000).

The success of using *in planta* transformation in the production of transgenic plants from the members of the Brassicaceae family encouraged researchers to investigate the usefulness of floral dip as a means of transforming radish (Curtis and Nam, 2001). *A. tumefaciens* strain AGL1 (Lazo *et al.*, 1991) was used in the study as the bacterial agent could transform seedling explants of the Korean variety “Jin Ju Dae Pyong” (Curtis, personal communication). This variety of radish was used in the investigation due to its commercial importance. The bacterial strain carried the binary vector pCambia3301, having the selectable marker for bialaphos resistance (*bar*) and the reporter β -glucuronidase (*gusA*) genes both under the control of the cauliflower mosaic virus (CaMV) 35S promoter located between the left and right transferred-DNA (T-DNA) borders, respectively. This dual-marker gene system would allow putative-transformed plants to be screened *in solium* through the use of herbicide sprayings and GUS expression to be monitored by histochemical staining. The concentration

of herbicide used, in this case Basta®, was determined experimentally by spraying different concentrations of the herbicide onto wild type plants and then monitoring the number of necrotic plants over a period of 30 days. Basta used at 0.03% (v/v) was the lowest concentration to cause necrosis on all wild type plants and so was used as the working concentration for selecting transformed plants. Unique to previous studies on *in planta* transformation, the effects of the type and concentration of surfactant used in the inoculation medium were studied in terms of transformation efficiency.

The floral-dip transformation system for radish has been described in detail (Curtis, 2004). Essentially, when radish plants are dipped into a suspension of *A. tumefaciens* containing a surfactant the plants are grown in the glasshouse and then hand pollinated to promote seed set. Prior to seed harvest, the origins of the seeds were noted from all plants. Following seed ripening, all seeds were sown in trays of compost. Plantlets were sprayed with herbicide at the four-leaf stage of development, then twice more at weekly intervals. Thirty days after sowing, all non-necrotic plants were deemed to be resistant to Basta and were subsequently assayed for GUS activity by histochemical staining to confirm their transgenicity.

In this same study, three stages of plant development were investigated in terms of their effect on transformation efficiency (Figure 5). These stages included plants exhibiting a primary bolt (single main stem with many immature floral buds), plants with branches from the main stalk (secondary bolts), and plants with side branches from the secondary bolts (tertiary bolts). In terms of the surfactants used in the inoculation medium, the presence of Silwet L-77, Pluronic F-68, and Tween 20 at concentrations 0, 0.01, 0.05, and 0.1% (v/v) were investigated. The study confirmed that the presence of a surfactant in the inoculation medium was necessary for the transformation of radish as treatments without the chemical failed to yield any transformants. Silwet L-77, a tri-siloxane, was the superior surfactant when used at 0.05% concentration in the inoculation medium. Primary bolted plants dipped into a suspension of *Agrobacterium* containing 0.05% Silwet L-77 and 5% sucrose (w/v) gave the optimal transformation efficiency of 1.4%

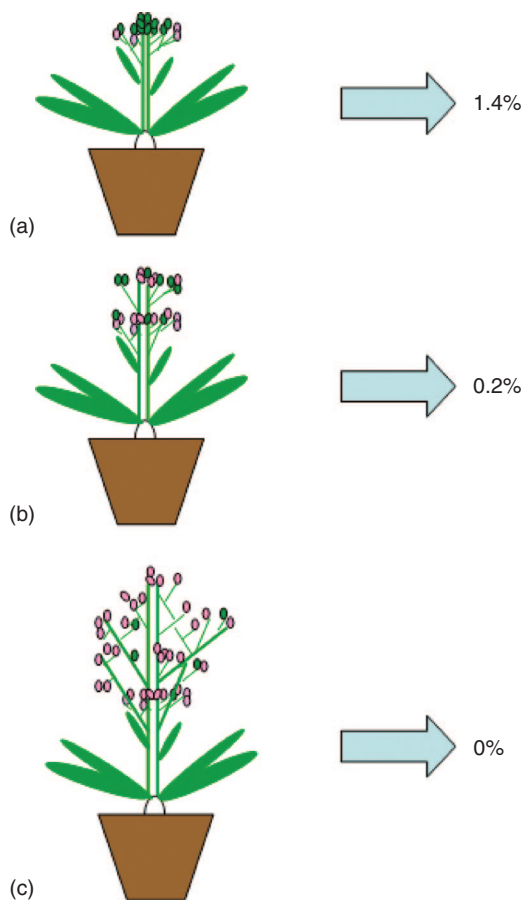


Figure 5 The effects of plant development on the transformation efficiency of the Korean ecotype “Jin Ju Dae Pyong” using floral dip; the three stages of development investigated were (a) primary bolt, (b) secondary bolted plant, and (c) tertiary bolt (percentage figures represent the optimal yield of transformed seeds with regards to the state of plant floral development at the time of dipping into inoculation medium)

(15 transformed seeds from a population of 1110). The stage of floral development of the dipped plant was found to be important in the transformation of radish. Tertiary bolted plants failed to produce any transformed seed regardless of which surfactant was present in the inoculation medium. Treatments involving secondary bolted plants gave a lower optimal transformation efficiency (0.2%) compared to primary bolted plants. Overall, this study confirmed that the ideal conditions for the transformation of Korean variety “Jin Ju Dae Pyong” was to dip primary bolted plants into a suspension of *Agrobacterium*

containing 5% (w/v) sucrose and 0.05% (v/v) Silwet L-77.

Molecular analysis of plants produced from the progeny of plants dipped into inoculation medium revealed some insight of the possible mechanism of how radish is transformed during floral dipping. Southern blot analysis showed that there were several populations of transformed plants with the same T-DNA insertion pattern, a phenomenon known as siblings. Observing the site where the seeds originated from the mother dipped plant, it was found that each individual population of siblings was derived from the same bolt. This discovery is very different from that of *Arabidopsis* as all transformed seeds are usually independent transformants (Clough and Bent 1998). However, studies made on a less-related plant, *M. truncatula*, revealed that between 77% and 87% of transformants were siblings (Trieu *et al.*, 2000). It has been postulated that *Agrobacterium* transfer their T-DNA into meristematic cells, which later develop into floral bolts (Trieu *et al.*, 2000). If such a meristematic cell is transformed, then that cell would carry the same T-DNA event into the whole developing bolt. However, in the case of *Arabidopsis*, the site of *Agrobacterium*-mediated transformation following floral dip is the ovule (Ye *et al.*, 1999; Desfeux *et al.*, 2000). Histochemical staining of pollen from several thousands of flowers from plants dipped into a suspension of *Agrobacterium* revealed no GUS activity and thus confirming that the pollen is not the site of transformation in radish. At present, the frequency of sibling transformants in radish following floral dip is between 50% and 60%. For this reason, researchers on radish improvement are now looking for alternative procedures to increase the yield of independently transformed plants.

2.3.2 Infiltration of germinating seeds

To date, there have been several reports on the transformation of recalcitrant plants through non-tissue culture approaches. During the late 1980s, it was reported that germinated seeds of *Arabidopsis* when co-cultivated with a disarmed *A. tumefaciens* strain carrying neomycin phosphotransferase II (*nptII*) gene resulted in treated plants producing seeds with resistance to the antibiotic (Feldmann

and Marks, 1987). Seeds imbibed for 12 h prior to being inoculated with *Agrobacterium* gave the highest transformed progeny. In a later study, this approach was also proved successful in the transformation of *M. truncatula* (Trieu *et al.*, 2000). In this particular study, seeds were imbibed and cold treated for 14 days prior to the seedlings being infiltrated with a suspension of *Agrobacterium* under vacuum. Following transferring these plants to the glasshouse, the number of transformants was determined in the following generation. The frequency of transformants ranged from 2.9% to 27.6% and, similar to the floral-dip procedure, such a population of transgenics was made up of independent transformants and sibling transformants. Southern analyses confirmed that the percentage of independent transformants arising from the seedling transformation was between 67% and 86% compared to 13% and 23% for the flower infiltration procedure. Although these two methods for transforming *M. truncatula* were successful for several strains of *Agrobacterium*, the widely used strain, LBA4404 failed to yield any transformed seeds. The seed yield from plants infiltrated at flowering and at seedling stage of development was poor compared to nontreated plants.

Sonication-assisted *Agrobacterium*-mediated transformation has been shown to improve the efficiency of *Agrobacterium* infection by introducing many small wounds into target plant tissues in soybean (Santarém *et al.*, 1998; Trick and Finer, 1998). In a recent study, examining the value of sonication followed by vacuum infiltration on germinated seeds in the presence of *A. tumefaciens* enabled transgenic radish plants to be produced (Park *et al.*, 2005). Seeds of radish cultivar “Kosena” were germinated on damp filter paper at 25 °C for 24 h in darkness. Germinated seeds were inoculated with *A. tumefaciens* strain LBA4404 carrying the *nptII* gene under the control of nopaline synthase (*nos*) promoter and terminator along the T-DNA. The inoculation was performed in a vessel with sonication for 0–12.5 min with a 2.5 min interval. Following sonication, the germinated seeds were placed into a vacuum chamber for 0–12.5 min. After vacuum infiltration, the seeds were blotted dry then co-cultivated in the dark at 25 °C for 3 days. Seeds were washed in Claforan®, washed in water, and then finally blotted dry prior to transfer to the soil.

After 4 weeks, the number of surviving plants was analyzed at the molecular level to determine their transgenicity. The optimal yield of transformed plants was obtained with the treatment comprising of a 5 min sonication period followed by a 5 min vacuum infiltration (8% transformation efficiency) as confirmed by polymerase chain reaction (PCR) and Southern blotting. Transformants carried 1 or 2 copies of the transgene and were derived from independent transformation events. This study confirmed that an alternative procedure exists for the production of novel germplasms of radish by an *in planta* method of gene transfer.

2.4 Transfer of Agronomic Traits into Radish

To date, two *Agrobacterium*-mediated transformation systems exist for the production of transgenic radish. These systems have recently been employed for the generation of important germplasms to improve the agronomic performance of this important crop.

2.4.1 Late flowering

Korean people are very proud of their own varieties of radish in terms of the root being hard, crisp in texture making this root crop the country's national vegetable. In terms of the percentage, growing area used for the cultivation of root crops in Korea, the cultivation of radish crop constitutes 85% of this total (Curtis, 2004). However, despite its popularity in Korea, such varieties are cold sensitive causing the cropping season to be restricted. Cool temperatures during the fall season (Figure 6a) results in the growth habit of the radish to change from a rosette plant to bolt and the root to become shrunk and inedible (Figure 6b). Although sexual crosses between cold-tolerant Japanese varieties and commercial Korean varieties produced late-flowering hybrids, such plants had poor textured roots (see Section 1.4). For this reason, other approaches were investigated to overcome this problem. Using the recently developed “floral-dip” technique for transferring foreign genes into radish (Curtis and Nam, 2001), such a system was investigated for the transfer of a late-flowering trait into the

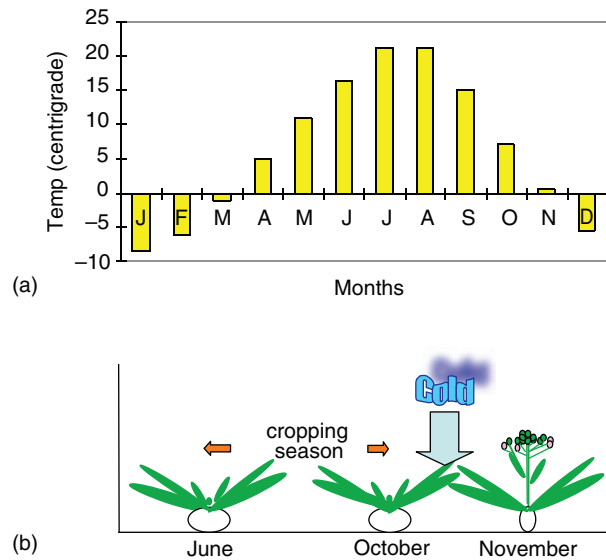


Figure 6 The restriction of cropping season of the native Korean radish due to cool temperatures: (a) average minimum temperatures in Suwon, a popular region for the cultivation of radish in Korea; (b) prevailing low temperatures in Korea restrict the cropping season from June to October

commercially important Korean variety “Jin Ju Dae Pyong”.

The change of the shoot apical meristem from the vegetative to the flowering form occurs in response to a change in the environment such as daylength (photoperiod) and reductions in temperature (vernalization). The genes involved in these developmental changes have been reviewed in substantial detail in the model plant *Arabidopsis* (Reeves and Coupland, 2000; Sheldon *et al.*, 2000). *GIGANTEA* (*GI*) is important in regulating photoperiodic flowering and controlling circadian rhythms (Fowler *et al.*, 1999; Park *et al.*, 1999). Several *gi* mutants have been identified in *Arabidopsis*, which exhibit photoperiod-insensitive flowering and a breakdown of the circadian clock (Koornneef *et al.*, 1991). Hence, under long days, such mutants exhibited delayed flowering time compared to wild type. In addition, overexpression of the *GI* gene under the control of the constitutive promoter, CaMV 35S, in *Arabidopsis* resulted in early flowering (Park, personal communication). These observations suggest that down-regulation in the expression of the *GI* gene may cause late flowering. In order to investigate whether a down-regulation in the expression of the native *GI* gene in radish could delay flowering, the model cultivar

“Jin Ju Dae Pyong” was transformed with an antisense *GI* gene under the control of CaMV 35S promoter by the floral-dip technique (Curtis *et al.*, 2002). *A. tumefaciens* strain AGL1 carrying pCambia3301 with an antisense *GI* gene from *Arabidopsis* was used in this study. From the seeds of 25 floral-dipped plants, a total of 16 T₁ plants were found to be transformed as confirmed by *in solium* selection, GUS staining and by Southern blot analysis. Northern analysis revealed that the expression of the *GI* gene in the antisense *GI* T₁ plants was much reduced compared to wild type and plants transformed with only *gus* and *bar* genes (positive control). The progenies of the antisense *GI* plants (T₂ generation) were compared phenotypically with wild type and positive control plants. All antisense *GI*-transformed lines showed a delay in bolting and flowering time compared to wild type and plants transformed with marker genes only (Figures 7a and b). The longest delay in bolting time by a T₂ antisense *GI* line compared to wild type was 17 days. In terms of time to anthesis (time to reach first flower opening), this same T₂ line showed a delay of 18 days compared to wild type. However, the heights of 10 of the 11 T₂ lines investigated, exhibited a significantly smaller height compared to wild type and positive

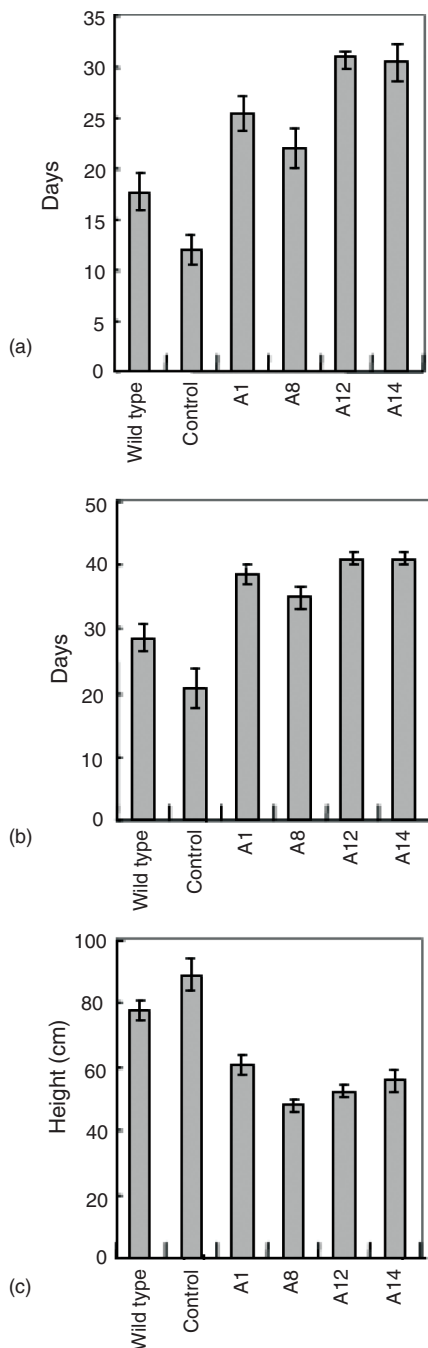


Figure 7 Phenotypic responses of T₂ plants of antisense *GI* transformants: (a–c) the times to (a) bolting, (b) flowering, and (c) the heights of transgenic and nontransformed plants is given as mean \pm standard error of the mean (plants A1, A8, A12, and A14 represent antisense *GI*-transformed lines; the control is the positive control line carrying *gusA* and *bar* genes; wild types are nontransformed plants)

control plants (Figure 7c). It is not clear whether a reduction in *GI* expression could affect stem elongation but further studies are necessary to confirm this observation. Nevertheless, this study demonstrated that by down-regulating the expression of *GI* in radish resulted in a delay in both bolting and flowering times, which ultimately could allow the growing season of this commercial variety to be extended into the autumn season.

2.4.2 Drought tolerance and salt stress

One of the main breeding objectives in radish is to produce roots out-of-season. Through the recent development of gene transfer systems now available for radish, such useful germplasm could be generated through the expression of drought and salt tolerance genes. One possible group of genes that could induce such tolerance are the late embryogenesis abundant (*LEA*) genes, which may be induced to high levels of expression by abscisic acid (ABA) and/or desiccation (Bray *et al.*, 2000). *LEA* proteins are classified into five major groups based on their amino acid sequence (Dure, 1993). In terms of their function, the wheat *LEA* protein was reported to act as an osmoprotective molecule in yeast (Swire-Clark and Marcotte, 1999). In addition, the expression of the *hva1* gene from barley, a group 3 *LEA* gene in rice (Babu *et al.*, 2004) and wheat (Sivamani *et al.*, 2000) further confirmed their value in providing tolerance to stress conditions in transgenic plants. In order to determine whether the expression of a group 3 *LEA* gene in radish could induce drought and salt tolerance, transgenic plants were produced through sonication and vacuum infiltration of germinated seeds (Park *et al.*, 2005).

In this report, *A. tumefaciens* strain LBA4404 carrying pBI121 with a group 3 *LEA* gene from *B. napus* was used in this study. The *LEA* complementary-DNA (cDNA) clone *ME-leaN4* was under the control of the CaMV 35S promoter with an *nptII* gene linked to a NOS promoter and terminator sequence. Studies on drought tolerance and salt stress were performed on the progeny of plants (T₁ generation) from the *Agrobacterium*-inoculated seedlings. Seeds from transgenic lines were sown on semi-solidified

Murashige and Skoog (MS, Murashige and Skoog, 1962) medium containing 50, 100, 200, 300, and 400 mM mannitol to study osmotic stress. For the salt stress experiment, seeds were sown on MS medium supplemented with 50, 100, and 150 mM NaCl. Following 10-days culture at 25°C under a 16 h photoperiod, both the length and fresh weight of plantlets were compared with nontransformed plants. Transgenic plants cultured on medium containing 50–300 mM mannitol exhibited a faster growth rate compared to nontransformed plants. In terms of the salinity stress experiment, the transgenic lines showed a longer plant length and a greater fresh weight for the treatments 50–150 mM NaCl compared to wild type. These experiments implied that the *LEA* gene from *B. napus* improves the tolerance of radish to both water deficit and salt stress in culture.

3. FUTURE ROAD MAP

3.1 Expected Products

Due to the recent developments of two gene transfer systems for radish, namely, “floral dip” (Curtis and Nam, 2001) and the infiltration method into germinated seeds (Park *et al.*, 2005), one expects an increase in the production of novel germplasm in this crop. Over the last 5 years, such methods of producing transgenic plants have resulted in a late-flowering Korean radish (Curtis *et al.*, 2002) and the transfer of drought and salt tolerance (Park *et al.*, 2005). In terms of future products, the large-sized 3-month-old radishes from Asia has the attraction through gene technology of expressing antibodies or synthetic vaccines, which would have value in the Third World where the crop is extensively cultivated. In addition, the complete nuclear and organellar genome sequences of *Arabidopsis* (Unsel *et al.*, 1997; Sato *et al.*, 1999; The Arabidopsis Genome Initiative, 2000) and the future determination of their function will allow a host of useful traits to be transferred from a related plant into radish. Here, an alternative strategy of producing a late-flowering radish will be discussed using a novel approach by a chemical-inducible promoter system, which has recently been achieved in the model plant *Arabidopsis* (Curtis *et al.*, 2005).

The production of a late-flowering Korean radish offers the possibility of the growing season

to be extended into the autumn season. Expression of an antisense *GI* gene in radish resulted in bolting and flowering time to be extended by a few weeks compared to wild type (Curtis *et al.*, 2002). However, phenotypic characterization of T₂ plants showed that the down-regulation of endogenous *GI* could affect other plant-development processes including plant height. In a recent report, delayed bolting and flowering time in *Arabidopsis* was achieved through the site-specific inactivation of gibberellin (GA) through the expression of a GA 2-oxidase gene from bean *PcGA2ox1* under the control of an estrogen inducible system, XVE bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E; ER) (Zuo *et al.*, 2000). A single application of 17- β -estradiol, an analogue of estrogen, to the apical shoot of rosette plants of *PcGA2ox1* overexpressors showed delay in bolting (46 days) and flowering time (62 days) compared to wild type (36 and 41 days, respectively). Quantitative reverse transcription (QRT)-PCR results showed that GA down-regulated genes were up-regulated and that GA up-regulated genes were down-regulated in a site-specific manner. These results suggest that the *PcGA2ox1* under the control of an estrogen-inducible promoter could delay bolting and flowering time by localized 2 β -hydroxylation. Future research is needed to investigate whether such a system could be applied to crops, such as radish, which may result in a further extension of the growing season in commercial Korean varieties, especially if repeated applications of inducer are used.

3.2 Addressing Risks and Concerns

Recent advances in the transfer of novel genes into crops by genetic manipulation techniques have created new possibilities for crop improvement. However, the introduction of genetically modified crops into field trials opens the possibility of transgene escape into the environment via pollen dispersal. The concern, as with all genetically modified crops, is the likelihood and consequence of a transgene being transferred through pollen dispersal to wild relatives or nontransgenic crops. For pollen-mediated gene transfer to occur, pollen from transgenic plants must be dispersed toward

such plant populations and successfully fertilize an ovule. Transgenes for herbicide, insect, disease, and stress resistances could benefit weeds to become a new troublesome problem for farmers and affect the ecological balance in natural habitats (Darmency, 1994).

Studies on the hybridization between radish and other related plants in the field have focused on sexual crosses between oilseed rape (*B. napus*) and a wild radish, *R. raphanistrum*. Such reports have mainly focused on the possibility of transgenic pollen from *B. napus* being used to fertilize wild radish to produce hybrid seed (Darmency *et al.*, 1998; Chevre *et al.*, 2000; Guéritaine and Darmency, 2001; Rieger *et al.*, 2001). Wild radish is a self-incompatible species (Sampson, 1964) and so cannot produce seeds by itself. Early studies using *B. napus* as the seed parent produced partially fertile hybrids with *R. raphanistrum* but hand pollination and embryo rescue were necessary (Kerlan *et al.*, 1992). In the field, a large amount of hybridization was reported when a male-sterile *B. napus* was used (Chevre *et al.*, 1996). However, experiments conducted under commercial agronomic conditions showed that two from over 52 million seedlings from male-fertile *B. napus* were hybrids with wild radish (Rieger *et al.*, 2001). This study confirmed that under field conditions there is a very remote possibility (4×10^{-8}) of hybridization from pollen of wild radish fertilizing fertile oilseed rape.

Wild radish is considered to be within the top 100 economically damaging weeds worldwide. Recently, there have been concerns from environmentalists that biotechnology companies should refrain from releasing transgenic radishes to the field to avoid resistances to viruses, insect pests and herbicides being transferred into this troublesome weed. In a report produced at the University of California, researchers studied the incidence of gene flow from cultivated radish into wild radish using a test plot (N. Ellstrand, personal communication). Cultivated radishes were grown in the center of a field surrounded by a population of wild radishes. The cultivated radish carries a dominant marker *Lap6* gene, which is present as a recessive trait in wild populations. Thus, to monitor gene flow from the cultivated radish to wild populations was based on the presence or absence of this marker in the progeny of wild radish. All plants analyzed 1 m away from the

cultivated radish population produced a hybrid carrying the allozyme allele. In addition, the presence of the allele was also detected up to 1 km away from the cultivated crop. In terms of seed yield, the hybrid population produced 15% more seeds compared to the wild radish. Although this is the only study using both cultivated and wild radishes together in a field trial, there appears to be genuine concern that transgenes may be released into wild radish and appropriate management strategies need to be enforced to prevent the production of so-called “super weeds”.

3.3 Expected Technologies

Over the last 20 years, advances in gene technology have enabled a wide diversity of crop species to be successfully transformed. In the case of radish, the poor responsiveness of cultured explants to regenerate shoots was a major stumbling block toward generating transgenic plants through tissue culture approaches. However, the application of *in planta* systems toward the transfer of agronomic useful traits has allowed important novel germplasms to be created at transformation efficiencies up to 8% being reported (Park *et al.*, 2005). However, it is not clear how applicable such technologies would be to a wider gene pool of radish germplasms. At present, only two varieties of radish have been successfully transformed by *in planta*-mediated transformation techniques. One possible area for both widening the accessibility of radish varieties to be transformed and also improving transformation efficiency is through a tissue culture-based system. Over the last 10 years, there have been major improvements in the regeneration of seedling explants through the use of ethylene inhibitors in culture (Pua *et al.*, 1996; Curtis *et al.*, 2004) and shoot regeneration frequencies of up to 60% have recently been reported (Curtis *et al.*, 2004). At present, the major delay in the production of transgenic plants in culture has been the mismatch between a useful shoot regeneration system and the incorporation of a gene transfer system. Radish seedling explants are not amenable to shoot regeneration in the presence of antibiotics in the culture medium (Curtis, personal communication). However, the use of a nonantibiotic selection system may circumvent the problem.

The use of a nonantibiotic gene-marker system in radish may not only improve the transformation efficiency of radish in culture but also allow the future release of transgenics into field trials and prevent the possibility of “super weeds” being produced. One possibility is the use of the mannose-based positive selection system, which has been used successively for cassava (Zhang *et al.*, 2000). This system is based on the use of the phosphomannose isomerase (*pmi*) gene from *E. coli* being used as a selectable marker and mannose as the selectable agent. In nontransformed plant cells, mannose is phosphorylated by a hexokinase to mannose-6-phosphate, which can significantly impair cell growth. Cells carrying the *pmi* gene can convert mannose-6-phosphate to fructose-6-phosphate, which can be used as a carbon source and enabling transformed cells a metabolic advantage. Plants transformed with the *pmi* gene offers no risk to human, animal and the environment.

There is now a level of optimism in terms of improving the agronomic performance of radish by gene technology. If further commercial varieties of radish prove to be amenable to transformation then one expects the crop to enter field trials soon and provided appropriate management strategies are employed the crop should not pose a risk to the environment.

DEDICATION

This work is dedicated to the 2.5 million people who lost their lives during the North Korean famine of the mid 1990s. Radish is a major crop in North Korea and the work on the genetic improvement of the crop underlines the importance of genetic engineering to improve crops for the Third World.

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Carrot

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Carrot (*Daucus carota*) is a biennial crop, grown as an annual for its edible tap root. Eastern or Asiatic carrots are often called anthocyanin carrots due to their purple roots and are found predominantly in Russia, Afghanistan, Iran, and India. They are believed to be the ancestor of Western or Carotene-type carrot (Quiros, 2001). Domestication is thought to have taken place in these areas around the 10th century and the Carotene types were developed during the late 16th century and early 17th century. Orange-, red-, or white-rooted biennial carrots were derived from the Eastern types most likely through selection of hybrid progenies of yellow carrots and wild subspecies grown in the Mediterranean (Quiros, 2001). Farmers in the Netherlands were the first to cultivate orange carrots and today's cultivars are thought to have originated from varieties developed there (Rubatzky and Yamaguchi, 1997).

1.2 Botanical Description

Carrot is a monocarpic herb and a member of the family Apiaceae (formally Umbelliferae) (Pressman, 1997; Rubatzky and Yamaguchi, 1997). Native to Europe and parts of Asia, *D. carota* consists of 13 subspecies divided into two groups,

the Carota and Gingidium (Quiros, 2001). The Carota group contains the cultivated species (var. *sativum*). Wild forms of the Carota group are widespread throughout the world (var. *carota*). All subspecies of *D. carota* can cross freely with each other (Quiros, 2001).

1.3 Economic Importance

In excess of 13.5 million tons of carrot is grown for human consumption (FAOSTAT, 2006), accounting for approximately 3% of the world's vegetable production and considered one of the most important vegetables (Hardegger and Sturm, 1998). Carrot is the third most important vegetable crop produced in Australia after tomatoes and potatoes, comprising 8% of the value of Australia's total vegetable production (Seven Vegetables, 2000). In 2004, Australia produced 302 560 t of carrots on 73 195 ha with a production rate of 420 514 hg ha⁻¹ with an average export value of US\$432.18 per ton (FAOSTAT, 2006).

Victoria is Australia's highest carrot producing state, producing 113 310 t in 2002, predominantly for the local market (Australian Horticulture Statistics Handbook, 2004). Western Australia is Australia's leading exporter of fresh carrots, accounting for approximately 90% of the country's carrot exports (McKay and Pasqual, 2006). In 2002/2003 Western Australia produced 88 211 t of carrots and exported in excess of 75 000 t (Australian Horticulture Statistics Handbook,

2004). Western Australia's largest carrot export market is Malaysia, followed by Singapore and the United Arab Emirates (McKay and Pasqual, 2006; Australian Horticulture Statistics Handbook, 2004). Important carrot varieties in Australia are Nantes type, Ivor, Stefano, Red Count, and Crusader (McKay and Pasqual, 2006). Nantes type carrots (blunt-ended, straight to slightly tapered roots) are the most commonly grown variety Australia wide (Seven Vegetables, 2000).

The β -carotene content in the storage roots makes carrot a highly nutritious food source (Rubatzky and Yamaguchi, 1997; Hardegger and Sturm, 1998). Responsible for the orange color of storage roots, β -carotene is cleaved into two vitamin A molecules during digestion (McKay and Pasqual, 2006). Carrot is also an excellent source of sugar, vitamin C, and fiber (Quiros, 2001). The mature storage organ of a carrot comprises 88% water, 6–8% sugar, 1–2% fiber, 0.7–1.2% protein, 1% ash, and 0–3% fat (Hardegger and Sturm, 1998). Glucose, fructose, and sucrose make up total sugar content (Peterson and Simon, 1986). Of commercial interest are cultivars with a high yield, a high sugar level, and high resistance to pests and diseases (Peterson and Simon, 1986). As well as using carrot raw, cooked, canned, frozen, pickled, or dehydrated, carotene can be extracted for use as food coloring in margarine, or in poultry feed to intensify skin and egg yolk color (Rubatzky and Yamaguchi, 1997).

1.4 Traditional Breeding: Breeding Objectives, Tools, and Strategies

Genetic improvement of *D. carota* has been previously achieved through plant breeding (Simon, 1984; Peterson and Simon, 1986; Ammirato, 1996). Carrot hybrids are most often generated via three-way crosses and selection is made via the root to seed method (Quiros, 2001). Cultivated carrots are classified into two major groups, temperate and subtropical, with breeding efforts focused on temperate types due to their higher value and larger market share. Temperate carrot types include Nantes, Imperator, Danvers, and Chantenay and the subtropical class includes Kurada, Brasilia, and Tropical Nantes types (Quiros, 2001).

Madjarova and Bubarova (1983) were the first to investigate the application of genetic crosses for improved root color. More recently, breeding for specific characteristics such as male sterility (Nothnagel *et al.*, 2000), bolting resistance, and disease and pest resistance (Bonnet, 1983; Boiteux *et al.*, 1993, 2004) have become a priority. Other important traits being introduced into carrot genotypes are improved flavor, eating quality and color and increased carotene content (Quiros, 2001).

Sufficient genetic variation may exist among cultivated classes of carrot to derive any root type or regional adaptation; however, crosses between domesticated and wild-type carrots and subsequent backcrosses have been necessary to introduce pest and disease resistance in elite breeding lines (Quiros, 2001). Tissue culture techniques such as *in vitro* anther culture (Ammirato, 1996), somaclonal variation (Sung and Dudits, 1981; Ammirato, 1996), and protoplast fusion (Sung and Dudits, 1981) have been used to enhance resistance to pests and diseases.

1.5 Limitations of Conventional Breeding

Pathogenic and environmental pressures on modern crop production practices have increased as a result of broadening growing regions outside those that plants are naturally adapted to. Conventional breeding technology has historically enabled breeders to develop more robust plant genotypes that incorporate genes and hence traits of interest. However, some argue that dwindling genetic resources are making conventional breeding practices increasingly difficult (Quiros, 2001). Where no feasible natural biological method is available, alternative methods must be developed to enable crop improvement and overcome specific abiotic and biotic stress. Genetic transformation has been developed as an attractive tool for the transfer of useful traits into genetic backgrounds lacking those traits. By incorporating the genetic material that provides the trait into the plant genome, the favorable trait can be passed onto the plant's progeny. This technology has been widely exploited to obtain commercial crops with improved agronomic traits.

2. DEVELOPMENT OF TRANSGENIC CARROTS

2.1 Transformation Methods Employed

A variety of techniques exist for the direct introduction of new genetic material into carrot host cells. These include electroporation (Fromm *et al.*, 1985), chemicals that increase uptake of DNA (Paszowski *et al.*, 1984), microinjection (Crossway *et al.*, 1986), and DNA delivery via microparticle bombardment (Klein *et al.*, 1987). However, it is widely believed that *Agrobacterium*-mediated transformation has distinct advantages over direct transformation methods (Horsch *et al.*, 1985; de la Riva *et al.*, 1998). *Agrobacterium*-mediated transformation of plant tissue generally results in a low transgene copy number and minimal rearrangements compared to direct transformation methods, potentially leading to fewer problems with transgene co-suppression and instability (Flavell, 1994; Koncz *et al.*, 1994; Hansen *et al.*, 1997; Vaucheret *et al.*, 1998). Direct transformation frequently gives rise to mosaic plants, whereas *Agrobacterium*-mediated transformation, being a single-cell transformation system, avoids this problem (Enriquez-Obregón *et al.*, 1998). It is also believed that a higher transformation efficiency can be achieved by using *Agrobacterium*-mediated transformation (Pawlowski and Somers, 1996; Gelvin, 1998).

Carrot was first reported to be susceptible to infection by *Agrobacterium* wild-type strains and disarmed strains of *Agrobacterium tumefaciens* by De Cleene and De Ley (1974). Subsequently, from the late 1980s onwards, carrot tissues have been routinely transformed with *A. tumefaciens* strains (Hardegger and Sturm, 1998). The development of *Agrobacterium*-mediated transformation during the late 1980s and early 1990s, opened up the potential for the genetic manipulation of carrot phenotypes. Using *Agrobacterium*-mediated technology, the selectable marker gene neomycin phosphotransferase (*nptII*), conferring kanamycin resistance or the reporter gene β -glucuronidase (*GUS*), was introduced and expressed in carrot genomes (Scott and Draper, 1987; Liu *et al.*, 1992; Gilbert *et al.*, 1996; Hardegger and Sturm, 1998; Tang and Sturm, 1999). The use of antibiotic resistance genes and reporter genes allowed studies on the factors affecting transformation efficiency.

Hardegger and Sturm (1998) also made direct comparisons between two *A. tumefaciens* strains harboring different genetic backgrounds (LBA4404: Ach5 and GV3101: C58) and binary plasmids containing the *nptII* gene conferring resistance to kanamycin. Although in this instance hypocotyl segments of 7-day-old seedlings were inoculated with *A. tumefaciens*, the transformation efficiency was high for both strains following 2 days of co-cultivation (Hardegger and Sturm, 1998). Interestingly, inoculation of callus cells with LBA4404 was 20 times less effective than GV3101 (Hardegger and Sturm, 1998), indicating that the use of different *A. tumefaciens* strains results in variable transformation efficiencies dependent on the plant tissue type inoculated.

Many reports exist for the successful transformation of carrot tissues, however, only four state transformation efficiencies (Scott and Draper, 1987; Wurtele and Bulka, 1989; Liu *et al.*, 1992; Hardegger and Sturm, 1998), making it difficult to draw meaningful comparisons. Generally though, for *Agrobacterium*-mediated DNA transfer, selection of transformed cells with the antibiotic kanamycin and plant regeneration via somatic embryogenesis have been the preferred methods (Scott and Draper, 1987; Wurtele and Bulka, 1989; Balestrazzi *et al.*, 1991; Pawlicki *et al.*, 1992).

2.2 Transgenes and Transgenic Traits

One of the first reports of carrot transformation described the high-frequency transformation of carrot proembryogenic suspension culture with a Ti-plasmid vector carrying a kanamycin resistance gene (Scott and Draper, 1987). Transformed tissues expressed readily detectable levels of *nptII* as confirmed by Western blotting (Scott and Draper, 1987). In an effort to improve transfer-DNA (T-DNA)-mediated transformation, Liu *et al.* (1992) investigated the effect of multiple copies of *virG* genes contained in *A. tumefaciens* strains on the transient transformation of carrot tissue. Histochemical GUS assays determined that multiple copies of *virG* in *A. tumefaciens* greatly enhanced the transient transformation of carrot calli (Liu *et al.*, 1992). Hardegger and Sturm (1998) investigated transformation efficiency of different cell types and tissues of carrot using *A. tumefaciens*

harboring a binary vector that contained the *GUS* marker gene and the *nptII* gene. They found the highest T-DNA transfer rates were achieved by co-cultivating bacteria with hypocotyl segments and subsequent regeneration of somatic embryos via liquid culture (Hardegger and Sturm, 1998).

The ability to induce somatic embryos from carrot tissue and the application of embryos in transformation systems have recently led to the development of transgenic carrot genotypes with resistance to *Erysiphe heraclei* and *Alternaria dauci* (Takaichi and Oeda, 2000), and *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Chen and Punja, 2002; Punja and Chen, 2004). Herbicide tolerance (Chen and Punja, 2002) and resistance to viral infections (Zheng *et al.*, 2002) have also been developed in carrot genotypes using the somatic embryogenesis/*Agrobacterium*-mediated transformation partnership. *Agrobacterium* was also employed to incorporate an edible vaccine for the measles virus (Marquet-Blouin *et al.*, 2003) and to increase calcium content (Park *et al.*, 2004). For this, a range of explant tissue types, *Agrobacterium* strains, and antibiotic selection regimes were used.

Although there are many reports of carrot transformation that provide a sound basis for future transformation experiments, they are generally genotype specific. The development of optimal conditions for co-cultivation and regeneration as well as the use of an optimal type and concentration of a selectable marker is required for each carrot genotype.

2.3 Whole Plant Regeneration

Somatic embryogenesis was first identified in cultures of *D. carota* tissue derived from the storage tap root by Steward *et al.* (1958) and Reinert (1958, 1959). Since 1958, a substantial number of investigations involving somatic embryogenesis in carrot cultures of various tissue types in both wild and cultivated species have been undertaken (Nomura and Komamine, 1986; Wake *et al.*, 1995). Subsequently, somatic embryogenesis has become the 'model system' for carrot regeneration because embryogenic cultures can be easily obtained and regenerated (Komamine *et al.*, 1992).

For the rapid production of large numbers of plants, where spatial constraints exist, the greatest potential of clonal multiplication is through

somatic embryogenesis, with a single isolated cell possessing the ability to produce an embryo then a complete plant (Taji *et al.*, 1997). Minocha and Minocha (1995) reported the potential for multiplying millions of superior plants using this method of asexual propagation for both horticultural and forestry crops. Combined with genetic engineering, tissue culture through somatic embryogenesis provides an efficient means of producing a large number of elite or transgenic plants (Osuga and Komamine, 1994).

Carrot tissue is readily manipulated *in vitro*, thus has been used for decades as a 'model system' for regeneration via somatic embryos (Quiros, 2001). Regeneration from carrot callus cultures, via somatic embryogenesis, has been achieved from seedling-derived hypocotyl tissues (Kamada and Harada, 1978; Takaichi and Oeda, 2000; Jiménez and Bangerth, 2001; Chen and Punja, 2002), mature petiole tissue (Drew, 1979), and root tissue (Smith and Street, 1974; Kitto and Janick, 1985). Somatic embryogenesis can be divided into an auxin-requiring (embryo induction) and an auxin-inhibited (embryo expression) stage for carrot and most other plants (Nissen and Minocha, 1993; Jiménez and Bangerth, 2001) and a basic three-step formula has been found to be successful for the induction of somatic embryos and regeneration of whole carrot plants from callus. Firstly, callus is induced from explant material incubated in the dark on Murashige and Skoog (MS) (1962) nutrient medium supplemented with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Figure 1), most often at 5 μ M (Kamada and Harada, 1978; Kitto and Janick, 1985; Jiménez and Bangerth, 2001; Chen and Punja, 2002). A low level of kinetin in combination with 2,4-D was also shown to successfully induce callus in root, petiole, and hypocotyl explants (Smith and Street, 1974; Drew, 1979; Kitto and Janick, 1985; Chen and Punja, 2002).

The second step is embryo maturation and proliferation following excision of callus from explant material, also an auxin-requiring stage (Figure 2). This was achieved from root and hypocotyl explants on an auxin-rich medium (Fujimura and Komamine, 1975, 1979; Nomura and Komamine, 1985; Roustan *et al.*, 1989; Nissen and Minocha, 1993; Nissen, 1994; Munksgaard *et al.*, 1995; Jiménez and Bangerth, 2001; Ceccarelli *et al.*, 2002; Chen and Punja, 2002),



Figure 1 Four-week-old petiole explants with varying amounts of callus initiation

and on a medium containing both auxin and cytokinin in similar concentrations (Smith and Street, 1974; Drew, 1979; Kitto and Janick, 1985). Although suspension culture is often used for proliferation of callus cultures (Smith and Street, 1974; Fujimura and Komamine, 1975; Kamada and Harada, 1978; Drew, 1979; Fujimura and Komamine, 1979; Kitto and Janick, 1985; Nomura and Komamine, 1985; Roustan *et al.*, 1989; Nissen and Minocha, 1993; Nissen, 1994; Munksgaard *et al.*, 1995; Ceccarelli *et al.*, 2002; Chen and Punja, 2002), other published methods include the use of solid medium for callus proliferation and maintenance (Smith and Street, 1974; Kitto and Janick, 1985; Jiménez and Bangerth, 2001).

The third step is germination or regeneration of embryos and subsequent growth into plantlets. This step has been most commonly achieved for carrot via the removal of auxin from the nutrient medium (Smith and Street, 1974; Drew, 1979; Kitto and Janick, 1985; Roustan *et al.*, 1989; Nissen, 1994; Jiménez and Bangerth, 2001;

Takaichi and Oeda, 2000; Chen and Punja, 2002), although the inclusion of 0.5 μM zeatin in the nutrient medium has resulted in successful embryo regeneration (Fujimura and Komamine, 1975, 1979; Fujimura *et al.*, 1980; Nomura and Komamine, 1985). Kamada and Harada (1978) reported that 5 μM 2,4-D was successful for regeneration of plantlets from a callus intermediary via somatic embryos, which contradicted the commonly held belief that auxins inhibited somatic embryogenesis (Nissen and Minocha, 1993; Jiménez and Bangerth, 2001). Germination of somatic embryos has been achieved on both MS (Murashige and Skoog, 1962) and Lin and Staba (1961) nutrient media, while regeneration was achieved using both suspension and solid medium culture.

3. FUTURE ROAD MAP

3.1 Risks and Concerns

Before commercial release, the potential risks of genetically modified carrots must be evaluated using an integrated multidisciplinary approach incorporating molecular biology, toxicology, nutrition, and genetics (Kuiper *et al.*, 2001). Safety considerations include: the effects of any production processes applied to the novel food; the effect of the genetic modification on the properties of the host organism; the genetic stability of the modified organism used as the source of the novel food; the specificity of expression of the novel genetic material, and the ability of the modified organism to survive in and colonize the human gut microflora. The anticipated intake and extent of the use of the novel food; nutritional information, allergenicity and toxicological information, and

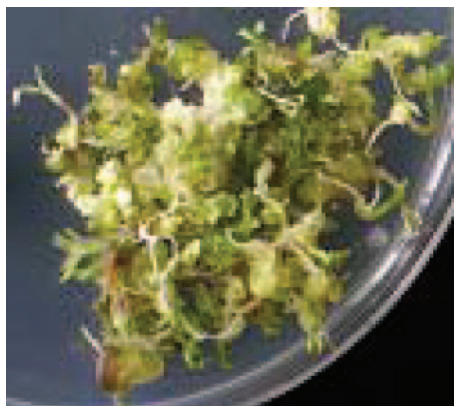


Figure 2 Somatic regeneration of carrot cv. Crusader

ecological impact must also be considered (Kuiper *et al.*, 2001; GMO Compass, 2006). Any potential risks of genetically modified carrots should be compared with the associated environmental benefits. It has been proposed that the increase in adoption of insect-resistant and herbicide-resistant corn, cotton, and soybean crops resulted in 8.2 million fewer pounds of active pesticide used in 1998 compared to 1997 (USDA, 2000).

Novel technologies are continually being developed which are addressing potential risks associated with genetically modified food. Potential risks such, as horizontal gene flow associated with the use of antibiotic selectable markers, are subject to controversy (Kuiper *et al.*, 2001). Recently, a gene linked to kanamycin resistance in *Arabidopsis* was discovered and was proven as a robust selectable marker for plant transformation (Rommens, 2006). The plant origin of this resistance gene, opposed to the bacterial origin of the *nptII* kanamycin resistance gene, has advantages from a public perception point of view (Rommens, 2006). Public survey has shown that consumers would be more likely to eat a vegetable that contains an extra plant gene rather than one that contains a gene from an alternate source (Lusk and Sullivan, 2002; Rozin, 2005). Alternatively, gene excision systems such as cre/lox are providing a means of removing superfluous genes following the introduction of expression cassettes into plant genomes (Wang *et al.*, 2005).

3.2 Public and Industry Perspectives

The success in creating transgenic carrot lines has created new considerations for further assessment and future commercial release. Although high-value crops such as maize, cotton, and canola form the majority of genetically modified food and feed authorized worldwide, horticultural crops are also being assessed and grown commercially (FDA, 2006; GMO Compass, 2006; OGTR, 2006). In the European Union, which has 84 genetically modified crops registered at present, potato varieties with altered starch composition and sugar beet with herbicide tolerance are currently being assessed (GMO Compass, 2006). The United States currently has 98 bioengineered foods registered, including insect-resistant tomatoes, male-sterile radicchio, potatoes resistant to

Colorado potato beetle, cantaloupe with delayed fruit ripening and virus-resistant potato, squash, and papaya (FDA, 2006).

Australia currently has 65 genetically modified organisms or genetically modified products under examination or approved for intentional release by the national biotechnology governing body, the Office of Gene Technology Regulator (OGTR, 2006). Of these only two, namely insect-resistant cotton and color-modified carnation are currently being grown commercially (OGTR, 2006). In 2003, national regulatory approval was given for the release of herbicide-resistant canola (OGTR, 2006), however, a number of Australian state governments imposed moratoriums of commercial planting of this crop (OGTR, 2006). Australia, together with Europe, has been slow to approve commercial planting of transgenic crops compared to both Canada and the United States (Linacre *et al.*, 2006). Australia has planted only 0.2 million hectares of transgenic crops, principally insect-resistant cotton (Linacre *et al.*, 2006), whereas Canada and the United States have 4.4 and 42.8 million hectares, respectively of transgenic crops under cultivation (James, 2003). The regulatory framework currently employed to approve commercial releases in Australia is believed to be one reason for the slow approval rate of transgenic crops (Linacre *et al.*, 2006), however, public perception concerning the risks associated with genetically modified food still remains a major hurdle for further release of transgenic crops (Biotechnology Public Awareness Survey, 2003).

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Cucurbits

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1. INTRODUCTION

1.1 *Cucurbita* Species

1.1.1 History, origin, and distribution

There are five cultivated species of the genus *Cucurbita*. These include *C. pepo*, *C. moschata*, *C. maxima*, *C. argyrosperma*, and *C. ficifolia*. Because most of the wild species of *Cucurbita* are found in Mexico in an area extending south of Mexico City to the border with Guatemala, this area is considered as the center of origin or distribution of the genus (Whitaker and Robinson, 1986; Robinson and Decker-Walters, 1997).

The most extensively cultivated species is *C. pepo* and, therefore, much more is known about its origins and areas of domestication than the other cultivated species. *C. pepo* is indigenous to North America and consists of numerous subspecies and varieties (Andres, 1987; Wilson, 1990). This crop was probably independently domesticated in Mexico and the eastern United States between 10 000 and 30 000 years ago (Decker, 1988; Smith *et al.*, 1992). The current view of the infraspecific taxonomy of the species has been developed by Decker-Walters and coworkers (Decker, 1988;

Decker-Walters *et al.*, 1993; Decker-Walters *et al.*, 2002): Pumpkins, marrows, and some of the ornamental gourds belong to *C. pepo* ssp. *pepo*, while cultivated crookneck, scallop, and acorn squashes as well as most of the ornamental gourds belong to *C. pepo* ssp. *ovifera* var. *ovifera*. Free-living (i.e., noncultivated) forms are placed in other taxa. The free-living populations in Texas belong to *C. pepo* ssp. *ovifera* var. *texana*; free-living populations occurring in the Mississippi Valley and the Ozark Plateau belong to *C. pepo* ssp. *ovifera* var. *ozarkana*; free-living populations occurring in northeastern Mexico belong to *C. pepo* ssp. *fraterna*.

While cultivated *C. pepo* can be found throughout the world wherever the climate is suitable, and escaped populations may become established, free-living populations naturally occur in North America from northeastern Mexico and in the United States from Texas to Alabama, through the Mississippi Valley to Illinois (Smith *et al.*, 1992; Cowan and Smith, 1993). Some of these free-living populations can occur as weeds in soybean and cotton fields especially in Arkansas, Louisiana, and Mississippi (Oliver *et al.*, 1983; Boyette *et al.*, 1984; Smith *et al.*, 1992; Bryson and Byrd, 1998). There is anecdotal evidence that the advent of genetically engineered herbicide tolerant

soybean and cotton has reduced the weed problem posed by these weedy populations of *C. pepo*.

Numerous studies have documented the present and historical ability of free-living and cultivated populations to exchange genes (Kirkpatrick and Wilson, 1988; Smith *et al.*, 1992; Decker-Walters *et al.*, 1993, 2002). Other studies have shown that some free-living populations may have been the result of individuals that have escaped from cultivation, became established, and subsequently have crossed with other nearby cultivated or free-living populations (Decker and Wilson, 1987; Wilson, 1990; Asch and Asch, 1992; Cowan and Smith, 1993; Decker-Walters *et al.*, 1993).

1.1.2 Botany

Cucurbita is considered to be one of the most morphologically variable genera in the plant kingdom (Robinson and Decker-Walters, 1999). *Cucurbita* cultivars are categorized as summer squash or winter squash. Summer squash are eaten immature when tender and seeds are small and soft. Winter squashes are generally eaten when rind and seeds are fully mature. Summer squash cultivars are *C. pepo* while winter squash cultivars can be *C. pepo*, *C. maxima*, *C. moschata*, or *C. argyrosperma*.

Variation in fruit shape within *C. pepo* is the basis for 10 horticultural classifications of this species (Paris, 1986, 1996). These include eight edible-fruited cultivar groups designated pumpkin, vegetable marrow, cocozelle, zucchini, acorn, scallop, crookneck, and straightneck and two nonedible-fruited ornamental cultivar groups designated orange gourd and ovifera gourd. Pumpkin cultivars produce round, spherical, globular, oblate, ovate, obovate fruit shapes; smooth or warted, with or without longitudinal grooves, ribs, or furrows. Fruit rind color of this group varies from orange to green; solid in color to those with stripes. Vegetable marrow has short, tapered cylindrical fruit with a length-to-width ratio of 1.5–3.0. Rind colors include yellow, orange, light to dark green, solid or striped. Fruit can be smooth to warty. Cocozelle has cylindrical, long fruit with length-to-width ratios of greater than 3.5. Rind colors range from light to dark green, solid or striped with some ribbing. Zucchini

is uniformly cylindrical, or nearly so, with a length-to-width ratio of 3.5–5.0. It is considered to be the most popular *C. pepo* cultivar. The most common rind color is green but brilliant yellow cultivars are available since the introduction of the precocious yellow gene. Acorn is turbinate in shape with deep furrows, broad at the peduncle end and convex at the styler end. Rind colors include white, orange, yellow, green, tan that are solid, striped, or blotched. Scallop fruit, also known as pattypan, is flattened disk shaped with scalloped margins. Rind colors include white, yellow, orange, and green, solid or variegated. Crookneck cultivars produce fruits that are elongated with slight to very curved necks and broad blossom ends. Straightneck cultivars, derived from crooknecks, produce fruits with shorter and straighter necks. The rind color of crooknecks and straightnecks are predominantly yellow. The two ornamental gourd groups include various shapes and colors, mostly bitter and inedible. The botanical and horticultural classifications of *C. pepo* have been integrated. *C. pepo* L. subsp. *pepo* contain the pumpkin, vegetable marrow, cocozelle, zucchini, and orange gourd groups, while *C. pepo* L. contain the acorn, crookneck, scallop, straightneck, and ovifera gourd groups.

There is considerable morphological diversity in *C. moschata* with regard to its seeds and fruit color, shape, and thickness of rind (Bates *et al.*, 1990; Robinson and Decker-Walters, 1999). Horticultural groupings based on market-type cultivars include neck, cheese, tropical, and japonica. The neck group produces fruits that are curved or straight, have an enlarged blossom end, and the rind is usually buff colored. Cheese-type cultivars produce fruits that are oblate in shape and buff colored. Fruits of the tropical group are round or irregularly shaped with buff, yellow, green, spotted or patchy, solid or bicolored rinds. The japonica group is generally bluish-black in rind color, warty, and wrinkled.

C. maxima exhibits the greatest diversity of fruit types after *C. pepo* (Bates *et al.*, 1990; Robinson and Decker-Walters, 1999). Several horticultural groups based on market type describe this species based on fruit shape, color, or size. These include Australian blue, banana, buttercup, Hubbard, mammoth, and turban. Australian blue are blue-rinded squash produced mainly in Australia. Fruit size and shape vary. The banana group produces

fruits that are elongated, tapered at the stem, and blossom ends. Rind color varies from orange and light to dark green. The buttercup group has small drum-shaped fruit with a “button” or protuberance at the blossom end. Rind colors include deep orange to dark green. The Hubbard group produces oval fruit, tapering to curved necks at both ends. Fruits are smooth or warty and range in color from orange, green, and gray. The mammoth group produces mainly orange fruits that are 100 pounds or more and grown for exhibition at contests. Cultivars in the turban group are similar to buttercup but the protuberance at the blossom end is more prominent. The rind of this group is very colorful.

Cultivars of *C. argyrosperma* and *C. ficifolia* are produced primarily for their seed, which provides a considerable amount of oil and protein. Both these species are produced and consumed in Central America.

1.1.3 Economic importance

Cucurbita species (pumpkin, squash, and gourds) are grown throughout the temperate, subtropical, and tropical regions of the world. They collectively rank among the top 10 vegetable crops produced worldwide with nearly 1.6 million ha devoted to production (FAO, 2006). In 2005, approximately 20 million metric tons of pumpkin, squash, and gourds were produced; an increase of almost 15% compared to 2000. China and India lead the world in production providing close to 30% and 18% of the world output, respectively. Other major producers include Ukraine, the United States, Egypt, Mexico, Cuba, Iran, Italy, and South Africa.

Cucurbita spp. are most often grown for human consumption of their fruits (Paris, 1989; Bates *et al.*, 1990; Robinson and Decker-Walters, 1999). Summer squashes are generally eaten immature when fruits are relatively small and tender, requiring no cooking for use in salads, or they can be boiled, fried, or microwaved. Winter squashes are eaten mature and can be baked, boiled, or microwaved. Both summer and winter squashes are low in saturated fat, cholesterol, and sodium, and are a good source of dietary fiber, vitamins, and minerals (NutritionData, 2006). Fruits with yellow or orange flesh generally

have high concentrations of carotenes, some of which are the precursors of vitamin A (e.g., β -carotene) and play a significant role in human nutrition, especially in tropical countries where their consumption is high. *C. moschata* and *C. maxima* cultivars producing fruits with the strongest flavor, highest soluble solids, and deepest flesh color are preferred for canned and frozen winter squash. The commercially canned pumpkin pie mix is made up of *C. pepo*, *C. maxima*, or *C. moschata* cultivars. Deep orange, carotene-rich fruits of *C. moschata* and *C. maxima* are processed for use in the baby food industry. The stringy flesh of *C. ficifolia* can be boiled to create “angel’s hair” candy. In the United States, various sized and shaped pumpkins are traditionally carved for Halloween festivities in the autumn.

In addition to the fruit, other parts of *Cucurbita* plant are edible (Bates *et al.*, 1990; Robinson and Decker-Walters, 1999). Seeds can be eaten whole, roasted, or toasted, and are a good source of protein, vitamins, and minerals. *Cucurbita* seeds can also be ground into a paste or pressed for vegetable oil. The oils of squash generally contain 40–60% linoleic acid, 20–40% oleic acid, 10–20% palmitic acid, and 0–15% stearic acid. The shoots, leaves, and tendrils of *Cucurbita* plants can be eaten as greens. Recipes using *Cucurbita* staminate and pistillate flowers, harvested on the day of or day after anthesis, are used in many countries.

1.1.4 Breeding achievements

Breeding of *Cucurbita* in the past has played a key role in the variety and productivity of cultivars available to growers and consumers today (Bates *et al.*, 1990; Robinson and Decker-Walters, 1999). Several examples include disease resistance (based on inter- and intraspecific hybridization), bush (short internode) plant habit, precocious yellow fruit pigmentation, and the development and introduction of specific varieties based on growers’ and consumers’ preferences. Genetic engineering has been used in this species to introduce commercial virus-resistant varieties (Quemada and Tricoli, 1992, 1995; Tricoli *et al.*, 1995, 2002).

Interspecific hybridization to transfer disease resistance alleles from wild to cultivated species of *Cucurbita* has proven beneficial. *Cucurbita ecuadorensis* is resistant to a number of viruses,

including zucchini yellow mosaic virus (ZYMV), the watermelon strain of papaya ringspot virus (PRSV-W), and watermelon mosaic virus (WMV) (Cutler and Whitaker, 1969). Multiple virus-resistant *C. maxima* cultivars derived from *C. ecuadorensis* have been developed at the Cornell University, USA, since 1985. “Redlands Trailblazer” and “Dulong QHI”, winter squash resistant to ZYMV, WMV, and PRSV, were bred in Australia from the same interspecific cross (Herrington *et al.*, 2001). Certain interspecific *Cucurbita* crosses can be difficult to obtain (Whitaker and Robinson, 1986). These difficulties can be overcome by embryo rescue methods or by use of a bridging species; e.g., crossing *C. pepo* to the hybrid of *Cucurbita okeechobeensis* with *C. moschata*. By using the hybrid of *C. okeechobeensis* with *C. moschata*, resistance to cucumber mosaic virus (CMV) and powdery mildew has been transferred from *C. okeechobeensis* to *C. pepo* and *C. moschata*. Intraspecific hybridization has also proven to be beneficial especially with regard to virus resistance. High levels of resistance to ZYMV, PRSV-W, and WMV were identified in a landrace of *C. moschata* “Nigerian Local” (Munger and Provvidenti, 1987). This source of virus resistance has been successfully introgressed into *C. moschata* and *C. pepo* cultivars.

Bush (short internode) plant habit, conferred by the allele *Bu*, in cultivars of summer and winter squash has maximized growing space and ease of harvest. In *C. pepo*, the *Bu* allele is incompletely dominant during the latter stage of development (Shifriss, 1947). In *C. maxima*, the *Bu* allele is completely dominant during early growth but loses this effect during the latter stage of growth (Grebenscikov, 1958). Denna and Munger (1963) characterized the bush habit in both *C. pepo* and *C. maxima*, and found it to be allelic in both species. Many cultivars of *C. pepo*, *C. moschata*, and *C. maxima* with a bush plant habit are available to growers.

Precocious yellow fruit pigmentation from a bicolored gourd (Shifriss, 1981), expressed by the allele *B*, has been bred into crookneck and straightneck squash and zucchini squash (*C. pepo*). Introgression of this gene, along with several modifiers (*Ep-1*, *Ep-2*, and *Ses-B*), results in fruit with a very deep yellow golden color and high vitamin A content. Another benefit of the *B* allele is in its ability to mask the unsightly green ringed

fruit symptoms of WMV. Fruits from WMV-infected plants can still be marketed compared to other nonprecocious yellow summer squash.

In addition to the traditional plant breeding methods of transferring virus-resistance alleles from wild to cultivated species of *Cucurbita*, scientists have genetically engineered virus resistance into squash (Quemada and Tricoli, 1992, 1995; Tricoli *et al.*, 1995, 2002). The first commercially available transgenic cucurbit was “Freedom II” (Asgrow Seed Co.), a yellow crookneck summer squash (*C. pepo*). *Agrobacterium tumefaciens* was used to transfer the coat protein genes of WMV and ZYMV into this crookneck squash. In 2004, 10% of the squash acreage grown in the United States was biotechnology derived, providing a little over 19 million dollars in revenue (Sankula *et al.*, 2005).

Breeding achievements of *Cucurbita* include the introduction of specific varieties or cultivars based on grower and consumer preferences (Bates *et al.*, 1990; Robinson and Decker-Walters, 1999). The following are just a few examples. Butternut-type (*C. moschata*) squash with consistent fruit size and shape, buff-colored rind, deep orange flesh, and disease tolerance is now grown and consumed throughout the United States. This winter squash was originally selected from the heirloom cultivar “Canada Crookneck” for bell-shaped fruit and introduced by the Breck Seed Company in 1936. Another butternut-type squash, “Waltham Butternut” (*C. moschata*), has been very popular since its introduction in 1970. This butternut was obtained from crossing “New Hampshire Butternut” with an African plant introduction producing fruit of greater uniformity, shape and color, and better flavor. The acorn squash “Table Queen” (*C. pepo*) was introduced in 1913 by Iowa Seed Company, and is believed to have been selected from a Native American landrace. This vine winter squash produces a smooth dark green ribbed rind with deep orange sweet flesh. Acorn squash cultivars with similar fruit shape and color, but with bush plant habit, have since been bred (e.g., Table Ace). “Delicata”, a *C. pepo* within the acorn group introduced by Peter Henderson Company in 1894, is still grown in both home gardens and commercially. Its cylindrical fruits are cream colored with green strips and their flesh is fine grained, sweet, and starchy. “Vegetable Spaghetti” is a member of the

vegetable marrow group of *C. pepo*, originating in northern Manchuria and first commercialized by Sakata Seed Company in 1934, and then in the United States in 1936. This yellow oval fruit when cooked produces thin fibrous strands resembling spaghetti. Yellow crookneck and straightneck summer squash (*C. pepo*) are very popular in the United States. “Early Prolific Straightneck” was introduced by the Ferry Morse Seed Company in 1938 and is said to have been derived from “Summer Crookneck”, which is listed in seed catalogs as early as 1807. Zucchini (*C. pepo*) is now considered the most consumed squash in the world. First introduced in Italy around 1901, it found its way to the United States in 1918. Since then, it has been bred for fruit uniformity, quality, and disease resistance. With the identification of the precocious yellow fruit pigmentation gene, *B*, working in concert with several modifying genes, a yellow-colored zucchini was bred and appeared on the market as “Golden Zucchini” in 1973. Other golden-type zucchini are now available to home gardeners and commercial growers.

1.1.5 Current breeding efforts

The goal of *Cucurbita* breeding programs is to release new cultivars having elite combinations of many desirable horticultural characteristics (Robinson and Decker-Walters, 1999). These desirable traits include earliness, increased productivity or yield, environmental adaptation, resistance or tolerance to diseases and pests, small, upright, open plant habit, shortened internode length for reduced vining or for bush plant habit, spineless foliage, consistent fruit shape and color, high fruit gloss, a small blossom scar, peduncles that separate easily from fruit or easily fracture away from fruit, and enhanced nutritional value such as β -carotene.

Many of the desirable characteristics of *Cucurbita* have complex inheritance and are influenced by a number of genes and their interactions (Robinson and Decker-Walters, 1999). Breeding to select for traits of importance requires population development, appropriate experimentation, and statistical analyses to understand the genetics governing these traits. Mass, recurrent, and pedigree selection as well as backcross breeding methods have been employed successfully to

introgress desirable traits into summer and winter squash and continue to be used in breeding programs (Munger *et al.*, 1993).

The development of hybrid cultivars instead of open-pollinated cultivars has been a major change in breeding summer and winter squash. This change has resulted in cultivars that can be harvested earlier and exhibit uniformity in fruit shape, as well as increased yield (Robinson and Decker-Walters, 1999). Current trends in breeding also include the use of wide crosses within or among species to identify beneficial genes. Wild *Cucurbita* species and germplasm in gene banks continue to be evaluated as important sources of resistance to pathogens and pests, variation in fruit and plant characteristics, and nutrition.

1.1.6 Genetics

Cucurbita species have 20 pairs of chromosomes ($2n = 2x = 40$), more than any other species in the *Cucurbitaceae*. Many *Cucurbita* genes and alleles have been identified and a list of genes has recently been published (Paris and Brown, 2005). A limited number of linkage maps exist for *Cucurbita* species. On the basis of an F_2 population derived from *C. maxima* and *C. ecuadorensis*, Weeden and Robinson (1986) were the first to create a linkage map containing 11 isozyme loci placed in five linkage groups. In 1995, Lee developed a map based on random amplified polymorphic DNA (RAPD) markers of a *C. pepo* \times *C. moschata* F_2 population consisting of 28 markers placed in five linkage groups. No morphological traits were included. Recently, a BC_1 population derived from *C. pepo* and *C. moschata* was used to develop a map placing five morphological traits and 148 RAPD markers in 28 linkage groups (Brown and Myers, 2002). This map covered 1954 cM, which is estimated to be 75% of the *Cucurbita* genome. Compared to other cucurbit genera such as *Citrullus* (watermelon) and *Cucumis* (cucumber and muskmelon), additional genetic linkage maps of *Cucurbita* species are needed to facilitate breeding efforts and increase our knowledge of *Cucurbita* genetics. Markers linked to genes that control fruit quality or confer resistance to diseases and pests are still required. A combination of traditional breeding methods and biotechnology

will likely play an important role in the genetic improvement of summer and winter squash.

1.2 *Cucumis* Species

1.2.1 History, origin, and distribution

1.2.1.1 Melon

Melon (*Cucumis melo* L.; $2n = 2x = 24$) is a member of the economically important family *Cucurbitaceae*. Molecular markers have been used in melon for genetic diversity analysis, elucidation of taxonomic relationships, determination of sites of origin and domestication, assessment of market class relationships in different melon backgrounds, and the definition of melon taxonomic groups and market classes (Stepansky *et al.*, 1999; Akashi *et al.*, 2002; Monforte *et al.*, 2003). Although the center of origin for *Cucumis* species is likely Africa for the wild species, initial sites of domestication for melon are likely in the Middle East (Dane *et al.*, 1980). During its domestication, melon received wide use and was distributed from points of origin along well-defined trade routes (e.g., the Silk Road and international shipping routes) (McCreight *et al.*, 1993; Robinson and Decker-Walters, 1997). Wild and feral *C. melo* populations can still be found in Africa, America, Asia, Australia, Europe, and the Pacific Islands (Jeffrey, 1980; Kirkbride, 1993).

1.2.1.2 Cucumber

Cucumber (*Cucumis sativus* var. *sativus* L.; $2n = 2x = 14$) may have originated in Africa, China, India, or in the Near East with domestication occurring later throughout Europe (Tapley *et al.*, 1937; Harlan, 1975). It was domesticated about 3000 years ago, and is indigenous to India (primary center of diversity; Jeffrey, 1980). Genetic relationships among accessions with regard to geographical origin are consistent with the generally accepted historic dispersal patterns of cucumber (Horejsi and Staub, 1999). Being initially domesticated in either India or Africa, cucumber was carried to the Middle East, then to the Mediterranean, and finally to Europe (Tatlioglu, 1993). Cucumber was brought to Greece and Italy by the Romans (2nd century

BC; Mesopotamia), and it appeared in France in the 9th century, in England in the 14th century, and in North America by the mid-16th century. The Spanish brought cucumber to Haiti in 1494, and cucumber was reported in Montreal, Canada (by Cartier), in Florida, US (by Desoto), and in Virginia, US (by Amidas and Barlow) in 1535, 1539, and 1584, respectively (Robinson and Decker-Walters, 1997; Lebeda *et al.*, 2006).

1.2.2 Botanical description: taxonomy, habit, genome size, and cytological features

1.2.2.1 Melon

In 1753, Linnaeus coined the genus name, *Cucumis*, and classified five cultivated melons as independent species (Stepansky *et al.*, 1999). Subsequently, Naudin (1859) made the first detailed classification of *C. melo*, and divided it into nine tribes of cultivated melons and one tribe consisting of wild forms. More recently, *C. melo* classifications were performed mainly by Russian researchers at the Vavilov Institute in the early 20th century (Stepansky *et al.*, 1999; Pitrat *et al.*, 2000).

The extensive morphological variation found in *C. melo* has led botanists to propose numerous infraspecific taxonomic classifications during the last 150 years (Stepansky *et al.*, 1999; Pitrat *et al.*, 2000). By modern consensus, melon belongs to the *Cucurbitaceae*, which is subdivided into two subfamilies, Zanonioideae and the Cucurbitoideae (Jeffrey, 1980; Kirkbride, 1993; Eurosid I clade of the Rosids). The latter subfamily is further partitioned into eight tribes of which the Melothrieae includes the genus *Cucumis*. *Cucumis* is partitioned into two subgenera designated as *Cucumis* ($2n = 2x = 14$ and 24) and *Melo* ($2n = 2x = 24$) that contain five cross-sterile species groups (Jeffrey, 1980). *Melo* ($x = n = 12$), the second subgenus, is divided into two sections (Melo and Aculeatosi). Section Melo contains four series (Humifructuosi, Melo, Hirsuti, Metulifrei, Angurioidei, and Myriocarpi) (Kirkbride, 1993). Series Melo houses melon and series Metulifrei contains metuliferus ("horned cucumber").

Melon consists of a diverse array of morphotypes differing in leaf, vine, plant, and fruit characters. Wild and cultivated melon in the

series Melo is subdivided into two subspecies that is based on assessments of Jeffrey (1980) according to the flower ovary hairiness. Typically, ssp. *melo* (cultivated) has lanate ovaries while ssp. *agrestis* (wild) has sericeous ovaries. According to ovary morphology, Munger and Robinson (1991) subdivided *C. melo* L. into ssp. *agrestis* (Naud.) Pangalo and ssp. *melo*. Based on their classification, ssp. *melo* is currently further subdivided into six cultivar groups including: (1) Cantalupensis—cantaloupe or muskmelon; (2) Inodorus—winter melons, honeydew, casaba; (3) Flexuosus—snake melon; (4) Conomon—pickling melon; (5) Chito and Dudaim—mango melon, vine peach, and other similar names for the former; pomegranate melon, Queen Anne's Pocket melon for the latter; and (6) Momordica—"phut" or snap melon.

Group Cantalupensis (e.g., Earl's, House, Galia, Charentais, and Ogen), Group Inodorus (e.g., Honeydew and Casaba), and Group Conomon (e.g., Oriental) have differing fruit morphologies and are valued worldwide for their unique flavor, aroma, and variable shelf life. While groups Cantalupensis and Inodorus are of commercial importance in the United States and Europe as well as in Mediterranean and Asian countries; group Conomon types have their origin and are widely grown in Asia (McCreight *et al.*, 1993; Robinson and Decker-Walters, 1997; Lebeda *et al.*, 2006).

1.2.2.2 *Cucumber*

Cucumber belongs to the subgenus designated as *Cucumis* ($2n = 2x = 14$ and 24 ; Jeffrey, 1980). The subgenus *Cucumis* comprises three or four Sino-Himalayan species, including *C. sativus* ($2n = 2x = 14$) and *Cucumis hystris* Chakr. ($2n = 2x = 24$). *C. sativus* consists of several botanical varieties including var. *sativus*, the cultivated cucumber (hereafter referred to as *C. s.* var. *sativus*), and the wild, free-living var. *hardwickii* (R.) Alef. (hereafter referred to as *C. s.* var. *hardwickii*) (Kirkbride, 1993). Wild African *Cucumis* species (mostly $2n = 2x = 24$) are cross-incompatible with cucumber and melon, which are themselves cross-incompatible (Kroon *et al.*, 1979). Likewise, the wild, free-living *C. hystris* is only sparingly fertile with cucumber (Chen *et al.*, 1995, 1997a, b). This

species is found only in the Yunnan Province of Southern China, and has unique genetic attributes that make its taxonomic determination complex.

1.2.3 Economic importance

1.2.3.1 *Melon*

Melon is the most economically important member of the *Cucurbitaceae*. Worldwide, more than 18 million metric tons of melons were produced in 1999, with China, Turkey, Iran, the United States, and Spain being the major producers (FAO, 1999). In the United States in 2003, over 1 million tons of Western Shipping and Eastern Market type melons, having a market value of almost 400 million US dollars, were produced (NASS, 2003).

The horticultural groups Cantalupensis (i.e., cantaloupe) and Inodorus (i.e., honeydew) are of particular commercial importance in the United States, Europe, and Asia (McCreight *et al.*, 1993). Although historic production records for some major growing areas are not available, consumption of cantaloupe has increased steadily in the United States, reaching approximately 5.5 kg per person in 2001. In contrast, demand for honeydew melon in the United States has fluctuated from 0.5 to 1 kg per person between the late 1970s and 2001.

In the United States, the Western Shipping and Eastern Market cantaloupe types of the Cantalupensis group are commercially the most important, being primarily grown in Arizona, California, Texas, Georgia, and Indiana (NASS, 2003). In 2003, US farmers grew more than 36 450 ha of cantaloupes for a total production in excess of 1 million tons and having a market value of almost 400 million US dollars. Cantaloupe yield in the United States has increased from 3 t ha⁻¹ in 1992 to 4.6 t ha⁻¹ in 2003.

1.2.3.2 *Cucumber*

After tomato and watermelon, cucumber and melon are cultivated more broadly than any other vegetable species. Approximately 2 427 436 ha of cucumber were harvested in 2004 producing 40 860 985 t under field and greenhouse culture

(FAO, 2004). Production of cucumber is the second largest of all cucurbits, with China (553 Mcwt or 25 084 080 t), Iran (30 Mcwt or 1 360 800 t), Turkey (38 Mcwt or 1 723 680 t), and the United States (23 Mcwt or 1 043 280 t) representing 71% of the world production in 2003.

Cucumber usage in the United States has climbed steadily since the 1960s, reaching 4.4 kg per capita in the 1990s (NASS, 1998). Average annual farm value was \$361 million (~2.4 billion kg) during 1997–99. Production of US fresh market cucumber (about 60% of all cucumber production) is highest in Florida, Georgia, California, and Michigan. In 1997, approximately 26 000 ha of fresh market cucumbers were harvested having a total value of \$185 194 000. These production levels have been maintained, and Michigan, Florida, North Carolina, Texas, Ohio, California, and Wisconsin continue to lead the production of pickling (processing) cucumber (33% used in fast foods).

1.2.4 Traditional breeding: breeding objectives; tools and strategies; and achievements

1.2.4.1 Melon

Melon quality has been improved specific to market demands and culinary practices depending on market class. Most of the yield improvement in melon (as with cucumber) can be credited to cultural practices, breeding for relatively simple traits such as resistance to diseases and pests, and the use of hybrids created from sparingly few elite lines (McCreight *et al.*, 1993; Robinson and Decker-Walters, 1997; Lebeda *et al.*, 2006). Continued yield and quality increases in melon will depend on the preservation, availability, and use of genetic variability (e.g., exotic germplasm), and breeding for quantitative traits that are directly related to yield.

Common breeding methods for population improvement of quantitative traits (e.g., fruit yield and quality) involve recurrent selection in diverse populations where gain from selection is made through mass or more structured family (half- or full-sib) selection. For introgression of qualitatively inherited traits (e.g., disease resistance, sex expression) simple backcross

and/or pedigree selection are often employed. For instance, studies assessing the superiority of hybrids and sex expression (important in hybrid seed production) are common, and by simple backcrossing procedures have resulted in the current predominance of melon hybrids in cultivation (Robinson and Decker-Walters, 1997; Rubatzky and Yamaguchi, 1997).

Disease resistance, high yield, and uniform fruit shape, size, and excellent quality are prerequisites for the release of superior melon varieties. Breeding for resistance to diseases (i.e., bacterial, fungal, and viral) and pests (e.g., aphids, beetles, and white flies) as well as for excellent quality melons (e.g., high sugar content, acceptable shape, and size) have been priorities of melon improvement programs (Robinson and Decker-Walters, 1997; Rubatzky and Yamaguchi, 1997; Lebeda *et al.*, 2006).

Historically, group *Cantalupensis* market classes have been uniformly enhanced by increasing carotene and sugar content, as well as improving internal (e.g., seed cavity size and total soluble solids) and external (fruit size and shape, netting) morphological attributes. Few studies, however, have investigated the genetics of yield or its components (e.g., days to anthesis, plant architecture, yield, and fruit maturity) (Lippert and Legg, 1972; Lippert and Hall, 1982). Quantitative trait loci (QTL) for external and internal traits have, however, recently been identified and mapped (Périn *et al.*, 2002a, b, c; Monforte *et al.*, 2004).

Yield- and quality-associated traits in melon are quantitatively inherited and typically have low heritability (Zalapa *et al.*, 2006). Thus, selection for yield often requires extensive evaluation over multiple years, locations, and replications. Indirect selection for yield and quality using correlated traits (i.e., fruit yield and quality components) has been successfully used for the improvement of several crop species, but has not been extensively applied to melon. Andrus and Bohn (1967) reported that in a heterogeneous cantaloupe melon population that had undergone nine generations of mass selection for a selection index including 18 fruit traits, the mean values for most characters increased without any apparent loss in genetic diversity. Furthermore, inbred lines developed from advanced mass selection cycles performed better for the selected fruit characters than inbreds



Figure 1 Melon (*Cucumis melo* L.) plant architectural types: dwarf indeterminate (vining; a), standard indeterminate with mainly distal fruit set (vining; b), crown set on fractal type indeterminate (vining; c), and internode differences among standard indeterminate and dwarf indeterminate (vining; d)

developed from earlier cycles. Subsequently, they studied pair-wise correlations among the 18 fruit characters under mass selection, and concluded that negative and positive correlations exist among these fruit characters studied (Bohn and Andrus, 1969). Thus, strategically implemented index selection of correlated yield and quality traits may be the best approach for melon improvement in such populations.

The varied growth habits of melon provide for a wide array of architectural plant types (Figure 1). The three primary architectural types recognized in melon include vining (Rosa, 1924), dwarf (syn. short-internode or bush) (Mohr and Knavel, 1966), and bird-nest (Paris *et al.*, 1981). These architectural types feature various combinations of vining habit (determinate or indeterminate), internode length (standard or shortened), and branch number (unilateral or multilateral).

In the 1970s and 1980s, both dwarf and bird-nest types were considered to have potential for increasing melon yield in once-over or mechanized harvesting operations (Paris *et al.*, 1981; Nerson *et al.*, 1983; Mohr and Knavel, 1966). Dwarf melon plant types were proposed as a means of increasing yield because their small-sized vines could be planted at higher densities (e.g., in close row spacing with staggered double rows), and each dwarf plant would theoretically support a single fruit. It was believed that higher yield would be achieved by the increased number of plants and that fruit development would be synchronized across the field, thus making once-over or mechanical harvesting practical (Knavel, 1988, 1990, 1991). The initial breeding efforts of dwarf cultivars proved difficult and required over 10 years to complete (Mohr and Knavel, 1966; Davis *et al.*, 1976; Zink, 1978, 1980; Halsey, 1980). In the

United States, the breeding of dwarf melons (Mohr and Knavel, 1966; Davis *et al.*, 1976; Zink, 1978, 1980; Halsey, 1980) has not resulted in superior yielding cultivars, and their fruit are often rather small for commercial acceptance (Nerson and Paris, 1987). The deficiencies of the dwarf types are probably related to their relatively small total stem and leaf area, which affect net photosynthetic capacity and efficiency (Knavel, 1988, 1990).

Case study for plant improvement The case study presented below typifies breeding procedures for the introgression of exotic genes into elite melon germplasm, and genetic assessments used to define work plans for plant improvement. It models the

introgression of multiple lateral branching and fruit set for changed plant architecture, which is the result of a co-operative project between J.D. McCreight (USDA, ARS, Salinas, CA) and J.E. Staub (USDA, ARS, Madison, WI).

Initially, horticulturally unique germplasm designated CR was received from Mr. Claude Hope, Cartago, Costa Rica (Figure 2). This accession, *C. melo* ssp. *agrestis* (Naud.) Pangalo, is characterized by a “fractal” or radiant growth habit (Smith, 1984; Prusinkiewicz and Harlan, 1989). CR is early flowering, monoecious, fast growing, indeterminate, possesses standard size internodes, abundant branching (6–12 primary branches), and bears many small fruits (up to 100 fruits/plant), 3–6 cm in diameter (Zalapa



Figure 2 Fruit and vine characteristics of a monoecious fractal exotic melon (*Cucumis melo* L.) accession (CR-1) originating from Costa Rica (a and b) and an extreme fractal indeterminate monoecious line USDA 846-1 (c and d) derived from a cross between CR-1 and indeterminate andromonoecious cultivar Top Mark

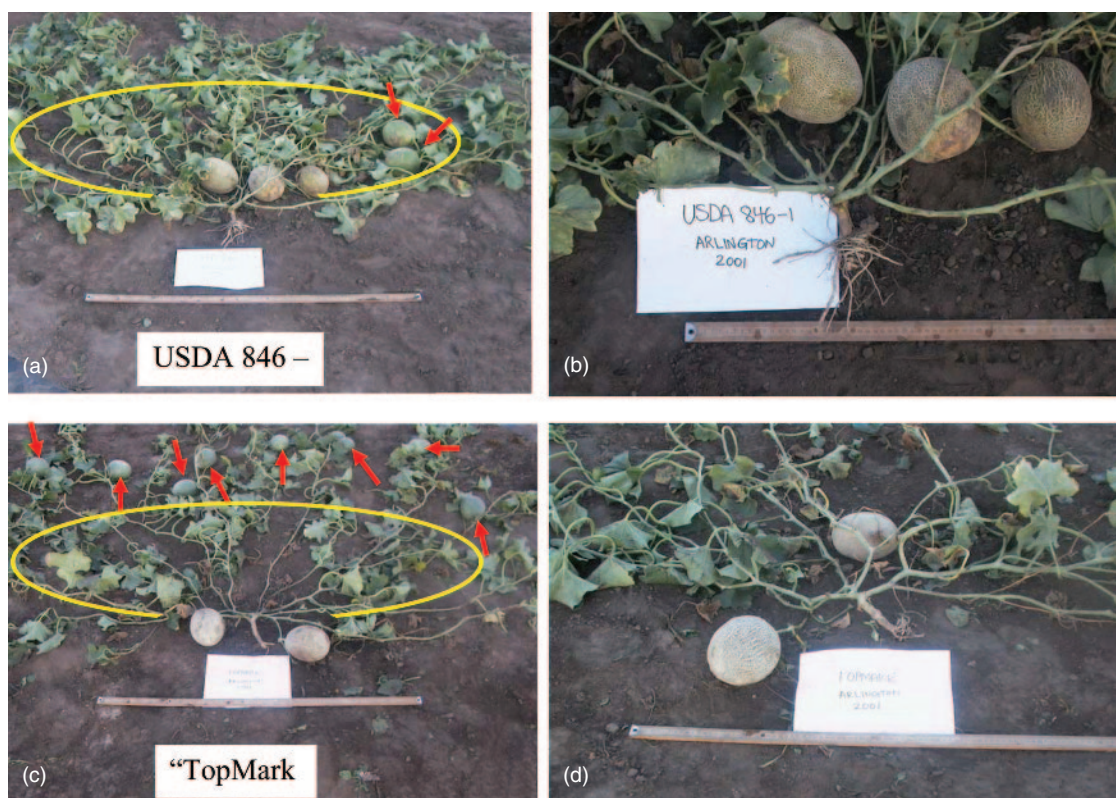


Figure 3 Vegetative growth habit, branching, and fruiting patterns in melon (*Cucumis melo* L.) lines USDA 846-1 (a and b) and cultivar Top Mark (c and d)

et al., 2006). The fractal architecture of CR is distinct from the vining, dwarf, and bird-nest plant habit, and its unique habit is a function of internode length (standard size) coupled with a comparatively high number of primary, secondary, and tertiary branches.

A monoecious, early flowering CR plant having 12 primary branches was selected in 1996, and was subsequently crossed to an F_1 plant derived from a cross between andro-monoecious USDA lines FMR#8 and SC#6. A monoecious, early flowering plant was then selected and self-pollinated four times to produce an inbred line designated USDA 846-1 (Figure 3). This monoecious, fractal, highly branched (5–8 primary branches) line produces a concentrated fruit set (2–5 fruits near the crown of the plant), and is capable of multiple fruiting cycles (Zalapa *et al.*, 2006).

USDA 846-1 (P1) was crossed to “Topmark” (P2), which is andro-monoecious, possesses between two to four lateral branches, and produces

a diffuse, distal fruit setting habit typical of commercial vining melon types (Figures 2 and 3). A single F_1 plant from this initial cross was self-pollinated to generate F_2 individuals, which were subsequently used to produce several BC1 and 119 F_3 families. A generation mean analysis was performed to estimate gene action, trait heritabilities, and least effective factors contributing to expression of yield components (Zalapa *et al.*, 2006). The F_3 families were assessed for variance of yield components (i.e., days to anthesis, primary branches number, fruit number and weight per plant, average weight per fruit, and days to fruit maturity (Zalapa *et al.*, 2007a), and subsequently used to develop recombinant inbred lines (RIL) for detection of QTLs controlling yield and quality component traits (Zalapa, 2004; Zalapa *et al.*, 2007b).

Generation mean and variance component analyses indicated that significant phenotypic variation existed in advanced families for

continued selection (Zalapa *et al.*, 2006, 2007a). Examination of the RILs in multiple environments (California and Wisconsin) detected location-independent QTLs that were distributed across nine linkage groups (Zalapa *et al.*, 2007b). Thirteen (43%) of these QTLs were detected consistently across locations. These results suggest that marker-assisted selection (MAS) will likely have utility in breeding programs directed toward improving yield through alterations in plant architecture.

Melon breeding has provided superior cultivars for improved human health. Historic achievements include the following: (1) development of pest-resistant germplasm through seedling screening and mature plant techniques; (2) uniform external characteristics (e.g., netting, ribbing, etc.); (3) improved internal characteristics (e.g., sweetness, carotenoid content, texture, etc.), and (4) characterization of sex types to produce primarily andro-monoecious commercial hybrids. With the advent of molecular tools, dissection of complex traits (disease, yield, quality, and abiotic stress) will allow for gene identification and manipulation through biotechnology (e.g., MAS and transgenic introgression). Such manipulation will likely provide more rapid release of unique disease resistant, high yielding genotypes that possess high fruit quality.

1.2.4.2 *Cucumber*

There are two basic cucumber types: those that are eaten fresh (i.e., fresh or slicing market types; Wehner and Horton, 1986) and those that are consumed as a processed product (processing or pickling types; Staub and Bacher, 1997). The major fruit types are the American processing and fresh market types, the Dutch gherkin and greenhouse types, the German Schalgurken type, the Mideast Beit Alpha type, and the Oriental trellis (burpless) type.

Fresh market types are field or greenhouse grown, and are usually between 15 cm (i.e., US and Mediterranean) to 40 cm (i.e., European) in length. Less common fresh market types include Sfran (compact fruit types marketed in the Persian Gulf) and “lemon” cucumber (shape similar to a lemon with pale, greenish-yellow skin; hermaphroditic). Processing types differ depending on cultural preferences (e.g., the United States vs. Europe).

Breeding objectives are determined by the requirements associated with cucumber market classes (e.g., US processing (pickling), US fresh market, European glasshouse, Mediterranean, Asian glasshouse). Breeding plans are driven by historically proven procedures and emerging technologies. Typically, breeding follows a series of steps that consist of population development and improvement, line extraction, and hybrid evaluation. Population development via recurrent selection (e.g., yield and quality components), pedigree selection (e.g., disease resistance), and backcross introgression (e.g., sex expression) are characteristic of most cucumber breeding programs. The breeding process results in the refinement of populations derived from intercrossing elite and/or exotic (unadapted) germplasm, the development of inbred lines from such populations, and the identification of commercially acceptable F₁ hybrids.

Early genetic enhancement of cucumber (1850–1980) focused mainly on the incorporation of disease resistance and changes in plant architecture (e.g., sex expression, growth habit) that were augmented by improved cultural practices (Lower and Edwards, 1986; Tatlioglu, 1993; Staub and Bacher, 1997). The genetic control for resistance to scab (*Ccu*), downy mildew (*dm*), bacterial wilt (*Bw*), angular leaf spot (*psl*), anthracnose (*Ar*, *cla*), target leaf spot (*Cca*), *Corynespora* leaf spot, and *Fusarium* (*Foc*) is conditioned by few genes (Robinson *et al.*, 1976; Pierce and Wehner, 1990). Seedling cotyledon tests have been developed to screen for resistance to the pathogens of each of these diseases allowing for the release of a wide array of resistant cucumber market types (Lower and Edwards, 1986). Seedling screening procedures are amenable to simple backcrossing and selfing strategies for line development. In contrast, the genetics of resistance to viruses, such as CMV (*Cmv*), WMV (*Wmv*), Potyvirus, and ZYMV (*zymv*), or to powdery mildew (*pm-1*, *-2*, *-3*, *pm-h*), green mottle mosaic virus (GMMV), gummy stem blight, belly rot, cottony leak, *Phytophthora* rot, and gray mold is complex. Thus, breeding for resistance to these diseases, as well as many architectural traits, requires exacting test protocols (often in the mature plant stage) and extensive replicated testing (field and greenhouse) in multiple environments.

Yield and quality are a major focus of cucumber improvement and consist of many extensively reviewed, interrelated traits that are often the focus of the cucumber breeder (Lower and Edwards, 1986; Tatlioglu, 1993; Lebeda *et al.*, 2006). These quantitatively and qualitatively inherited traits range from disease resistance to plant and fruit architecture and habit. Conventional breeding for yield involves selection for total yield or index selection for yield components, and can be enhanced by MAS (Fazio *et al.*, 2003a; Fan *et al.*, 2006). Correlations among traits are important when manipulating plant architecture for yield improvement, since source-sink relationships impose practical constraints on fruit development. Correlations between and among yield and quality component traits have been investigated, and this information is used during recurrent selection procedures. Typical fruit quality traits considered for selection are fruit size, external and internal quality, and for US processing cucumbers, characteristics associated with processing methods. Common traits selected for yield improvement include fruit number and weight, sex expression, days to anthesis, lateral branch number, and fruit length and diameter.

Case study for plant improvement *C. hystrix* Chakr. ($2n = 2x = 24$) has been collected only in the Yunnan province of China (Chen and Adelberg, 2000; Chen and Kirkbride, 2000). It is sparingly sexually compatible with *C. sativus*, and harbors some degree of resistance to CMV and ZYMV and comparatively strong resistance to PRV (Chen *et al.*, 1997a, b, 2004). In addition, it possesses root-knot nematode resistance (Chen and Lewis, 2000; Chen *et al.*, 2001), gummy stem blight resistance, downy mildew resistance, unique nutritional qualities, and tolerance to growth under low irradiance and temperature not known in *C. sativus* (Chen *et al.*, 2004).

Fertile, interspecific hybrid progeny ($2n = 19$, with 12 and 7 chromosomes contributed by *C. hystrix* (H) and *C. s. var. sativus* (S), respectively) have been obtained through F_1 embryo rescue by Jin-Feng Chen (Nanjing Agricultural University, Nanjing, China; Chen and Staub, 1997; Chen *et al.*, 2002) (Figure 4). Subsequently, an amphidiploid (HHCC, $2n = 4x = 38$)

was produced via chromosome doubling, and was designated as a new synthetic species, *C. hystivus* Chen (Chen *et al.*, 2003). Partially fertile allotriploids ($2n = 3x = 26$; HSS), fertile diploids ($2n = 2x = 14$; SS), and partially fertile monosomics ($2n = 15$; SS + 1H) have also been derived from *C. hystivus* \times *C. s. var. sativus* crosses (Chen *et al.*, 2004; Chun-Tao *et al.*, 2005). These experiments demonstrate the unique introgression of exotic germplasm into elite cucumber germplasm.

The “proof of concept” for use of *C. hystrix* germplasm for cucumber improvement has been made (i.e., fruit with acceptable quality; Figure 4). However, during backcrossing many of the unique qualities of *C. hystrix* have likely been lost (e.g., pest resistance). The introgression of such traits will require rigorous selection of backcross families for economically important traits.

Historic cucumber breeding achievements include the following: (1) development and use of disease screening technologies to develop resistant cultivars; (2) identification of biochemical pathways, which regulate sex expression; (3) development and implementation of controlled pollination procedures, and (4) characterization of genetics, which stabilize gynoeious sex expression (Lower and Edwards, 1986). More recently, the development of sophisticated breeding strategies, the creation of sophisticated computer algorithms, and the development of molecular marker technologies has allowed for an in-depth quantification of some economically important metric traits, the development of unique genetic stocks, and an improved understanding of the cucumber genome (Serquen *et al.*, 1997; Fazio *et al.*, 2003b). Future contributions may include the introgression of exotic genes for improved disease resistance, yield, and improved human health (Figures 4 and 5).

1.2.5 Limitations of conventional breeding and application of biotechnology

Genetic improvement of both melon and cucumber (most particularly cucumber) has been impeded by their narrow genetic base (3–8% (cucumber) and 15–20% (melon) on a per band basis). As molecular marker technologies become more efficient, effective, and affordable, they will be increasingly used to augment



Figure 4 Fruit and vegetative habits of *Cucumis hystrix* (Chakr.) (a; $2n = 2x = 24$), a BC_3S_1 line derived from a cross between *C. hystrix* and *C. sativus* L. ($2n = 2x = 14$) (b; $2n = 2x = 38$), an F_1 derived from a cross between the BC_3S_1 and an elite *C. sativus* line (c), and vine biomass differences between the $BC_3S_1 \times C. sativus$ line (d; left is the F_1 and right is the standard indeterminate *C. sativus* commercial hybrid)

and enhance conventional phenotypic selection during population development and/or inbred line development.

1.2.5.1 Application of marker-assisted selection

Melon The genome size of melon has been estimated to be $4.5\text{--}5.0 \times 10^8$ bp, about three times that of *Arabidopsis* (1500–2000 cM; Arumuganathan and Earle, 1991). Genetic analysis of melon has led to the description of roughly 200 major genes that control morphological traits (Pitrat *et al.*, 2000). However, only a few of these genes have been assigned to linkage groups.

Molecular markers (e.g., amplified fragment length polymorphism, cleaved amplified polymorphic sequences, inter-simple sequence repeat,

random amplified polymorphic DNA, restriction fragment length polymorphism, sequence characterized amplified region, single nucleotide polymorphism, and simple sequence repeat) have been used to identify and map disease and pest resistance genes in melon. Several moderately saturated genetic maps have been developed using various molecular markers (RAPD, RFLP, and SSR) (Danin-Poleg *et al.*, 2002; Périn *et al.*, 2002b; Gonzalo *et al.*, 2005). Genes of economic importance that have been mapped include those for disease resistance (e.g., *Fom-1*, *Fom-2*, *Nsv*, *Pm*, *Pvr*, *CMV*, and *Vat*), flowering characters (e.g., *a* and *ms*), plant architecture (e.g., *si* and *lmi*), and ethylene production during fruit maturation (e.g., *CM-aco1*) (Dogimont *et al.*, 2000; Périn *et al.*, 2002a; Pitrat *et al.*, 2000). More specifically, marker-trait associations have been identified for *Fusarium* wilt (*Fom-1* and *Fom-2*; Wechter *et al.*,



Figure 5 A high carotene (vitamin A) cucumber line (*Cucumis sativus*) line derived from a cross between an exotic *C. sativus* accession originating from China and an elite USDA US processing cucumber line

1995, 1998; Wang *et al.*, 1998, 2000; Zheng *et al.*, 1999; Karsies *et al.*, 2000; Zheng and Wolff, 2000; Garcia-Mas *et al.*, 2001; Brotman *et al.*, 2004), ZYMV (Zym 2 and Zym 3; Danin-Poleg *et al.*, 2002), PRSV (Prv; Garcia-Mas *et al.*, 2001; Brotman *et al.*, 2002, 2004), virus aphid transmission resistance (Var; Brotman *et al.*, 2002), powdery mildew (Pm; Fukino *et al.*, 2002), and melon necrotic spot virus (Nsv; Garcia-Mas *et al.*, 2004; Morales *et al.*, 2004). Additionally, the map positions of complex traits contributing to fruit quality (Périn *et al.*, 2002b; Monforte *et al.*, 2004) and yield (Zalapa *et al.*, 2006) controlled by QTL have been determined. This information provides

opportunities for increasing breeding efficiency and effectiveness through MAS.

Cucumber Cucumber possesses a rather small genome size (~880 Mbp (mega base pair), 750–1000 cM; Staub and Meglic, 1993), low chromosome number, and has a rapid life cycle (three cycles per year). In addition, fairly saturated genetic linkage maps have been developed, and QTL analyses have identified several genomic locations involved with important traits (Serquen *et al.*, 1997; Fazio *et al.*, 2003b).

The pyramiding of simply inherited genes (e.g., disease resistance) during germplasm enhancement is common, and has proven useful in the improvement of many crop species. In cucumber, the pyramiding of disease resistance genes resulted in important inbred lines and populations. MAS has potential for augmenting conventional breeding programs to increase breeding efficiency and effectiveness during pyramiding selection. MAS can be applied to the improvement of cucumber and melon if marker-trait associations are robust and stable. This has, in fact, been the case with cucumber where MAS has proven effective in selection for yield component traits during backcross introgression (Fazio *et al.*, 2003a; Fan *et al.*, 2006).

1.2.5.2 Application of transgenic technologies

New melon and cucumber varieties have been traditionally released by conventional breeding. With the exception of *C. hystrix* introgression, transfer of target traits has been limited within many cucurbit species. The development of plant regeneration techniques and gene transfer via particle bombardment and gene specific *in vitro* transfer (transformation) provide opportunities for augmenting conventional breeding strategies (Fang and Grumet, 1990, 1993; Ezura *et al.*, 2000). For instance, ethylene promotes climacteric melon fruit ripening and genes that encode ripening operate to modulate the ethylene synthesis pathway, 1-aminocyclopropane-1-carboxylate synthase oxidase. “Knocking out” such genes by antisense can result in reductions in ethylene production (Ayub *et al.*, 1996) and thus could potentially increase fruit self-life (Bauchot *et al.*, 1998; Silva *et al.*, 2004). Disease resistance has also been introduced through transgenic techniques. Fang and Grumet (1993) have introduced resistance to ZYMV infection in melon. Yoshioka *et al.* (1992, 1993) have introduced genes conferring CMV resistance into melons. One group has been able to introduce genes conferring resistance to ZYMV, WMV2, and CMV, PRSV, and squash mosaic virus (SqMV) into melon and cucumber (Fuchs *et al.*, 1997; Tricoli *et al.*, 2002). All of these workers have achieved their results through

the transformation using constructs containing the coat protein genes of the target viruses. They indicate that transgenic technology could be used successfully in production agriculture, if negative socio-political barriers were to be removed, and if the significant regulatory hurdles to commercialization could be overcome. To date no transgenic melon or cucumber has been commercialized.

1.3 Watermelon

1.3.1 History, origin, and distribution

Watermelon was thought to have originated in southern Africa (Jeffrey, 2001) and has a long history of cultivation in Africa and the Middle East. Recently, however, several watermelon seeds that are 5000 years old have also been discovered in northern Africa (Wasylikowa and Van Der Veen, 2004). Therefore, southern or northern African origins are still unresolved. Nevertheless, it is commonly believed that watermelon originated from that continent and spread from there to the rest of the world. It spread to China and southern Russia by the 10th Century AD. By the 13th century, watermelon was grown in Europe and from there it was introduced to North America by the Spaniards in the 16th century, where it rapidly became popular with Native Americans (Jeffrey, 1975; Robinson and Decker-Walters, 1997). Watermelon grows in areas with temperatures warmer than 20 °C. Under suitable climatic conditions, its semi-tap root system can grow 2 m or more below the soil surface (Jeffrey, 2001). In the United States, watermelons are mainly grown in the southern and western states, such as Florida, Georgia, California, and Texas (Wehner and Maynard, 2003). Worldwide top watermelon-producers are listed in Table 1.

1.3.2 Botanical description: taxonomy, habit, habitat, genome size, and cytological features

Most cultivated or edible watermelons belong to the species *Citrullus lanatus*, which includes two morphological subspecies, *C. lanatus* subsp.

Table 1 Production of watermelon in 2005 and 1990^{(a)(b)}

Country	Area (ha)		Production (t)	
	2005	1990	2005	1990
China	2 014 500	574 045	69 315 000	10 955 371
Turkey	137 000	102 845	3 800 000	3 300 000
Iran	100 000	136 275	2 150 000	2 645 989
Brazil	82 000	67 986	1 850 000	437 202
USA	55 200	82 000	1 718 920	1 100 000
Egypt	62 000	49 150	1 500 000	1 007 000
Mexico	42 979	29 705	970 055	404 077
S. Korea	23 000	25 681	850 000	593 228
Kazakhstan	43 000	Na	660 000	Na
Syria	25 000	28 800	620 000	249 700

^(a)Source: FAO, 2006

^(b)The top 10 watermelon producing countries in 2005 are ranked according to total production in 2005; 1990 data are included for comparison

lanatus (Thunb.) Matsum. & Nakai (cultivated watermelon; although *Citrullus vulgaris* Schrad. is still found in the literature) and *C. lanatus* subsp. *vulgaris* Schrad (Jeffrey, 2001). The latter contains wild annual forms that today grow only in the Kalahari Desert in southern Africa and are known under the local name *tsamma*, which is considered the ancestor of cultivated watermelon (Jeffrey, 2001). This subspecies also contains other botanical varieties such as citron, which is a source of *Fusarium* wilt resistance in watermelon (Robinson and Decker-Walters, 1997).

Cultivated watermelon is an annual herb with long (up to 10 m) stems creeping on the ground, with curly tendrils at each node. Stems are thin, hairy, angular, and highly branched. Leaves are hairy and deeply palmate with 3–5 lobes, on long petioles (Jeffrey, 1978). Most watermelon plants produce separate male (staminate) and female (pistillate) flowers, which are easily distinguishable due to their watermelon-like ovary. This condition of monoecy is controlled by a single dominant gene (Poole and Grimball, 1945). Andro-monoecious plants are not as common and have both male and perfect flowers, the latter housing both male and female floral organs. The separation of the male and female flowers means that proper pollination has to be carried out by insects, mostly honeybees (Fehér, 1993). This is true even for perfect flowers. Male flowers also appear earlier than the female flowers in both monoecious

and andro-monoecious plants. However, most modern varieties are monoecious, which facilitates commercial seed production of inbred lines and hybrid varieties. The ratio of male and female flowers under field conditions varies from 4:1 to 15:1 and most varieties have a ratio of 7:1, although 4:1 is more desirable for higher yield (Fehér, 1993; Wehner *et al.*, 2001). Watermelon fruits are usually round or oval/ovate. Fruits can weigh from 2–20 kg. Fruit sizes are divided into five categories: icebox (<5 kg), small (5–8 kg), medium (8–11 kg), large (11–15 kg), and giant (>15 kg) (Wehner *et al.*, 2001). Seed color varies from black to white. Fruit rind color can vary from pale yellow to dark green, with or without stripes, and flesh color can be yellow, pink or red, rarely white (Jeffrey, 1978).

Watermelon is usually diploid and has 11 pairs of chromosomes ($2n = 22$, $x = 11$) (Fehér, 1993; Wehner *et al.*, 2001). Because of its small genome size (4.25×10^8 bp), similar to melon and cucumber (Arumuganathan and Earle, 1991), the chromosomes are small and stain poorly for cytological studies, although an improved staining procedure has been developed (Skorupska and Allgood, 1990). Different ploidy levels exist in cultivated watermelons but diploids (which may contain 0.5–1% polyploids), triploids, and tetraploids have been commercially important (Fehér, 1993). Tetraploids may produce smaller fruits than diploids, but their fruit quality is excellent and germination is typically better than their diploid counterparts. In addition, tetraploids are critical in producing triploid seedless watermelons.

1.3.3 Economic importance

Watermelon production was more than doubled from 34 million tons in 1991 to 77.5 million tons in 2001 (Taylor and Brant, 2002). Production in top countries producing watermelon in 2005 increased tremendously even though in some countries such as the United States, where production has increased by 56% since 1990, the growing area has remained relatively constant (Table 1). The United States produced 1 718 920 t of watermelon in 2005 with a market value of over US\$ 400 million (NASS, 2006). But this by no means reflects the total consumption, because there are significant

imports from other countries. For example, the United States imported 248 057 t of watermelon in 2004 (FAO, 2006).

Delicious as a dessert eaten raw, watermelon is a rich source of vitamin A, β -carotene/lycopene and potassium (Robinson and Decker-Walters, 1997; Holden *et al.*, 1999; Wehner and Maynard, 2003). Although known for its high lycopene content, tomato actually contains less lycopene than red-fleshed watermelon on fresh weight basis (Perkins-Veazie *et al.*, 2001). In fact, it has been reported that on average, watermelon contains 40% more lycopene than raw tomatoes (Arnold, 2002). Yellow-fleshed watermelon contains a little lycopene, a potent antioxidant and anticancer agent (Rao *et al.*, 2006) but has higher carotene (vitamin A) content (Wehner and Maynard, 2003). Watermelon juice can also be fermented into alcoholic beverages (Robinson and Decker-Walters, 1997). In addition to fruits, watermelon seeds yield an edible oil, and are consumed roasted.

1.3.4 Conventional breeding: breeding objectives, tools and strategies, and achievements

Objectives of most watermelon breeding programs have been yield, quality, and disease resistance. Over the past century, conventional breeding has tremendously improved earliness, plant architecture (dwarfness), fruit yield (larger fruit), quality (increased soluble solids/sugar and lycopene content), diversity (e.g., seeded and seedless), disease resistance (*Fusarium* wilt, anthracnose and viruses including WMV, cucumber green mottle virus, telfairia mosaic virus, alfalfa mosaic virus, and ZYMV), and shipping quality (resistance to cracking) (Fehér, 1993; Wehner *et al.*, 2001; Gusmini and Wehner, 2006). Improved resistance to diseases such as bacterial fruit blotch and gummy stem blight remains a challenge (Gusmini *et al.*, 2005). However, sources of resistance have been identified for all important diseases in watermelon (Fehér, 1993; Wehner *et al.*, 2001). It is possible through conventional breeding to integrate these resistance traits into a desirable horticultural background, especially for resistances controlled by single genes. Over 500 diploid cultivars were developed in the United States

during the last two centuries, but there is still an ongoing need to improve watermelon, particularly in disease resistance and quality (Levi *et al.*, 2001).

Conventional breeding usually involves crossing between elite varieties, or an elite variety with another line that has one or more desirable traits. These varieties/lines may be selected based on the breeding objectives. The crossing has to be performed in an enclosed or an isolated area to minimize pollen contamination by insects. Progeny from the crosses are subsequently evaluated and re-evaluated in a variety of environments according to the breeding objectives. It is therefore critical to develop practical evaluation (field, greenhouse, or laboratory) criteria and selection (pedigree, recurrent, or backcross) strategies. After years of testing and selection, the best performers are released as certified varieties. Genetic diversity is a key to meet the needs of the growers/consumers, because not all varieties are going to be high yielding and of good quality in all growing areas.

The method used for trait evaluation has to be rapid and robust, such that a large number of lines can be screened within a certain period of time. For example, evaluation of sugar (soluble solids) content can be based on taste and refractometer readings (Wehner *et al.*, 2001). Refractometer readings are easily made in the field using a hand-held unit, which provides data on percentage of soluble solids ($^{\circ}$ Brix). For a variety to be acceptable, its soluble solids content should be at least 10% ($^{\circ}$ Brix) although newer varieties can have higher value of 14% (Wehner *et al.*, 2001). High sugar content and sweetness are the critical factors in determining the quality of many cultivars of watermelon. However, there may also be need for low sugar but red-fleshed watermelons for people on a low-carbohydrate diet, or for people with diabetes (Pons, 2004).

There are several selection strategies used in conventional watermelon breeding: backcross, pedigree, and recurrent selections (Wehner *et al.*, 2001). Backcross selection is used to transfer a single-gene trait from a donor to an otherwise superior inbred recipient, which is the recurrent parent. It may take more than five generations of selection and backcrossing to recover the genotype of the recurrent parent and much longer if the trait is recessive. The use of genetic markers linked to the trait has proven to shorten the generation time. In pedigree selection, two or more adapted and

complementary parents are crossed to form the hybrid (F_1) generation, which is then self- or sib-pollinated to form a segregating (F_2) population. The F_2 is self- or sib-pollinated while selecting for highly heritable (qualitative) traits to form the F_3 generation. Selections in later generations should focus more on quantitative traits when the plants are more uniform. Single-seed-descent is a modified pedigree selection in which selection is not practiced until later generations when quantitative traits such as yield and earliness are selected. Recurrent selection is used for population improvement. For example, an elite population can be developed by intercrossing more than two selected genotypes and select for qualitative traits in early and quantitative traits in late generations.

Many varieties have been developed in the latter part of last century through conventional breeding (Wehner *et al.*, 2001): “Charleston Gray” (USDA, Charleston, 1954), “Crimson Sweet” (Kansas State University, 1963), “Calhoun Gray” (Louisiana State University, 1965), and “Dixielee” (1979), “Jubilee” (1963), and “Smokylee” (1971) (all from the University of Florida) have high resistance to *Fusarium* wilt. “Dixlee” (University of Florida, 1979) and “Sangria” F_1 (Novartis, 1985) have dark red flesh. “Millionaire” F_1 , 3x (Harris Moran, 1992) and “Royal Jubilee” F_1 (Seminis) have consistently high yields. “Crimson Sweet” (Kansas State University, 1963) and “Sugarlee” (University of Florida, 1981) have high soluble solids. “Kengarden” (University of Kentucky, 1975) has dwarf vines. “Tri-X-313” F_1 3x (Novartis, 1962) is seedless. “Minilee” (University of Florida, 1986), “Mickylee” (University of Florida, 1986), “New Hampshire Midget” (University of New Hampshire, 1951), “Sugar Baby” (M. Hardin, Oklahoma, 1955), and “Tiger Baby” (Seminis) are icebox size. “Yellow Doll” (Seminis, 1977) has canary yellow flesh. Commercially, the most popular seeded varieties have dark red flesh, blocky (elongated) shape, and weigh 8–11 kg while popular seedless fruits have to be oval shaped and medium sized (5–8 kg), in addition to being red flesh and sweet (Wehner and Maynard, 2003). Varieties with dark red flesh include “Dixielee”, “AU-Sweet Scarlet”, “Red-N-Sweet”, and “Sangria”.

Although most earlier varieties have been open-pollinated, almost all watermelon varieties used

in the United States are now diploid hybrids (Kapiel *et al.*, 2004). A survey of watermelon varieties found that hybrids and sweet varieties are popular with growers in the United States due to their wider adaptation and quality. Another reason is that hybrids can be used to combine traits inherited in a dominant fashion from the two parents, such as red or yellow flesh, resistance to *Fusarium* wilt and anthracnose, and resistance to powdery mildew because their parents can accumulate those traits through inbreeding (Robinson, 1999; Wehner *et al.*, 2001). Like corn, watermelon is monoecious; but unlike corn, it can undergo inbreeding indefinitely without inbreeding depression. Hybrids have provided increased profitability to growers. (Rhodes and Zhang, 2000).

Eating watermelon with seeds is apparently a nuisance, an important reason why seedless watermelons (Figure 6) are produced and becoming popular with consumers (Kapiel *et al.*, 2004). Although watermelon with empty seeds has been produced by pollination with pollen irradiated with soft x-ray (Sugiyama *et al.*, 2002), the most practical method to produce seedless watermelons is through triploid hybrids, which are produced by crossing a tetraploid female parent with a diploid male parent to produce a triploid (the reciprocal cross does not produce offspring, for reasons that are yet unclear). A tetraploid line with desirable traits has to be developed first from a diploid using a chromosome-doubling agent, such as colchicine (Zhang *et al.*, 1995). It takes extra time and effort to produce this tetraploid parent due to low fertility and frequent fruit abnormality. Triploid hybrids produced in such way are female sterile and fruits induced by pollination from viable pollen are therefore seedless. As a result, about one-third of the field has to be used for diploid plants to provide a pollen source. As in other hybrid production, the quality of the hybrid (triploid in this case) depends on the combinations of the parents. As with other hybrid seeds, production is now mostly done in developing countries, where the low cost of labor allows hand pollination to produce seeds for triploid watermelons to be economically viable (Kapiel *et al.*, 2004; Rhodes and Zhang, 2000).

Triploids have other advantages besides seedlessness. They are generally more resistant to many diseases for reasons mentioned above, have a fruit

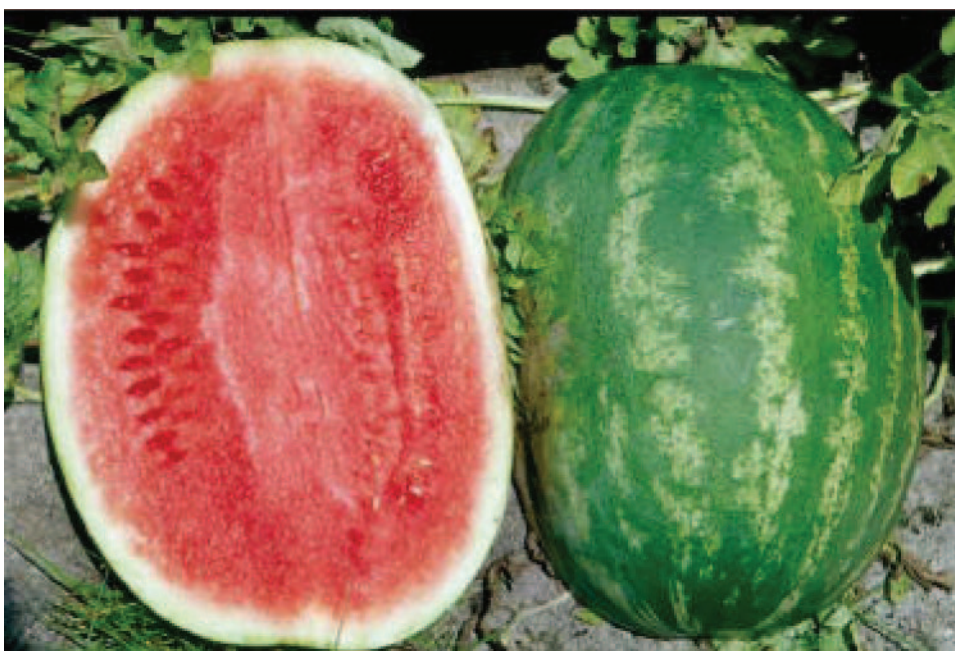


Figure 6 An example of a seedless variety, Tri-X-Carousel: triploid seedless, oval, broad green stripes in light-green background [Reproduced with permission of Donald N. Maynard]

with a longer shelf life, with higher soluble solids than the mid-parent. Triploids also outyield their diploid parents (Kapiel *et al.*, 2004).

1.3.5 Limitations of conventional breeding and rationale for transgenic breeding

Conventional breeding has been very successful in exploiting genetic variability within the cultivated watermelon. However, the low genetic diversity among watermelon cultivars has generated the need to expand the genetic base of cultivated watermelon (Levi *et al.*, 2001; Gusmini and Wehner, 2005). Traits controlled by single genes, such as *Fusarium* wilt resistance, can be transferred to cultivated watermelon from wild relatives through backcross selection, a process expedited by MAS. This process still takes a long time and can be very expensive.

Another feature of watermelon breeding is the selection for seedless watermelon varieties, which have become popular in recent years. Generating tetraploids from diploid varieties by colchicine treatment is difficult, because the frequency of success is less than 5%, and is genotype dependent

(Rhodes and Zhang, 2000). This is in addition to the long process of selecting a tetraploid plant with decent fertility and fruit traits. To produce the triploid seeds, at least six skilled workers are needed for 3–4 weeks to pollinate 1 acre of a hybrid watermelon seed production field (Rhodes and Zhang, 2000). Triploid seeds also are difficult to germinate because of their thick and hard seed coat (Rhodes and Zhang, 2000; Kapiel *et al.*, 2004) although this can be improved by tumbling the seeds (Rhodes *et al.*, 1997).

Gene transfer through genetic transformation is not limited by species boundaries because no sexual cross is made. Traits are transferred through the introduction of specific genes, a process ultimately determined by the availability of genes of interest (not just traits of interest). Resistance to a particular disease can be introduced from wild species without long backcross selection. Furthermore, genes underlying any traits can be introduced into watermelon, thereby enhancing existing traits or creating new traits and increasing the genetic diversity. Genetic engineering overcomes hurdles of conventional breeding outlined above. However, its success ultimately depends

on consumer acceptance of genetically engineered food.

2. TISSUE CULTURE AND TRANSFORMATION OF CUCURBIT SPECIES

2.1 Plant Regeneration

The ability to regenerate whole plants from tissue explants or cell cultures is crucial to the development of any transformation protocol. Advances in tissue culture of cucurbits over the last 30 years laid the platform for transformation of the four major cultivated species of the Cucurbitaceae. Regeneration via embryogenesis and organogenesis has been reported for all the four cultivated species (Debeaujon and Branchard, 1993; Kathiravan *et al.*, 2006). However, despite the ability to manipulate cucurbits *in vitro*, regeneration and transformation of cucurbits remain challenging.

2.1.1 *Cucurbita pepo*

Nearly 40 years ago, Schroeder (1968) reported on embryogenesis in *C. pepo*. Embryos were observed forming from the pericarp wall of zucchini squash. Jelaska (1972, 1974) produced embryogenic callus from pumpkin by culturing hypocotyl sections on Murashige and Skoog (1962) medium (MS) supplemented with indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). Callus remained embryogenic for up to 15 years; and Juretic and Jelaska (1991) were able to regenerate whole plants from this callus. Squash plants have also been regenerated from shoot apices (Chee, 1991) and cotyledons (Chee, 1992) by culturing tissue on MS medium supplemented with 2,4-D or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 6-benzylaminopurine (BAP), and kinetin. Leljak and Jelaska (1995) induced somatic embryo formation on zygotic embryos without application of exogenous hormones by first wounding the zygotic embryos and then plating them on MS medium with NH_4Cl as the only source of nitrogen. Gonsalves *et al.* (1995) were able to regenerate squash from somatic embryos induced

on cotyledon of six squash cultivars including three zucchini hybrids, two yellow squash hybrids, and a Casserta inbred.

Regeneration of plants from inbred parental lines is important for the development of transgenic commercial hybrid lines. Tricoli *et al.* (1995) produced embryogenic callus from *in vitro* leaf explants from inbred parental lines of zucchini, yellow crookneck, and Lebanese squash by culturing leaf explants on MS medium supplemented with 1.2 mg l^{-1} 2,4,5-T and 0.4 mg l^{-1} BAP. Kintzios *et al.* (2002) showed that pretreating squash leaves with $186 \mu\text{M}$ kinetin for 48 h followed by transfer to hormone-free medium induced somatic embryo formation on leaf explants. More recently, advances have been made in regenerating *C. pepo* (Kathiravan *et al.*, 2006) and *C. maxima* (Lee *et al.*, 2003) through organogenesis. Adventitious shoots were produced from a very specific segment of the seedling existing at the junction between the cotyledon and hypocotyl (Ananthakrishnan *et al.*, 2003; Kathiravan *et al.*, 2006). Although the number of explants that produced shoots using this method reached 65%, the number of shoots produced per explant for most cultivars was low, ranging from 1.2 to 1.6 shoots per explant. To date, there are no published reports of transgenic squash plants being produced using organogenesis. The probability of transforming tissue capable of regeneration via organogenesis may be low given the highly specific location of the tissue capable of regenerating and the low number of shoots regenerating per explant.

2.1.2 *Cucumis melo*

Stimulation of *de novo* shoot formation from tissues harvested from *in vitro* grown seedlings is the principal method used to regenerate melons. Cotyledon (Moreno *et al.*, 1985; Dirks and van Buggenum, 1989; Niedz *et al.*, 1989; Roustan *et al.*, 1992; Adelberg *et al.*, 1994), hypocotyl (Kathal *et al.*, 1986), roots (Kathal *et al.*, 1994), callus (Bouabdallah and Branchard, 1986), and leaf (Kathal *et al.*, 1988; Dirks and van Buggenum, 1989; Chee, 1991; Yadav *et al.*, 1996) explants have been used for shoot regeneration. BAP in combination with IAA or NAA with or without the addition of abscisic acid (ABA) are the most



Figure 7 *Cucumis melo* regeneration from cotyledon explants. Organogenic callus developing from cotyledon explant (upper left), shoot-bud formation (upper right), shoot elongation (lower left), and root formation (lower right)

common plant growth regulators used to stimulate organogenesis, especially when cotyledon tissue is employed (Figure 7). Regeneration is also observed when BAP is replaced with kinetin or 6- γ,γ -dimethylallylaminopurine (2ip). However, regeneration frequencies are often reduced (Kathal *et al.*, 1988). Shetty *et al.* (1992) found that when added to the regeneration medium, 10 mM proline enhances shoot regeneration and salicylic acid at 50–200 μ M stimulates BAP-induced shoot regeneration. For cotyledon-based regeneration, most reports use cotyledons from *in vitro*-germinated seedlings that are younger than 4 days old, with the peak regeneration response seen on cotyledons 1–3 day(s) after germination (Tahar and De Both, 1989). Guis *et al.* (2000) regenerated plants from leaf explants using a combination of BAP and 2ip. Regeneration through somatic embryogenesis has also been achieved in melon. Somatic embryos can be produced from various explant tissues on medium containing 2,4-D, with or without NAA, in combination with

BAP or thidiazuron (TDZ). Somatic embryos have been produced from cotyledons (Oridate and Oosawa, 1986; Trulson and Shahin, 1986; Branchard and Chateau, 1988; Gray *et al.*, 1993; Guis *et al.*, 1997; Kintzios and Taravira, 1997), hypocotyls (Blackmon and Reynolds, 1982), hypocotyl-cotyledon border (Homma *et al.*, 1991), petioles (Tabei *et al.*, 1991), mature seed and cell suspensions (Oridate and Oosawa, 1986), and protoplasts (Moreno *et al.*, 1985; Debeaujon and Branchard, 1992). Nakagawa *et al.* (2001) found that addition of 0.5 μ M ABA enhanced somatic embryogenesis from cotyledon explants. They also observed that the addition of 150 mM mannitol in combination with 100 mM sucrose or 50 mM mannitol in combination with 200 mM sucrose increased embryo formation compared to 200 mM sucrose alone, possibly by inducing osmotic stress.

Development of somatic embryos into plants with normal morphology can be problematic. Kageyama *et al.* (1991) showed that washing cultures in medium containing 0.5% activated charcoal resulted in the production of greater numbers of normal somatic embryos. Despite substantial research, melon regeneration remains genotype dependent (Oridate *et al.*, 1992; Ficcadenti and Rotino, 1995; Molina and Nuez, 1995). Galperin *et al.* (2003) screened 30 melon genotypes for regeneration capacity and for 24 of these lines no normal shoot regeneration was observed. They identified a line with enhanced transformation regeneration capacity which they designated BU-21/3.

2.1.3 *Cucumis sativus*

Somatic embryos have been produced from leaves (Malepszy *et al.*, 1982; Malepszy and Nadolowska-Orczyk, 1983), cotyledon (Wyszogrodzka and Shahin, 1985; Trulson and Shahin, 1986; Cade *et al.*, 1988; Chee, 1990a), cell suspensions (Chee and Tricoli, 1988; Bergervoet *et al.*, 1989), hypocotyls (Rajasekaran *et al.*, 1983; Chee, 1990a), and protoplasts (Orczyk and Malepszy, 1985; Jia *et al.*, 1986; Colijn-Hooymans *et al.*, 1988; Punja *et al.*, 1990b). Malepszy *et al.* (1982) and Malepszy and Nadolowska-Orczyk (1983) produced embryogenic callus from leaf tissue in

cucumber using 2,4-D or 2,4,5-T in combination with BAP. Punja *et al.* (1990a) regenerated cucumber plants through both organogenesis and embryogenesis using petiole, cotyledon, and leaf tissue. Somatic embryos developed most prevalently on 2,4-D and BAP whereas NAA and BAP or NAA and zeatin resulted in the production of both somatic embryos and organogenic shoots. Preculturing the tissue in the dark was essential for achieving high frequency regeneration. Chee and Tricoli (1988) generated embryogenic cell suspensions from callus produced from leaf explants cultured on 1.2 mg l^{-1} 2,4,5-T and 0.8 mg l^{-1} BAP. The callus was produced on agar-solidified plates and transferred to liquid media of the same formulation to establish a suspension culture. The suspensions were subcultured every 7–14 days and periodically filtered through a 520-micron stainless steel screen to maintain a fine suspension. Washing cells once in MS basal medium with 0.5% charcoal, and twice in MS basal medium enhanced differentiation of embryos into plants. Plating cells and embryos on MS medium with 5% sucrose also enhanced embryo development. Lou *et al.* (1996) found that sucrose concentration influenced the morphogenic response in cotyledon explants. Sucrose levels of 131 mM induced adventitious shoot formation, 263 mM sucrose induced both adventitious shoots and embryos, and 394 mM sucrose produced only somatic embryos.

Regeneration of cucumber through organogenesis has been achieved from leaves (Seo *et al.*, 2000), hypocotyls (Selvaraj *et al.*, 2006), cell suspensions (Bergervoet *et al.*, 1989), and cotyledons (Wehner and Locy, 1981; Kim *et al.*, 1988). Kim *et al.* (2000) developed a high frequency shoot regeneration system from hypocotyls harvested 2 mm below the cotyledonary node of 3–5 days old seedlings cultured on 2.0 mg l^{-1} zeatin. Selvaraj *et al.* (2006) regenerated cucumbers from nodular callus induced from hypocotyl explants on 2,4-D and BAP. Cheng and Chen (2005) found that the addition of polyamines to the medium enhanced adventitious shoot formation from cotyledon explants.

2.1.4 *Citrullus lanatus*

Blackmon and Reynolds (1982) described shoot regeneration from half-cotyledons of “Charleston

Gray 133” using BAP or 2ip. Srivastava *et al.* (1989) regenerated plants from cotyledon and, to a lesser extent from hypocotyls, cultured on MS medium containing BAP. Krug *et al.* (2005) found that cotyledons harvested from 3-day-old seedlings were more responsive than cotyledons harvested from 1–5 day(s) old seedlings. Dong and Jia (1991) were able to regenerate plants from eight watermelon cultivars on MS medium supplemented with 5 mg l^{-1} BAP and 0.5 mg l^{-1} IAA. They found that regeneration potential dropped significantly in cotyledons from seedlings older than 7 days. Compton and Gray (1993a, 1994) extended this work to triploid and tetraploid watermelon. Compton (1999) found that germinating seeds in the dark improved organogenesis from excised cotyledons. For difficult genotypes, enhanced regeneration was obtained from explants that consisted of the whole cotyledon base (Compton, 2000). Somatic embryogenesis has been obtained in watermelon from immature cotyledons but the number of embryos produced per responding explant was relatively low (Compton and Gray, 1993b).

2.2 Plant Transformation

2.2.1 *Cucurbita pepo*

The only published accounts of transformation of *C. pepo* are in patents (Chee, 1997; Tricoli *et al.*, 2002). Chee (1997) describes both *Agrobacterium* and microprojectile-mediated transformation and uses either shoot tips or freshly cut mature seeds. Shoot apices consisted of the apical domes and some supporting tissues removed from young squash seedlings or from cotyledons. Alternatively, tissue from mature seeds was used. Tissue was submerged briefly in an overnight culture of C58 Z707 (Hepburn *et al.*, 1985), blotted dry, and co-cultured abaxial side down on MS medium supplemented with 1.2 mg l^{-1} 2,4,5-T, 0.8 mg l^{-1} BAP, and 0.1 mg l^{-1} kinetin or 5 mg l^{-1} 2,4-D. After 4 days, the tissue was transferred to fresh plates containing the same media formulation supplemented with 500 mg l^{-1} carbenicillin and $100\text{--}200 \text{ mg l}^{-1}$ kanamycin. After 9 weeks, developing transgenic callus was transferred to MS medium supplemented with 0.05 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin, 500 mg l^{-1} carbenicillin, and

100–200 mg l⁻¹ kanamycin. As soon as plantlets developed, the apical region was excised and transferred to half-strength MS basal medium to induce root development. Microprojectile-mediated transformation utilized somatic embryos that were subjected to bombardment with DNA-coated beads. After bombardment, the tissue was transferred to nonselective media for 2 days and then transferred to selection media containing 100–200 mg l⁻¹ kanamycin.

In the protocol used to create the virus-resistant transgenic squash lines ZW-20 and CZW-3 (Quemada and Tricoli, 1992, 1995) commercialized by Asgrow Seed Company (Tricoli *et al.*, 2002), the seed coats were removed and the seeds were surface-sterilized for 20–25 min in 20% solution of sodium hypochlorite containing Tween 20 (200 µl per 1000 ml). Disinfection was followed by three rinses in sterile distilled water (100 ml per rinse). Seeds were germinated in 150 × 25 mm culture tubes containing 20 ml of one-fourth strength MS medium solidified with 0.8% Difco Bacto Agar. After 57 days cotyledons were removed from the seedlings, and the shoot tips were excised and transferred to GA7 vessels (Magenta Corp.) containing 75 ml MS medium solidified with 1.5% Difco Bacto Agar. Cultures were incubated in a growth room at 25 °C, with a photoperiod of 16 h of light. Light was provided with both cool fluorescent (Phillips F40CW) and plant growth (General Electric F40-PF) lamps. Leaf pieces (0.5 cm) were collected from *in vitro*-grown plants and soaked in *A. tumefaciens* broth culture (OD₆₀₀ 0.1–0.2) and transferred to 100 × 20 mm petri dishes containing 40 ml of MS medium supplemented with 1.2 mg l⁻¹ 2,4,5-T and 0.4 mg l⁻¹ BAP (MS-I) with 200 µM AS. Plates were incubated at 23 °C. After 2–3 days leaf pieces were transferred onto MS-I medium containing 500 mg l⁻¹ carbenicillin, 200 mg l⁻¹ cefotaxime, and 150 mg l⁻¹ kanamycin sulfate (MS-IA). After 10 days, leaves were transferred to fresh MS-IA medium. Thereafter, tissue was transferred to fresh MS-IA medium every 3 weeks. After approximately 16–24 weeks, kanamycin-resistant embryogenic callus was harvested and transferred to roller tubes containing liquid MS minimal organics medium supplemented with 500 mg l⁻¹ carbenicillin and 150 mg l⁻¹ kanamycin sulfate and 1.0 mg l⁻¹ CaCl₂ · 2H₂O. Developing embryos were harvested and transferred to MS

minimal organics medium containing 20 mg l⁻¹ AgNO₃. Germinating embryos were subcultured to fresh medium until rooted shoots were obtained. Plantlets were transferred to soil for R₁ seed production.

Published accounts only describe selection of transgenic squash using kanamycin selection. At 150 mg l⁻¹, kanamycin provides effective selection of transgenic callus from leaf explants, and nontransgenic plants that escape selection are extremely rare.

2.2.2 *Cucumis melo*

Despite significant research in the production of transgenic melons, reliable high-throughput production of phenotypically normal transgenic melons remains challenging for a variety of reasons, including the fact that transformation of melon remains highly genotype dependent (Gaba *et al.*, 2004). In addition, although regeneration potential is an important factor contributing to the ability to transform for a particular cultivar, high regeneration frequencies do not necessarily correlate with high transformation frequencies. Nearly 20 years ago, Tahar and De Both (1988) reported on the development of transgenic callus expressing the β -glucuronidase and neomycin phosphotransferase (*nptII*) genes. However, they were unable to regenerate plants. Tahar *et al.* (1989), Fang and Grumet (1990), Dong *et al.* (1991), Valles and Lasa (1994), and Curuk *et al.* (2005) produced fertile transgenic melon plants using *Agrobacterium*-mediated transformation of cotyledon explants with efficiency ranging from 3% to 7%. However, a large percentage of transgenic plants regenerated from cotyledon explants are tetraploid. Bordas *et al.* (1997) compared transformation frequencies using cotyledon versus leaf explants. They were able to produce transgenic melon plants from leaf explants at a frequency of 1.3%; lower than the 3% frequency they obtained from cotyledon explants. Guis *et al.* (2000) produced transgenic melons using leaf explants from 10-day-old seedlings, and in contrast to cotyledon explants, a large percentage of these plants were diploid. Ezura *et al.* (2000) showed that ethylene is produced by excised cotyledons and that production of this hormone is increased further by inoculation with

Agrobacterium. The addition of aminoethoxyvinylglycine (AVG), during co-cultivation increases the frequency of transient expression, suggesting that transformation efficiencies in melon may be increased by inhibiting ethylene production. Curuk *et al.* (2005) found that wounding cotyledon explants by vortexing with carborundum prior to inoculation with *A. tumefaciens* was important for transforming recalcitrant melon cultivars. The strain of *Agrobacterium* can also affect melon transformation efficiency. Based on β -glucuronidase (GUS) expression, Guis *et al.* (1998) were able to produce twofold higher transformation frequency using *Agrobacterium* strain C58 compared to LBA4404. One drawback of using C58 in melon transformation is that counter selection against strain C58 is less effective than against LBA4404, and cultures can often be lost through *Agrobacterium* overgrowth, which can sometimes manifest itself several months after counter selection has been initiated.

Although organogenesis has mainly been used for production of transgenic melon, somatic embryos have also been generated. Oridate *et al.* (1992) examined somatic embryogenesis in 18 melon cultivars and found significant differences in their embryogenic potential. Akasaka-Kennedy *et al.* (2004) produced transgenic melon plants via embryogenesis by pretreating cotyledon explants in liquid MS medium supplemented with 3% sucrose, 1.0 mg l^{-1} 2,4-D, and 0.1 mg l^{-1} BA (EI) for 2 days followed by inoculation with *Agrobacterium*. Explants were then transferred to agar-solidified medium for 4 days in the dark at 25°C . After co-cultivation the tissue was returned to liquid EI medium containing 25 mg l^{-1} kanamycin and 375 mg l^{-1} augmentin. Kanamycin-resistant callus was harvested and transferred to MS medium without growth regulators and supplemented with 50 mg l^{-1} kanamycin. Using this procedure, three transgenic plants were produced from 130 explants.

Historically, the *nptII* gene, which confers resistance to the antibiotic kanamycin, has been preferentially used for melon transformation. However, kanamycin often allows nontransgenic plants to escape selection (Fang and Grumet 1990; Dong *et al.*, 1991). Kanamycin levels ranging from 150 to 750 mg l^{-1} have been used. However, even at elevated kanamycin concentrations, non-

transgenic shoots are often recovered. Recently, Nunez-Palencia *et al.* (2006) used the selectable glyphosate resistance marker gene *CP4*, encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and were able to produce two diploid transgenic melon events. However, they also experienced problems with the production of "escape" plants when selecting with $50 \mu\text{M}$ glyphosate.

Most reports of melon transformation involve the use of *Agrobacterium*. However, using microprojectile bombardment of 3–4 day-old cotyledon explants, Gonsalves *et al.* (1994) were able to achieve transformation frequencies comparable to *Agrobacterium*-mediated transformation in the production of transgenic *C. melo* cv. Topmark containing the coat protein gene of CMV.

Transformation of multiple genes into melon has been achieved. Tricoli *et al.* (2002) produced transgenic melons containing the seven transgenes including the CMV, PRSV, ZYMV, WMV2, SQMV21, SQMV41 coat protein genes, and the *nptII* gene. Seventy-five percent (27/36) of the transgenic melon lines produced using this construct contained all six coat protein genes (Tricoli *et al.*, 2002).

2.2.3 *Cucumis sativus*

Transformation of most of the major types of cucumbers has been achieved, including pickling, slicing, beet alpha, and greenhouse cucumber types. Plants have been transformed using *Agrobacterium rhizogenes* (Trulson *et al.*, 1986), *A. tumefaciens* (Chee, 1990b; Sarmiento *et al.*, 1992; Nishibayashi *et al.*, 1996; Tabei *et al.*, 1998; Tricoli *et al.*, 2002; Rajagopalan and Perl-Treves, 2005; He *et al.*, 2006), and microprojectile bombardment (Chee and Slightom, 1992). Trulson *et al.* (1986) were the first to report on transformation of cucumber. They inoculated hypocotyl sections with *A. rhizogenes* carrying the *nptII* selectable marker gene. Root development occurred on medium without exogenous hormones and by transferring roots to embryo-induction medium, a low percentage of embryos were produced. Transformed cucumbers have been produced using *A. tumefaciens*-mediated transformation of cotyledons (Chee, 1990b; Chee and Slightom, 1991; Tabei *et al.*, 1998), hypocotyls (Nishibayashi *et al.*, 1996) as

well as leaf and petiole tissue (Sarmiento *et al.*, 1992). Rajagopalan and Perl-Treves (2005) demonstrated that the way in which the cotyledon explant is dissected has a significant impact on transformation efficiency. Dissecting the proximal half of the cotyledon using a “V” shaped cut increased transformation efficiencies possibly due to increased exposure of meristematic cells to infection by *Agrobacterium*. Vasudevan *et al.* (2002) found transformation efficiencies in cucumber to be 2–3 times higher with *Agrobacterium* strain EHA105 compared to LBA4404. Chee and Slightom (1992) produced transgenic cucumber via microprojectile bombardment of embryogenic callus followed by regeneration of transgenic plants.

Kanamycin selection has been the most common plant selectable marker gene used for cucumber transformation. Sarmiento *et al.* (1992) found that 91% of the shoots produced from callus generated on 75 mg l^{-1} kanamycin were transgenic. Rajagopalan and Perl-Treves (2005) found that a step-wise increase in the level of kanamycin selection from 100 to 150 and finally to 200 mg l^{-1} reduced the number of false positive, and chimeric shoots regenerated from cotyledon explants. Tabei *et al.* (1994) compared hygromycin and geneticin to kanamycin selection and found that hygromycin selection, especially when used in liquid medium, was highly effective for the selection of transgenic cucumber. Nishibayashi *et al.* (1996) produced transgenic cucumber plants from callus derived from hypocotyl explants cultured on medium containing hygromycin B at 20 mg l^{-1} . Vengadesan *et al.* (2005) regenerated transgenic shoots from cotyledon explants using phosphinothricin (pmi) selection at $2\text{--}6 \text{ mg l}^{-1}$. Recently He *et al.* (2006) generated transgenic cucumbers shoots and plants with mannose selection using 10 g l^{-1} mannose and 10 g l^{-1} sucrose.

2.2.4 *Citrullus lanatus*

Choi *et al.* (1994) reported on *Agrobacterium*-mediated transformation of watermelon from cotyledon explants inoculated with LBA4404 carrying the *GUS* gene and the *nptII* gene, the latter being the most common selective agent used in watermelon transformation (Choi *et al.*, 1994; Tricoli *et al.*, 2002). However, Reed *et al.* (2001) were able to produce transgenic plants using the

pmi selection system and found that preculturing cotyledons on medium containing BAP for 5 days increases the competence of cells to be transformed. Ellul *et al.* (2003) produced transgenic plants from cotyledon explants containing the *Saccharomyces cerevisiae HAL1* gene and these plants exhibited enhanced salt tolerance. Park *et al.* (2005) transformed *C. lanatus* cv. Gongdae, a wild watermelon commonly used as rootstocks onto which commercial varieties of watermelon are grafted. Recently, Hema *et al.* (2004) used electroporation to introduce DNA into zygotic embryos and nodal buds of watermelon under both *in vitro* and *in vivo* conditions. They confirmed the presence of the *GUS* gene in progeny using Southern blot analysis, although complete Mendelian inheritance studies still need to be done in order to confirm stable integration. Chinese scientists have developed transformation procedures in watermelon that avoid the use of antibiotics by facilitating direct gene transfer by microinjection (Tao *et al.*, 1996; Chen *et al.*, 1998; Xiao *et al.*, 1999a, b, c). These and other approaches hold promise for the eventual incorporation of transgenic techniques into conventional breeding programs for plant improvement.

2.3 Regeneration of Whole Plants

2.3.1 *Cucurbita pepo*

Regeneration of whole squash plants from transgenic embryos can be labor intensive. Embryos produced and maintained for long periods on hormone media often are difficult to convert into whole plants. Even after the embryos begin to germinate, the developing plantlet can re-callus, become necrotic or vitrified, and fail to root. After establishment in soil, many transgenic squash plants exhibit a weak phenotype and often produce male flowers with poor pollen formation. These plants are however fully female fertile. Poor pollen set appears to be a byproduct of the long time the plant tissue spends *in vitro* under vigorous hormone treatment. To maintain the germplasm, transgenic female flowers can be pollinated with nontransgenic male pollen and seed set can be achieved. T_0 plants that produce healthy pollen can be self-pollinated or backcrossed to a nontransgenic inbred line and seed production is

normal. T₁ seed from self-pollinated plants or from plants in which the transgenic line serves as the female parent is often smaller than wild-type seed, but T₁ plants produced from these seeds exhibit normal phenotypes, are fully male fertile, and can be self-pollinated without lack of fertility. Seed set on T₁ plants is similar to nontransgenic lines.

2.3.2 *Cucumis melo*

Conversion of organogenic melon buds to shoots occurs at a high frequency, but vitrification of developing shoots can make shoot elongation and rooting problematic. However, the most significant problem associated with regeneration of melon is the occurrence of a high percentage of tetraploid plants (Ezura *et al.*, 1992; Guis *et al.*, 2000). These plants exhibit shortened internodes, increased stem girth, large flowers with deeper yellow pigment, larger pollen grains, and decreased pollen production. Fruits produced from tetraploid plants are flattened at the poles and bulge slightly at their equator. When self-pollinated, these plants produce poor seed set, typically 10% of their diploid counterpart. Successful hand pollination is difficult, and flowers often abort prior to fruit set. The application of lanolin paste containing BAP to the young ovaries at anthesis (Jones, 1965) can improve flower and fruit retention. The seed produced on polyploid plants, although viable, germinate poorly. Attempts to rescue this material via crossing pollen from tetraploid plants with diploid plants produce “hollow seed” that has seed coats but lack endosperm or an embryo (see discussion above on the production of seedless, triploid watermelon). The production of tetraploid plants is more prevalent when cotyledon tissue is employed. The ploidy level in the cotyledon may also be correlated with age. Guis *et al.* (1998) found that cotyledons from quiescent seeds contained mainly diploid cells, whereas cotyledons from 2-day-old seedlings contained 60% tetraploid cells. Tissue sources may increase the percentage of diploid plants. Curuk *et al.* (2003) regenerated shoots from hypocotyl explants from 4 days old seedlings of three melon cultivars, and 99% of the shoots were diploid. Guis *et al.* (2000) regenerated plants from leaf explants and recovered 85% diploid plants. The propensity for regenerating tetraploid plants appears to be independent of

melon type. For instance, inbred lines of western shipping melons can be identified that produce very few tetraploid plants while other cultivars produce a preponderance of tetraploid plants when regenerated from cotyledon explants.

2.3.3 *Cucumis sativus*

Conversion of cucumber somatic embryos into plants often requires further manipulation, including excision of the developing shoot tip from the embryo and transfer to fresh medium (Chee and Tricoli, 1988). Some developing embryos recallus and become impossible to convert into plantlets. As with other cucurbits, developing shoots can often display vitrification, making rooting and acclimatization to soil difficult. Colijn-Hooymans *et al.* (1994) found that the recovery of tetraploid plants increased with the age of the seedling when plants were regenerated through organogenesis from cotyledon tissue. The authors believed this phenomenon to be due to endoreduplication in the cells of the cotyledon prior to regeneration, as a result of a sudden increase in ploidy level in the cells of the cotyledons soon after germination. In contrast, plants produced via somatic embryogenesis from leaf tissue display a normal phenotype and readily produce seeds. Pollen production is normal and application of silver thiosulfate can be used to stimulate the production of male flowers, which can be used to self-pollinate the primary transgenic plant. Virus-resistant transgenic plants were regenerated through embryogenesis using leaf explants for pickling, slicing, and beet alpha type cucumbers and tested in field trials from 1989 through 2002 (ISB, 2007). These plants did not exhibit features indicative of polyploidy plants and other than their high level of virus resistance, appeared comparable to their nontransgenic counterparts in the field (D.M. Tricoli, unpublished data).

2.3.4 *Citrullus lanatus*

Transgenic plant recovery for watermelon can be compromised if cotyledon explants are maintained on medium containing cytokinin for an extended period. However, if cotyledons with developing buds are transferred to medium without plant

growth regulators, elongated shoots are produced. Once shoots reach 15 mm in size, they can be readily rooted on medium containing low levels ($1\text{ }\mu\text{M}$ IAA or $0.54\text{ }\mu\text{M}$ NAA) of auxin. Rooted plants are readily acclimated to soil (Compton *et al.*, 2004). Similar to *C. melo*, polyploid plants are commonly recovered from cotyledon explants of watermelon. Compton *et al.* (1996) found that 5–20% of shoots regenerated from diploid watermelon were tetraploid. In contrast to *C. melo*, tetraploid watermelons have commercial utility in the production of seedless triploids.

2.4 Testing for Activity and Stability of Inheritance of the Transgene; and Adverse Effects on Growth, Yield, and Quality

2.4.1 *Cucurbita pepo*

Tricoli *et al.* (1995) produced numerous transgenic squash lines containing viral coat protein resistance. A large percentage (over 80%) of these plants exhibited resistance to the virus from which the engineered coat protein gene was derived. Plants that were resistant in the T_1 generation remained

resistant in the subsequent generations. Most experimental lines were advanced through the T_2 – T_3 generation, exhibiting predicted Mendelian segregation and no evidence of gene instability. The deregulated lines ZW-20 and CZW-3 (Quemada and Tricoli, 1992, 1995) which were originally produced in yellow crookneck squash inbreds, were incorporated in breeding programs and backcrossed into many different squash types (yellow straightneck, green zucchini, casserta, English marrow, butternut, acorn, grey zucchini, and pumpkin) with no loss of efficacy (Tricoli *et al.*, 1995). Using embryo rescue, the alleles were also transferred from *C. pepo* to *C. moschata*, again with no loss of resistance. Nutritional composition of transgenic *C. pepo* remained unaltered (Quemada and Tricoli, 1992, 1995). Transgenic lines with single, double, and triple viral resistance were advanced to field trials and showed no adverse effects on yield or quality when grown under nonviral conditions and, as expected exhibited significantly greater yield and quality when grown under viral pressure (Fuchs and Gonsalves, 1995; Tricoli *et al.*, 1995; Fuchs *et al.*, 1998a, 1999; Figure 8). No difference was observed in time to flowering, fruit shape, susceptibility to powdery mildew, cucumber beetles, whiteflies, or



Figure 8 Asymptomatic transgenic squash segregant containing the CMV, WMV 2, and ZYMV coat protein genes (left) compared to a nontransgenic segregant (right)

aphids (Tricoli *et al.*, 1995). Percent emergence and days to flowering were similar for the transgenic and nontransgenic hybrids (Arce-Ochoa *et al.*, 1995). Nearly, all transgenic lines tested in the field exhibited normal plant architecture and growth. However, one line was observed in the field with increased bushy plant architecture. This trait appeared to be due to somaclonal variation and did not cosegregate with the transgene. The vast majority of transgenic squash lines produced exhibited a high level of virus resistance when the coat protein gene(s) were present in the hemizygous state, allowing virus-resistant hybrids to be readily produced. However squash line A127-1-2 transformed with the coat protein genes from SqMV mimicked the response of a single recessive gene (Provvidenti and Tricoli, 2002). Transgenic *C. pepo* has been in commercial production in the United States since 1995.

2.4.2 *Cucumis melo*

Field tests have been conducted with male sterile, long shelf life and virus-resistant transgenic lines (ISB, 2007). Field testing of virus-resistant transgenic melon lines showed no detrimental effects associated with the transgene. Nutritional analysis conducted on fruits harvested from transgenic and control lines showed no alteration in any of the nutritional components measured. However, in contrast to squash, high levels of virus resistance were only achieved in plants that were homozygous for the transgenes (Clough and Hamm, 1995; Fuchs *et al.*, 1997). Plants homozygous for the CMV, ZYMV, and WMV2 coat protein genes (designated CZW-30) never exhibited systemic symptoms of virus, and symptoms that did develop late in the season were the result of a single infection of one of the three viruses present in the field as opposed to mixed infections seen in the control lines. Hemizygous lines derived from CZW-30 developed systemic symptoms late in the season but still exhibited a 7.4-fold increase in fruit yields compared to control lines (Fuchs *et al.*, 1997).

2.4.3 *Cucumis sativus*

Field trials have been conducted on transgenic cucumbers engineered for viral resistance, herbicide

tolerance, and increased salt tolerance. Slightom *et al.* (1990) and Gonsalves *et al.* (1992) tested cucumber lines Poinsett 76 expressing the CMV coat protein gene for resistance to CMV. These plants were compared to nontransgenic Poinsett 76 and the traditionally bred CMV-resistant line, Marketmore. The level of CMV infection was determined at the end of the trial by enzyme-linked immunosorbent assay. The transgenic lines had significantly lower rates of infection than either Marketmore or nontransgenic Poinsett 76. Virus-resistant lines of pickling, slicing, and beet alpha types were tested for resistance to virus in the field using paired plot designs in which each transgenic line was paired with its nontransgenic counterpart. Transgenic lines containing the coat protein genes from CMV, ZYMV, WMV2, and the nuclear inclusion protein A and/or B from PRSV exhibited high field resistance against all four viruses, including PRSV (Tricoli *et al.*, 2002).

2.4.4 *Citrullus lanatus*

Field trials have been conducted on transgenic watermelons containing genes designed to confer parthenocarp and virus resistance (ISB, 2007). Virus-resistant lines containing the coat protein genes from ZYMV and WMV2 were mechanically inoculated with both viruses and displayed resistance to both ZYMV and WMV2 (Tricoli *et al.*, 2002). Control vines were highly symptomatic producing very few fruits and the fruits that were produced were very small in size.

2.5 Regulatory Status of Transgenic Cucurbits

Cucurbits are one of the few families of plants that thus far have examples of commercialized and commercially viable transgenic varieties. Two transgenic squash lines have been approved for commercial planting in the United States and have been reviewed for food safety in the United States and Canada. In the United States, the regulation of field trials is primarily the responsibility of the US Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS), and in some cases where plants are transformed with genes to confer pest and disease resistance and field trials exceed 10 acres, the Environmental

Protection Agency (EPA) as well. Food safety is assured by the consultation process established by the US Food and Drug Administration (FDA). In Canada, approval for use as food is granted by Health Canada.

The first transgenic line of *C. pepo* received deregulated status from the USDA-APHIS in 1994 (US Department of Agriculture, 1994), and has been on the market since 1995. This line was resistant to ZYMV and WMV2. A second transgenic line, resistant to CMV, ZYMV, and WMV2, received deregulated status 2 years later (US Department of Agriculture, 1996). Both lines also completed the US FDA consultation process (US Food and Drug Administration, 1995, 1997). Both lines of transgenic *C. pepo* were also approved for use as food in Canada (Health Canada, 1999a, b). Because the US EPA has jurisdiction over plants producing compounds that confer resistance to pests or disease, termed "Plant Incorporated Protectants" (PIPs), the virus coat proteins expressed in transgenic *C. pepo* also required an exemption from tolerance from that agency as well. A tolerance exemption for WMV2 and ZYMV coat proteins in one transgenic squash line was granted (US Environmental Protection Agency, 1994). The agency then granted a broader exemption for these coat proteins in or on all raw commodities in the United States (US Environmental Protection Agency, 1997a), along with similar broad exemptions for the coat proteins of CMV (US Environmental Protection Agency, 1997b) and PRSV (US Environmental Protection Agency, 1997c).

Because the squash hybrids developed from the two approved transgenic lines were not developed for Canadian conditions, only applications for food use have been applied for and granted in that country (Health Canada, 1999a, b). Therefore, while transgenic squash can be imported into Canada as a food product, it cannot be planted for production there. Elsewhere in the world, applications for regulatory approval of transgenic squash have not been submitted to any other regulatory agency. Therefore, import and planting of transgenic squash seed by other countries is not permitted without specific permits from those importing countries. International agreements covering the movement of genetically engineered organisms also apply to transgenic squash, as they would to other transgenic seeds or commodities.

Thus, the Cartagena Protocol on Biosafety, as well as other international agreements that have provisions that encompass transgenic crops, such as the International Plant Protection Convention, also cover the international movement of transgenic squash seed or squash fruit for human or animal consumption.

A detailed description of the safety data and evaluations of the two transgenic squash lines approved by US agencies has been published elsewhere (Quemada, 2002). Additional studies, while not submitted in connection with regulatory approvals, have further some potential risks posed by transgenic cucurbits. For example, Fuchs *et al.* (1998b) studied the potential for the coat proteins expressed in transgenic squash (and melons) to enable the transmission of nonaphid transmissible CMV. These studies showed that transgenic plants expressing coat proteins of aphid transmissible strains of CMV did not mediate the aphid transmission of nonaphid transmissible strains. Similar results were obtained in experiments involving transgenic squash plants expressing coat proteins of aphid transmissible ZYMV inoculated with nonaphid transmissible ZYMV strains (Fuchs *et al.*, 1998b). Further studies have shown that commercialized transgenic squash affect the pattern of the spread of virus infections in the field by preventing secondary plant-to-plant spread of viruses (Klas *et al.*, 2006).

3. FUTURE ROAD MAP

Previous sections in this chapter describe the considerable efforts spent by research groups in both public and private sectors to develop methods for insertion of genes into cucurbits via genetic engineering methods, and the limited examples of success in commercializing these plants. Nevertheless, the potential for significant contributions to the improvement of cucurbit crops via genetic engineering remains great. Whether this potential will be realized depends upon a number of factors. One important factor affecting the ability of the private and public sector, especially the latter, to develop further transgenic cucurbit varieties is the significant barrier presented by regulatory requirements that must be fulfilled before commercialization of these crops. The human effort and expense

required for regulatory compliance have resulted in an unfavorable cost/benefit picture for the development of transgenic varieties of small acreage, “specialty” crops (Fernandez and Smith, 2004). These crops, which include vegetables and fruits, do not generate sufficient profit to justify the significant investment in regulatory compliance, and as a consequence, they have not been the focus of commercial development in recent years.

Another possible barrier to realizing the full potential of transgenic technology for improving cucurbit crops may be the reluctance of the public to consume fresh or raw genetically engineered commodities, especially in countries outside the United States. This barrier has thus far not been a major problem in the commercialization of transgenic squash, but the low level of publicity surrounding this crop may be responsible for this situation. The reaction of the public to more popular cucurbit crops such as melons, watermelons, or cucumbers may prove to be different. This reaction will depend in part upon the success of researchers and educators in providing accurate, credible information about transgenic crops in general.

While the potential for transgenic varieties remains largely unrealized in this family of crops, awaiting a more favorable climate for commercialization, other agricultural biotechnologies such as marker-assisted breeding are making significant contributions. Because of the increasing need for crop improvement in order to help meet the needs of an increasing world population not only for increased food quantity but accompanying nutritional quality, researchers involved in crop improvement will need to use all tools available to them in order to meet these needs within the context of more sustainable agricultural practices.

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Alliums

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1. INTRODUCTION

1.1 History

The cultivation of onion (*Allium cepa*) was first documented 4000 years ago by the ancient Egyptian civilizations (Hanelt, 1990). Since then, over 750 species of *Allium* have been recorded (Stearn, 1992) and the *Allium* species that have become agriculturally important are cultivated extensively for consumption worldwide (Table 1). Their popularity is due to unique sulfur metabolism pathways, which give Alliums their characteristic odor and flavor along with numerous health and nutritional benefits (Randle and Lancaster, 2002). Typical features of this genus include fleshy bulbs, basally arranged leaves, and an umbel-like inflorescence. A wide spectrum of these features is present throughout the genus *Allium*. Among cultivated Alliums, bulb characteristics and shaft length in leeks are highly variable but the other organs remain largely undifferentiated. This reflects intensive selection for a variety of bulb types by breeders (Fritsch and Friesen, 2002).

1.2 Industry

In 2004, worldwide production of onion and garlic was 54.8 and 12.9 million tons, respectively, with the total volume of global Allium crops at 69.3 million tons (FAO, 2007). Production of these two major Allium crops occurs globally throughout 175 countries. However, China is by far the largest

production area, with 30% (16.3 million tons) of the world's onion produce and 69% (8.9 million tons) of the world's garlic produce (FAO, 2007). Other notable areas of Allium production include India (4.9 million tons) and the United States (3.8 million tons). Further statistical data for global Allium production can be found on the FAO Web site (www.fao.org).

Global consumption trends show marked differences, with Asian markets dominated by green onion (*Allium fistulosum*) types, while western markets prefer bulb onion. Also, traditional Asian cultures use all parts of the plant, including bulb, leaf, scape, flower, and occasionally roots, for culinary purposes. New Allium cultivars with unique characteristics and flavors developed through a variety of methods, including traditional breeding (e.g., Vidalia onion and red onion varieties) and genetic modification, will add further value to the industry in the future.

1.3 Allium Flavor Compounds

The unique sulfur metabolic pathway of Allium, highly valued for its health and nutritional benefits, is thought to have evolved for storage and transport of carbon, nitrogen, and sulfur and as a natural form of defense against pests and diseases (Lancaster and Boland, 1990; Ankri and Mirelman, 1999; Jones *et al.*, 2004). Most of the sulfur in Alliums is in the form of nonprotein amino acids (S-alk(en)yl-L-cysteine sulfoxides (ACSOs), γ -glutamyl

Table 1 Commercially important *Allium* species and their common names^(a)

<i>Allium</i> species	Common names
<i>A. cepa</i> L.	Onion Shallot Spring onion Potato onion
<i>A. ampeloprasum</i> L.	Leek
<i>A. porrum</i> L.	Kurrat Great-headed garlic
<i>A. fistulosum</i> L.	Japanese bunching onion Welsh onion Spring onion Shallot
<i>A. x proliferum</i> (Moench) Schrad.	Top onion Tree onion Egyptian onion Catawissa onion Wakegi onion
<i>A. chinense</i> G. Don	Rakkyo
<i>A. sativum</i> L.	Garlic
<i>A. schoenoprasum</i> L.	Chives
<i>A. tuberosum</i> Rottl. ex spr.	Chinese chives

^(a)Adapted from Brewster, 1994. © CAB International

peptides, *S*-substituted cysteines, and cycloalliin) rather than as cysteine and methionine as it is in other plants (Randle and Lancaster, 2002). The unique flavor and odor of Alliums is derived from the hydrolysis of ACSOs, which produces pyruvate, ammonia, and volatile sulfur compounds (Randle and Lancaster, 2002). This reaction is catalyzed by the enzyme alliinase (alliin alkyl-sulphenate-lyase, E.C. 4.4.1.4), which is contained in vacuoles within cells and released upon disruption of the tissue (Lancaster and Collin, 1981). Recently, lachrymatory factor synthase, responsible for the evolution of the lachrymatory factor propanthial *S*-oxide from disrupted tissue, has also been identified (Imai *et al.*, 2002).

Four different ACSOs have been identified in Alliums (Bernhard, 1970): (+)-*S*-methyl-L-cysteine sulfoxide, (+)-*S*-propyl-L-cysteine sulfoxide, *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide, and (+)-*S*-(2-propenyl)-L-cysteine sulfoxide (2-PECSO or alliin). Variations in the ratios of these volatile sulfur compounds are responsible for the difference in flavors and odors between *Allium* species (Randle and Lancaster, 2002).

Fructans and quercetins are also important flavor components of Alliums. Fructans are

water-soluble carbohydrates that, during low-temperature storage, are gradually hydrolyzed to fructose for synthesizing sucrose at the initiation of sprouting (Pak *et al.*, 1995). Varying ratios of the soluble carbohydrate component of onion bulbs influence the osmotic potential, sweetness, and storage qualities over a wide variety of cultivars (Sinclair *et al.*, 1995a, b). For example, fructan content is low in sweet and low dry matter varieties of onion and high in storage-type onions such as US cv. "Sentinel" (Salama *et al.*, 1990). Quercetins, such as quercetin 3,4'-*O*-diglucoside and quercetin 4'-*O*-monoglucoside, are the major flavonol glucosides in onion (Price and Rhodes, 1996). Along with health and nutritional benefits associated with these compounds (Keusgen, 2002), these polyphenols are also major contributors to the bitter taste of some onions (Lugasi *et al.*, 2003). The oxidation of quercetins produces the antifungal agent dihydrobenzoic acid in the protective skins of onion (Takahama and Hirota, 2000).

1.4 Allium Cultivation and Traditional Breeding

The intensive breeding regime that has led to the development of the cultivated *Allium* crops in routine agricultural production today has resulted in significant deviations from wild *Allium* relatives. As such, many cultivated *Allium* crops require highly specific agronomic conditions for maximum yields. Onion is predominantly a biennial crop and can be propagated by seeds, bulbs, or sets (Brewster, 1994). Seed is sown in spring or mid to late winter with seedlings emerging in the first 2 weeks after sowing. The first true leaf appears after 4–10 weeks. Bulb formation is initiated in response to specific temperature and photoperiod cues, which vary between cultivars (Brewster, 1994). Bulb onions are broadly divided into three classes (i.e., long, mid, and short day-length/temperature) based on this differentiation. Bulbs aestivate in summer then sprout in autumn to produce flowers the following summer, which set seed in late summer to early autumn. For a comprehensive coverage of onion agronomy the reader is referred to Bosch Serra and Currah (2002). Glasshouse cultivation of onion is possible. However, it is fraught with difficulties as the

hot humid conditions render the plants highly susceptible to disease and provide a predator-free environment for insect pests to thrive.

Although true garlic seeds have been generated (Etoh *et al.*, 1988; Pooler and Simon, 1994), the absence of large sources of true seed garlic has led to the development of an industry heavily reliant on clonal propagation, with its associated pitfalls for this crop (Etoh and Simon, 2002). On the other hand, leek production has, until recently, relied on open-pollinated commercial cultivars with poor uniformity (De Clercq and van Bockstaele, 2002). Recent improvements in leek seed quality have been achieved through the implementation of seed priming, which increases seed germination and the rate of seedling emergence, improves early plant growth, and increases the crop uniformity in the field (De Clercq and van Bockstaele, 2002). In the past, commercial onion breeding lines have been derived from open-pollinated populations with high levels of genetic variation. However, they are increasingly being produced from hybrid lines (reviewed by Sidhu *et al.*, 2004). The development of doubled haploid and inbred onion lines is also currently underway to produce cultivars with much higher levels of uniformity (Bohanec, 2002).

Sterility or poor fertility in interspecific hybrids makes it difficult to transfer characteristics between *Allium* varieties or species. Also, recovering hybrid lines that show the introduced trait while retaining the original desirable characteristics (e.g., bulb onions) can take many years due to the biennial nature of this crop (Cramer and Havey, 1999). Further advances in the development of interspecific hybrids in the future will enable many of these challenges to be overcome. Mapping of molecular markers linked to desirable traits such as specific day-length response, bulb size, pungency, storage ability, hardness, dry matter content, and resistance to pests and diseases in the onion genome has also helped to advance traditional breeding methods (King *et al.*, 1998; Heusden *et al.*, 2000; Martin *et al.*, 2005).

1.5 Justification for Germplasm Modification

The ability to genetically modify onion to express specific traits may overcome some of the difficulties associated with traditional breeding.

Genetic modification would allow the introduction of traits to *Allium* germplasm that would otherwise be extremely difficult or impossible to improve by traditional breeding techniques. The late development of the technology to achieve genetically modified *Allium* germplasm is beneficial, as researchers can take advantage of prior studies performed in other crop species since the 1980s, thereby reducing the risk that this technology will fail to deliver economic returns (Eady, 2001a).

2. CURRENT APPLICATIONS OF ALLIUM TRANSFORMATION

2.1 Herbicide Resistance

Herbicide-resistant onion germplasm has now been developed (Figure 1). This work has concentrated on the use of the *CP-4*-derived gene constructs to confer resistance to the systemic herbicide glyphosate (Eady *et al.*, 2003a). The enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is involved in the production of aromatic amino acids in plants, is inhibited by glyphosate. Tolerance to glyphosate is achieved either through the overexpression of the *CP-4* EPSPS enzyme (Hetherington *et al.*, 1999) or by detoxification of the glyphosate by either the glyphosate oxidoreductase (*GOX*) gene or the glyphosate acetyl transferase (*GAT*) gene. For a detailed review of glyphosate-resistant crops, the reader is referred to Dill (2005). Initial results in onion indicate that the *CP-4*-derived gene is stable and constitutively expressed and is functioning as expected in onion (Davis *et al.*, 2006). Savings in herbicide usage of up to 75% for this crop have been projected (Eady, 2001b). In addition, glyphosate is a short-lived, low-toxicity herbicide compared with many of the persistent toxic herbicides that are currently used.

For experimental purposes, gene constructs containing the *bar* gene have also been introduced to demonstrate that plants resistant to the contact herbicide phosphinothricin can also be produced (Eady *et al.*, 2003a). The *bar* gene encodes a phosphinothricin acetyltransferase enzyme from *Streptomyces hygroscopicus*, which detoxifies the herbicide (Vinnemier *et al.*, 1995).

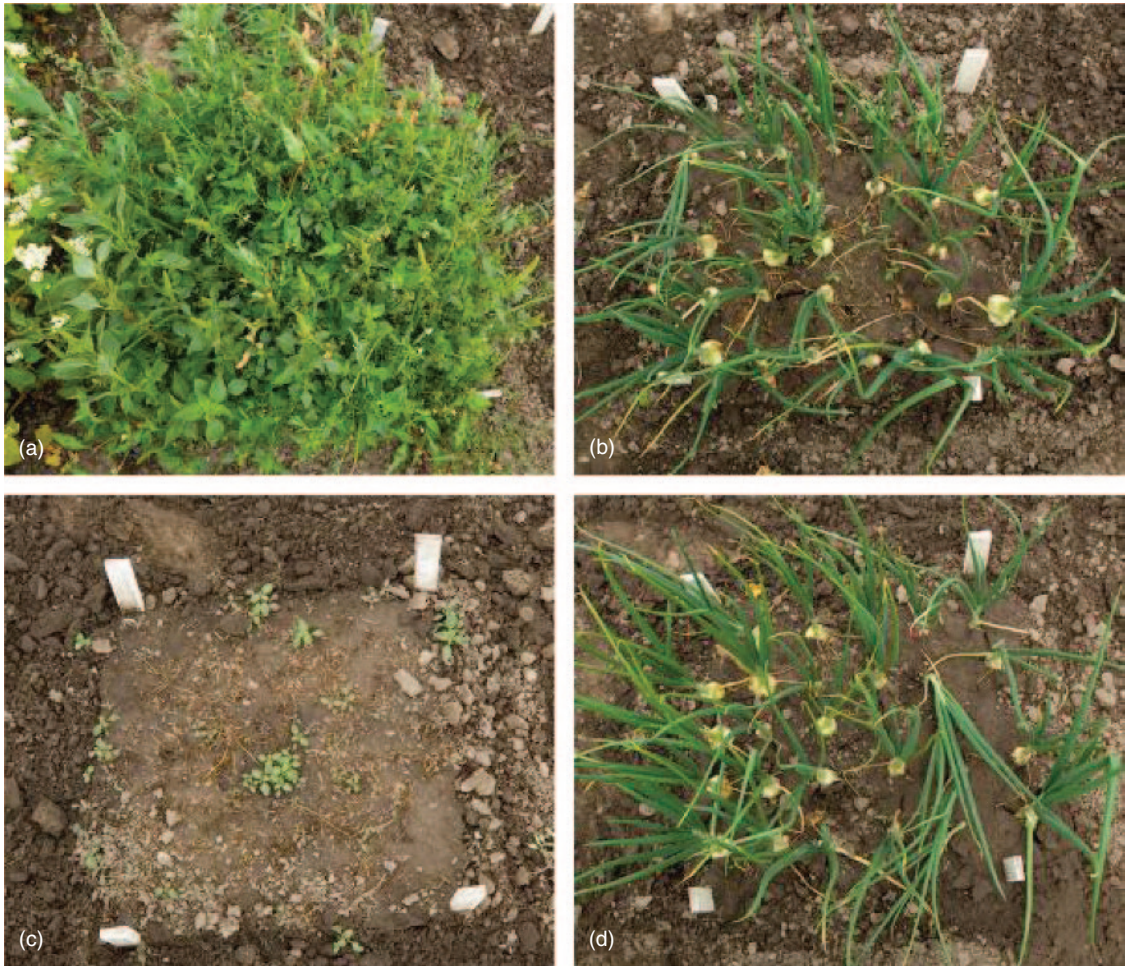


Figure 1 (a) Unsprayed nontransgenic onions; (b) herbicide-resistant germplasm sprayed with 2× recommended glyphosate rate for general weed control; (c) nontransgenic sibling plants sprayed with 2× glyphosate rate for general weed control; (d) nontransgenic siblings, hand weeded

Stable inheritance of this gene into the first filial generation has also been demonstrated (Eady *et al.*, 2003b).

2.2 Disease Resistance

Advances in biotechnology have led to the identification of many genes involved in plant defense mechanisms, and other genes from a broad range of organisms useful for improving plant disease resistance (reviewed by Punja, 2001). Recent research has focused on improving resistance to *Sclerotium cepivorum* (Berk.), the causative agent

of Allium white rot, through overexpression of a germin protein with oxalate oxidase (OXO) activity (Bidney *et al.*, 1999) and the antimicrobial magainin (MGD) peptide (Zasloff, 1987; Zasloff *et al.*, 1988) in onion. OXO (oxalate oxygen oxidoreductase, E.C. 1.2.3.4) degrades oxalic acid, the fungal toxin produced by *S. cepivorum* and many other plant fungal pathogens, to form carbon dioxide, hydrogen peroxide, and oxygen. The concomitant production of hydrogen peroxide further enhances the plant's resistance (Peng and Kuc, 1992; Brisson *et al.*, 1994; Levine *et al.*, 1994). MGD peptides act by preferentially integrating into acidic phospholipid bilayers of microbial

membranes (cf. zwitterionic lipids of mammalian cell membranes), thus destabilizing the cell membranes and causing cell lysis (Matsuzaki, 1998). Future research will use a rapid high-throughput assessment system that has also been developed (Hunger *et al.*, 2002) to demonstrate the effectiveness of these genetic modifications in onion against *S. cepivorum* (Figure 2).

The tospovirus Iris yellow spot virus is an emerging disease of Allium crops that is spreading rapidly (du Toit *et al.*, 2004; Schwartz *et al.*, 2007). DNA sequence information from this virus has been isolated (Pappu *et al.*, 2006), which, in combination with gene silencing technology, could be used to control this disease in the future. Indeed, multiple tospoviruses have already been targeted and successfully controlled in tomato using gene silencing (Bucher *et al.*, 2006).

Garlic mosaic disease, caused principally by the potyviruses leek yellow stripe virus and onion yellow dwarf virus, is another major viral disease of Alliums (Takaki *et al.*, 2005). The development of gene silencing technology to combat this disease in garlic would revolutionize this industry, which currently relies on costly continual regeneration of virus-free meristematic cultures in order to avoid the proliferation of garlic mosaic disease.

2.3 Insect Resistance

Crop Alliums can suffer damage from a variety of insect pests depending on their geographical location (Soni and Ellis, 1990). Some of these pests are generalist grazers, such as thrips, for which it is likely to be difficult to develop a simple genetic basis of resistance. However, for others, such as the beet armyworm (*Spodoptera exigua* Hübner) and onion maggot, it should be possible to develop effective transgenic approaches (Eady, 2002a; Ramputh *et al.*, 2002). Indeed, transgenic garlic containing the *Bacillus thuringiensis cry1Ca* and *H04* hybrid gene constructs produced at the Plant Research International (PRI), Wageningen, showed complete resistance to beet armyworm (Zheng *et al.*, 2004).

2.4 RNA Interference

The sulfur and carbohydrate pathways of the *Allium* genus cause some of the unique nutraceutical qualities ascribed to garlic and onions (reviewed by Block (1992), Hell (1997), Leustek and Saito (1999), and Randle and Lancaster (2002) for sulfur, and Darbyshire and Steer (1990)

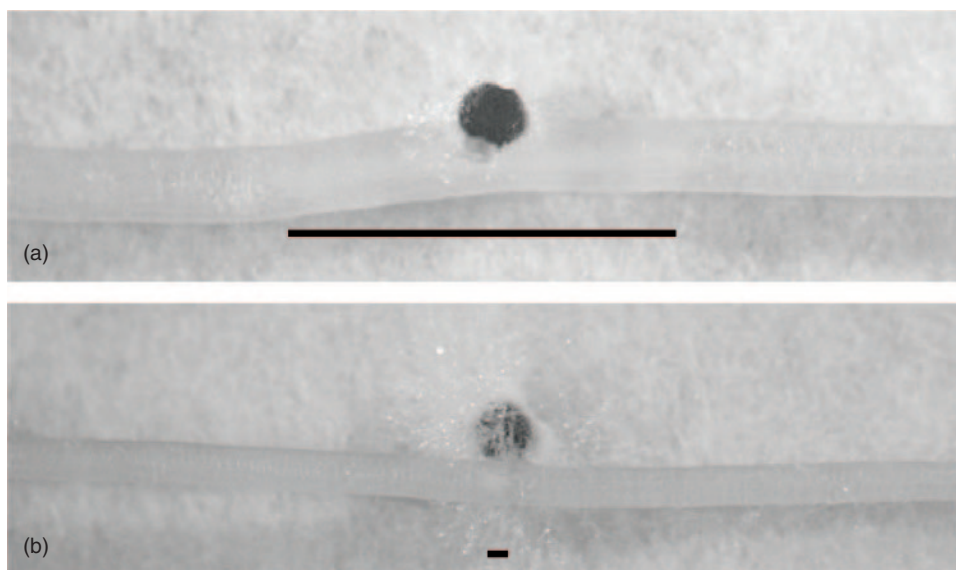


Figure 2 (a) Nontransgenic onion root 24 h after contact with a pregerminated sclerotia; the black bar indicates the oxalic acid induced necrotic zone; (b) transgenic onion root expressing the OXO gene 24 h after contact with a pregerminated sclerotia; the black bar indicates the much reduced necrotic zone

for carbohydrate). Gene discovery programs, dedicated to identifying the candidate genes playing a role in regulation of these pathways, have been established in several laboratories around the world (van Heusden *et al.*, 2000; Galmarini *et al.*, 2001; McCallum *et al.*, 2001). Candidate genes identified as being involved in these pathways are being used to build RNA interference (RNAi) constructs in order to silence specific genes in the onion genome so that their role in sulfur and carbohydrate regulation can be determined. Plants are currently being developed with RNAi constructs directed against serine acetyl transferase and γ -glutamyl cysteine synthetase genes in the S-pathway and sucrose phosphate synthetase in the carbohydrate pathway (C.C. Eady and J.A. McCallum, personnel communication). To date only one gene, the *alliinase* gene, has been silenced using antisense technology (Eady, 2002b). Although some of these lines appeared to have reduced pungency, the phenomenon was also linked to low solids content, which made quantifying pungency difficult.

2.5 Targeted Transgene Expression

Expression of transgenes in transgenic plants is generally driven by constitutive promoters of viral origin, which produce high levels of gene expression throughout all plant tissues, e.g., cauliflower mosaic virus 35S (CaMV 35S) promoter (Kay *et al.*, 1987), or cestrum yellow leaf curling virus promoter (Stavolone *et al.*, 2003). Our group is currently investigating a promoter that could potentially produce high levels of disease resistance transgene expression in the roots of onion plants at the site of *S. cepivorum* infection. The use of a native onion promoter to drive transgene expression in onion would be desirable because much of the debate surrounding the use of genetic modification to enhance food crops focuses on the introduction of foreign transgenes into an organism. The newly emerging field of intragenics, in which all of the genetic material used to enhance crop species by genetic modification derives from the host plant genome (Conner *et al.*, 2006), addresses this issue directly. Intragenics redefines genetically modified organisms so that they can no longer technically be considered “transgenic” (Conner *et al.*, 2006).

2.6 Overview of Allium Transformation

Allium species have proven recalcitrant to genetic transformation and regeneration (Eady, 1995; Eady *et al.*, 1996). This is attributed to their unusually large genome (15 290 Mbp/1C), which is 107 times larger than *Arabidopsis* (115 Mbp/1C; Arumuganathan and Earle, 1991), the limited tissue types from which plants will regenerate, low transformation efficiencies, and the precision required for post-transformation selection protocols (Eady, 2002a). As such, this genus is amongst the last commercially important vegetable genera for which gene transformation protocols have been developed. Despite these caveats, DNA has been successfully delivered into onion (Klein *et al.*, 1987; Dommissie *et al.*, 1990; Eady *et al.*, 1996). However, these techniques did not succeed in producing transgenic onion plants. To date, efforts to develop routine transformation protocols have largely focused on onion, the most economically important of the three main cultivated *Allium* species (Eady, 1995; Eady *et al.*, 1996; Barandiaran *et al.*, 1998). Transformation protocols have also been developed for leek (Eady *et al.*, 2005) and garlic (Kondo *et al.*, 2000; Eady *et al.*, 2005). However, these are still in their infancy.

Since monocotyledons are not natural hosts of *A. tumefaciens*, direct DNA delivery to onion tissue through biolistic transfer was initially investigated (Eady *et al.*, 1996). However, low transformation efficiencies and reports of unusual transfer-DNA (T-DNA) integration patterns using biolistics (Songstad *et al.*, 1995) resulted in limited success using this approach (Eady *et al.*, 1996). Later, more precise, stable T-DNA integration into monocotyledonous plants by *A. tumefaciens*-mediated transformation was developed (Hiei *et al.*, 1997). This was the catalyst for the development of the first successful routine transformation protocol for onion by Eady *et al.* (2000). Since then, there have been several further reports of *Agrobacterium*-mediated transformation of onion (Zheng *et al.*, 2001b; Bastar *et al.*, 2003; Aswath *et al.*, 2006). However, the protocol developed by Eady *et al.* (2000) is the only routine onion transformation protocol available for production of transgenic onion plants through the selection and proliferation of transgenic embryogenic cultures.

The consistent yield of transformants, albeit at low efficiencies (<2.75%), from the protocol of Eady *et al.* (2000) has been attributed to the use of immature embryos. Immature embryo cells are very plastic with regard to regeneration and differentiation (Eady *et al.*, 1998) and have a cell wall structure that is thought to be more amenable to *Agrobacterium* attachment than the cell walls of other monocotyledonous tissues (Mankarios *et al.*, 1980; Dommissie *et al.*, 1990). These cells are transformed after wounding and co-cultivation with *A. tumefaciens*. In this system, the initial transgenic cells are dependent on the surrounding nontransgenic tissue (Eady, 2002a). Thus, the stringency of the selective agents applied during the recovery of transgenic onion cultures is critically important (Eady and Lister, 1998).

Other researchers have transformed alternative tissue types with limited success. Zheng *et al.* (2001b) transformed 3-week-old cultures produced from zygotic embryos extracted from mature seed and achieved a maximum transformation rate of only 0.53% in onions and up to 1.95% in shallots. Bastar *et al.* (2003) transformed organogenic structures precultured from onion ovaries but obtained chimeric plants showing transgene expression in superficial tissue layers only (Bohanec, 2003). Aswath *et al.* (2006) transformed embryogenic callus from seedling radicle using both *Agrobacterium*-mediated and biolistic DNA transfer. Although these authors report a phenomenally high rate of plantlet regeneration (27 and 23%, respectively; Aswath *et al.*, 2006), they present no picture of phenotypically normal, mature transgenic onion plants.

2.6.1 Onion transformation protocol

The transformation procedure outlined below is used routinely in the Crop & Food Research Laboratory at present and is based on that developed by Eady *et al.* (2000). Although the rate of mature transgenic plant regeneration is low using this protocol (up to 2.7%), it is reliable and reasonably consistent on a seasonal basis. For other onion transformation protocols in the literature, the reader is referred to Zheng *et al.* (2001b) and Aswath *et al.* (2006).

2.6.1.1 Bacterial strain and plasmids

Agrobacterium tumefaciens strain LBA4404, with the binary vector pBIN or pCambia derivatives, and possessing within its T-DNA the selectable genes *nptII* (neomycin phosphotransferase II) or *hyg* (hygromycin) and the visual reporter genes *uidA* (β -glucuronidase) or *m-gfpER*, were used in Allium transformation experiments. Transgenic onion plants containing genes that confer resistance to the herbicides glyphosate or phosphinothricin were also introduced into onion (Eady, 2002b). To generate glyphosate-tolerant plants, *A. tumefaciens* strain ABI was used, containing a binary vector that included the *CP-4*-derived gene within the T-DNA (kindly supplied by Monsanto Corp.). To generate plants tolerant to phosphinothricin, the *A. tumefaciens* strain LBA4404 that contained a binary vector pCambia 3301 (from Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra) with the *bar* gene within the T-DNA was used. In experiments to date, expression was driven by either the CaMV 35S (Eady, 2002b) or *nos* (nopaline synthase) promoter (Zheng *et al.*, 2001b).

2.6.1.2 Transformation procedure

Day 1. *Agrobacterium* cultures were initiated by inoculating 50 ml of Luria broth (LB) media containing appropriate selective agents, in a 100 ml flask, with 1 ml of frozen stock (0.7 ml of actively growing culture plus 0.3 ml of 50% glycerol stored in 1 ml aliquots at -80°C), then incubated overnight at 28°C with agitation at ~ 125 rpm.

Field-grown umbels of *A. cepa* L. were used as a source of plant material from which approximately 12 g of immature seed was isolated. The isolated seed was stored overnight at 4°C in a 50-ml vial covered with wet tissue.

Day 2. The *Agrobacterium* cultures were replenished with an equal volume of LB containing antibiotic and $100\ \mu\text{M}$ acetosyringone (virulence gene inducing factor), then incubated at 28°C with agitation at ~ 125 rpm for a further 4–5 h. The optical density was adjusted to approximately 0.4 at 550 nm by the addition of LB. After the immature embryos were isolated (see below), *Agrobacteria* were pelleted in 15-ml falcon tubes

by centrifugation at 4000 rpm, then resuspended in an equal volume of liquid plant culture media, P5, containing 200 μ M acetosyringone.

Isolated immature seeds were washed for 1 min in ethanol and then surface sterilized in 50 ml of 30% household bleach solution, plus 1 drop of Tween-100, for 30 min with gentle agitation at \sim 25–50 rpm. After washing four times in sterile water and draining for 2 min, immature embryos were isolated from the seed by stereo microscope (Eady *et al.*, 1998). Large translucent embryos (2–5 mm in length), isolated from seed that had recently turned black with a liquid endosperm, were the most responsive to treatment. Embryos were isolated in batches of 50 then cut into \sim 1 mm lengths and stored in 50 μ l of liquid P5 (Eady *et al.*, 1998) in a 1.8-ml microfuge tube until \sim 1000 embryos had been isolated.

Four hundred microliters of *Agrobacteria* culture was added to each 1.8-ml microfuge tube, then the tube was vortexed for 30 s. The lid of the microfuge tube was pierced with a sterile scalpel blade, then the tube was placed under vacuum (\sim 25 mmHg) for 30 min. The excess liquid *Agrobacteria* culture was aspirated off using a 200- μ l pipette, then the immature embryo tissue pieces carefully transferred to filter paper and left to drain briefly, in a clump, before being transferred to P5 media and carefully being distributed with fine tweezers over the surface of a 90-mm petridish containing solid P5 medium (two batches per petridish). It was important to make sure that there was no excess moisture on the surface of the P5 media at this point as this caused liquid to pool around the embryo, which reduced embryo survival. After transfer, the petridishes were sealed with household polythene wrap and incubated in the dark at 24–26 $^{\circ}$ C in a growth room.

Day 8. After 6 days' co-cultivation, the embryogenic tissue was transferred to P5 containing timentin (200–250 mg l^{-1}) to kill the *Agrobacteria* and the appropriate selective agents to select for the transgenic tissue. The embryogenic sectors were cultured in the dark under the conditions described above for 12 weeks, transferring to fresh medium every fortnight. The use of timentin could normally be discontinued by week 6–8. However, it was necessary to watch carefully for any latent *Agrobacteria* overgrowth after this time. Clean tissue was immediately transferred to media containing 250 mg l^{-1} timentin in the

event of any observed *Agrobacteria* overgrowth. It was necessary to maintain timentin levels through to transfer to shoot media with experiments using the ABI strain as it was more virulent than LBA4404 and, thus, more prone to overgrowth.

Selection for 4 weeks on 12 mg l^{-1} of geneticin, followed by 8 weeks at 20 mg l^{-1} of geneticin, was found to be optimal for the regeneration of transgenic onion tissue sectors containing the *nptII* gene. The selection regime for transgenic onion tissue sectors treated with the *CP-4*-derived gene comprised 0.025 mM glyphosate and 250 mg l^{-1} timentin. The glyphosate level was raised to 0.05 mM after 6–8 weeks' selection and maintained at this level throughout selection and regeneration. For the selection of phosphinothricin-tolerant tissue, co-cultivated embryos were initially transferred to P5 media containing 5 mg l^{-1} phosphinothricin and 250 mg l^{-1} timentin. The phosphinothricin was maintained at this level throughout selection and regeneration.

From day 8 onward, it was possible to observe any transgenic material containing the *m-gfpER* gene using a fluorescence microscope by observation of the tissue under 440–480 nm excitation and 510 emission (Leffel *et al.*, 1997; Eady *et al.*, 2000). If *uidA* visual reporter gene was used then tissue had to be sacrificed and stained (Eady *et al.*, 1996; Zheng *et al.*, 2001b).

Week 13. After 12 weeks of selection, actively growing material was transferred to regeneration medium (Eady *et al.*, 1998) containing 20 mg l^{-1} geneticin. (This time period could be reduced to 8 weeks if the visual *m-gfpER* marker was also used to identify transgenic tissue.) Shoot cultures were maintained for 12 weeks under 16-h day-length through 3 weekly subculture.

Week 19 onward. Developing shoots were transferred to $1/2$ Murashige and Skoog (MS) media (Murashige and Skoog, 1962) plus selective agents and maintained as above to induce rooting. *In vitro* culture response was varied and some transgenic sectors proliferated to produce hundreds of shoots on regeneration media, whereas others failed to produce shoots or produced only a few. Maintenance for long periods (>6 months) *in vitro* resulted in a decline in regeneration capability and an increase in the degree of hyperhydricity shown by the cultures (Eady *et al.*, 1998). It was possible to induce *in*

vitro bulbs by subculturing the plantlets on $1/2$ MS medium plus 120 g l^{-1} of sucrose (Seabrook, 1994).

2.6.1.3 Ex-flasking and growth in containment

Transferring the primary transformant from *in vitro* culture to the glasshouse is often a technically difficult process. Fortunately, onion plantlets in culture are quite robust and there are numerous reports of successful transfer to the glasshouse (Novak, 1990; Eady, 1995). Rooted plants were carefully washed to remove any attached agar and transferred to a 1:1:1 perlite:bark:compost mix containing 400 g dolomite, 300 g Osmocote, 75 g superphosphate, and 50 g sulphate of potash per 50 l in the glasshouse (12 h, 12–23 °C day; 12 h, 4–16 °C night).

Although ex-flasking has proved relatively easy in onions, maintenance within transgenic containment facilities can be a problem. For correct chilling and flowering, it is preferable to grow them within a shade house. In wet winters and in spring in the wet, the still air in such shade houses is conducive to scarab fly infestations and infection by a host of bacterial and fungal pathogens. Bulb formation in glasshouse-grown plants was induced naturally by increasing the day-length. After 50% of the tops had fallen, bulbs were lifted and air dried. Bulbs were rested for 2 months in a dry environment and then either cold stored to induce floral meristems prior to planting in the spring or replanted in large pots and grown in a containment shade house over winter, spring, and summer, in order to produce flowers.

Transgenic plants were self-fertilized by bagging the umbel (within a microperforated plastic bread bag) as the flowers start to open. Approximately 20–50 flies or ready-to-hatch pupae (*Lucilia cuprina*) were then introduced into the bag and left until all flowers had finished opening (occasionally the introduction of additional flies was necessary). Crossing on to nontransgenic plants was achieved by flowering plants in $1 \text{ m} \times 1 \text{ m} \times 2 \text{ m}$ high cages with flies or bees introduced to effect pollination.

After all the flowers were pollinated, the umbel was left to ripen. As the umbels ripen, the ovary walls containing the seed start to crack open. At this point, the umbels were harvested and air dried

in large 1-mm mesh bags for 1 month. Seeds were then separated from the chaff and either stored (viability drops dramatically in onions unless stored properly) or germinated immediately for further investigation.

2.6.2 Leek transformation

The onion transformation protocol described above was, for the first time, successfully applied to leek immature embryos to demonstrate the stable transformation of leek (Eady *et al.*, 2005).

Immature umbels of leek are more difficult to extract because the ovary wall tissue is tougher and more slippery than onion ovary wall tissue. In addition, when the seeds are at the correct developmental stage, they bleach upon sterilization. This does not affect the viability of the embryo. Initial embryo treatment and co-cultivation followed the onion transformation protocol described in Section 2.6.1.2. Selection and regeneration was performed on geneticin using only one level of selection (12 mg l^{-1}). It should be noted that leek embryo response to P5 media was markedly different to that of onion. The majority of leek embryos rapidly elongated and failed to form an embryogenic culture. This reduction in embryogenic response most probably caused the observed reduction in transformation efficiency in leek compared with onion. Initial gene transfer, as determined by observations of transient *gfp* expression, appeared similar to that observed in commercial lines of onion, but less than that observed in the open-pollinated Canterbury Longkeeper (CLK) lines with which the onion transformation procedure was developed.

2.6.3 Garlic transformation protocol

The onion transformation protocol described above was also successfully applied to immature embryos extracted from true seed garlic (Eady *et al.*, 2005). Two plant lines were produced from four experiments, in which approximately 3200 embryos were isolated, equating to a transformation efficiency of approximately 0.06% (Eady *et al.*, 2005). Garlic immature embryos responded to selection and culture in a similar manner to onions (cf. leek). However, a large proportion

of the immature embryos (approximately 50% in some experiments) failed to produce embryogenic cultures, instead developing elongated cotyledons (similar to etiolated seedlings) in response to the P5 medium. Transgenic embryogenic cultures that regenerated successfully on selective medium eventually formed roots in *in vitro* culture (albeit at a much slower rate than transgenic onion cultures). These plantlets were easily transferred to the glasshouse and developed to maturity, producing both cloves and topsets (Eady *et al.*, 2005). For a garlic transformation protocol that is not reliant upon true seed garlic, the reader is referred to Kondo *et al.* (2000).

2.7 The Challenges of Allium Transformation

2.7.1 Embryogenic material

Although transformation rates in the New Zealand CLK onions are generally better than in other cultivars, it seems that only a small proportion of the highly genetically variable onion embryos used in the experiments have a genetic background that is receptive to T-DNA transfer. CLK is a heterozygous, open-pollinated cultivar in which every embryo is genotypically unique. Therefore, within this gene pool there are presumably certain genotypes that are more receptive to genetic transformation and regeneration. Isolation of receptive genotypes through the rescue of transformable cultures is currently underway. The production of doubled haploid lines from cultures with a receptive genetic background may permit the identification of homozygous lines that are highly receptive to genetic transformation. Genetic transformation into a homozygous genetic background permits detailed analysis of the effect of transgenes on the original plant phenotype, in the absence of naturally high levels of genetic variation, which otherwise make such investigations difficult. Currently, research using inbred dehydrator onion lines is underway to investigate more specifically the effects of introduced genes and the transformation procedure.

Embryo health and the development stage prior to harvest are important factors influencing the seasonal transformation rate. There appears to be an optimal stage of development at which

the embryo is most receptive to transformation. This optimal development stage is difficult to define and isolate specifically due to the low transformation frequency, rapid rate of embryo development *in vivo*, and limited ability to identify suboptimal embryos during the isolation process. Seasonal environmental variations have a large impact on these factors, as the maturation rate of embryos is very rapid in hot conditions, whereas wet, cool conditions promote the development of disease and poor embryo health. Also, onions are problematic to grow in the glasshouse, making it difficult to control seasonal variation between years by growing them in standard conditions.

2.7.2 External variables

External variables such as operator skill and environmental conditions also impact on the embryo transformation rate. Operator skill and minor changes in the wounding process have a significant impact on the rate of transformation as shown by a reduction in the initial rate of transient *gfp* expression after the co-cultivation period in embryos transformed by inexperienced operators. The location of the wound in the embryo is important because it is thought that the shoot meristematic cells and the cells surrounding this area are the most receptive to transformation and regeneration. Therefore, specific wounding of these cells is important for T-DNA transfer (Hiei *et al.*, 1997).

2.7.3 Selection systems

The development of a selection regime is difficult with embryogenic onion cultures, because the transgenic cells are initially dependent on other nontransgenic cell types within the culture. Thus, the selection regime must not kill the nontransgenic tissue in the culture until the transgenic cells can be “weaned” from the supporting nontransgenic cells to become independent transgenic cultures (Todd and Tague, 2001). To date, the herbicides L-phosphinothricin (PPT) and glyphosate (Eady *et al.*, 2003a), the antibiotics geneticin (G418) (Eady *et al.*, 2000) and hygromycin (Zheng *et al.*, 2001b) and the metabolic selectable marker phosphomannose isomerase (Hunger, 2007) have

been successfully used as selection agents to recover transgenic onions.

2.7.3.1 Antibiotic selection

Until recently, the selection of transgenic plants has largely been based on the use of antibiotic markers (Cheng *et al.*, 2004). These are predominantly aminoglycoside antibiotics, such as kanamycin, geneticin, neomycin, paromomycin, and hygromycin, which bind to ribosomes involved in protein synthesis in prokaryotic cells, mitochondria, and chloroplasts (Wilmink and Dons, 1993). Some public groups oppose the use of broad-spectrum aminoglycoside antibiotics in biotechnology. The other disadvantage with this approach is the “leaky” nature of the geneticin selection system in onion, which only works well in conjunction with the *gfp* visual reporter gene (Eady and Lister, 1998). These issues make the use of antibiotic resistance selectable marker genes undesirable in applied research.

Although selection on geneticin is leaky in onion, it is still used routinely as a selective agent in our research group. Geneticin acts by binding to the 30S ribosome, blocking protein synthesis in eukaryotic cells (Wilmink and Dons, 1993). Resistance to geneticin is conferred by the NPTII enzyme. When a chimeric version of the *nptII* gene (Bevan, 1984) is expressed in transgenic plants, members of the aminoglycoside group of antibiotics, which includes geneticin, are disabled by phosphorylation (Wilmink and Dons, 1993).

2.7.3.2 Herbicide selection

Herbicide-based selection is an alternative nonantibiotic, negative selection approach in which tolerance to the herbicides PPT or glyphosate is conferred to the transgenic tissue. Glyphosate is currently the only truly effective selection agent for the regeneration of transgenic onion plants without the concomitant regeneration of escapes (Eady *et al.*, 2003a). Herbicide-based selection is not popular with some public groups, owing to their concerns about the transfer of herbicide resistance traits to weedy plant species. These concerns are largely unjustified in onion due to

poor ability of onion to hybridize or breed with other species (Kik, 2002).

2.7.3.3 Mannose selection

The Positech[®] selection system enables plants expressing the phosphomannose isomerase (*pmi*) gene, derived from the *Escherichia coli manA* gene (Miles and Guest, 1984), to convert mannose-6-phosphate into the usable carbon source, fructose-6-phosphate; by contrast, the phosphorylation of mannose to mannose-6-phosphate by hexokinase in nontransgenic tissue depletes stores of phosphate and adenosine triphosphate (ATP) (Sheu-Hwa *et al.*, 1975). Thus, transgenic tissue proliferates on media containing mannose, whereas nontransformed tissue either stops growing or dies of starvation (Hansen and Wright, 1999). Selection on mannose has been reported to improve the transformation rate severalfold in numerous plant species (e.g., wheat (Wright *et al.*, 2001), maize (Negrotto *et al.*, 2000), and sugar beet (Joersbo *et al.*, 1999).

Optimal levels of selection are essential for a successful transformation protocol (Hiei *et al.*, 1997; Cheng *et al.*, 2004). This is especially true of recalcitrant crops from which the identification and selection of a small proportion of transgenic tissue from large amounts of nontransgenic tissue is required. To date, only negative selection systems that “kill” nontransgenic tissue (e.g., antibiotics and herbicides) have been used to recover transgenic onions. With negative selection, if the selection criteria are too moderate then the nontransgenic tissue will proliferate and outcompete the transgenic tissue, whereas if the selection criteria are too stringent they may be detrimental to the growth of transgenic tissue. Identifying appropriate stringency for selection is very important in the embryogenic culture system used in this research, in which transgenic tissue is initially dependent upon surrounding nontransgenic tissue (Eady *et al.*, 2000).

Selection on mannose is based on the premise that transgenic tissue with the ability to use this compound as a carbohydrate source will be positively selected for and outcompete nontransgenic tissue that is unable to metabolize mannose. The accumulation of mannose within the plant cell

itself is not directly toxic, but the depletion of phosphate stores as the mannose is phosphorylated to mannose-6-phosphate eventually starves the cell to death (Hansen and Wright, 1999). However, the presence of other metabolizable sugars in the growth medium ameliorates the starvation effect (Joersbo *et al.*, 1999; Wright *et al.*, 2001). Other nonantibiotic positive selection systems do exist, including those based on benzyladenine N-3-glucuronide (Joersbo and Okkels, 1996), 2-deoxyglucose (Kunze *et al.*, 2001), and xylose (Haldrup *et al.*, 1998). However, mannose-based selection is better than other methods as it is more economical, is functional as an additive in both *in vitro* growth medium and soil (Todd and Tague, 2001), is not toxic or allergenic (Reed *et al.*, 2001), and is reported to significantly increase transformation efficiencies (Wright *et al.*, 2001).

Determining the minimum concentrations of mannose required to prevent regeneration of nontransgenic onion plants was used to develop a selection regime for onion transformation experiments. Initially, 2.5–5 g l⁻¹ of sucrose was included with 10 g l⁻¹ mannose as in other mannose-based selection regimes (Joersbo *et al.*, 1999). However, although it was possible to discern a difference between embryogenic cultures and plantlets grown with various levels of mannose and sucrose and those grown on sucrose alone, it was not sufficient to identify transgenic tissue from nontransgenic tissue. Indeed, the repression of onion tissue growth by mannose appeared to have been reversed by the addition of sucrose as observed by Joersbo *et al.* (1999). The most effective growth inhibition of onion tissue was observed on growth medium containing 15 g l⁻¹ mannose without sucrose. However, growth of nontransgenic embryogenic cultures was still evident on this medium, albeit at a slower rate than that of clonal embryogenic cultures grown on sucrose. Also, there were no obvious phenotypic differences between the two sets of clonal cultures grown on each type of medium. Thus, it became apparent that nontransgenic tissue cultured on mannose was largely insensitive to this selection. Based on these observations, a selection regime of 15 g l⁻¹ mannose without sucrose from 2-week post-transformation to rooting was developed, which permitted the regeneration of transgenic plants with the concomitant regeneration of escapes.

The only apparent explanation for the growth of nontransgenic onion embryogenic cultures on mannose in the absence of other metabolizable sugars and photosynthesis is the metabolism of mannose. Thus, it seems feasible that endogenous enzymes may be active in onion tissue. However, the presence of such enzymes remains highly speculative in the absence of DNA sequence data. Tolerance of mannose has also been reported in other crop species such as carrot, tomato, tobacco, soybean, and a few other legumes (Aragão and Brasileiro, 2002). This was associated with endogenous PMI activity in the soybean and legumes (Gao *et al.*, 2005).

Many reports of selection of transgenic plants on mannose are favorable, with researchers reporting improved transformation efficiencies with this strategy in wheat (Wright *et al.*, 2001), maize (Negrotto *et al.*, 2000), sugar beet, (Joersbo *et al.*, 1999), pearl millet (O'Kennedy *et al.*, 2004), and sorghum (Gao *et al.*, 2005). However, our observations suggest that, while it is possible to recover transgenic onions using this system, it is problematic (Hunger, 2007). The Allium transformation research group from PRI, Wageningen, also reported problems with this system in onion and shallot (S.-J. Zheng, personal communication). A recent publication reports successful selection of transformed onion expressing the *pmi* transgene on mannose (Aswath *et al.*, 2006). However, Aswath *et al.* (2006) did not confirm the production of mature, healthy transgenic plants with this system.

2.7.3.4 Visual reporter genes

The use of a visual marker gene, such as the gene for green fluorescent protein (*m-gfpER*; Haseloff *et al.*, 1997), enables the development of transgenic tissue sectors to be monitored ensuring that cell proliferation in the transgenic sector has become established prior to exposure to more stringent selection conditions. The *m-gfpER* gene cassette targets GFP (green fluorescent protein) to the endoplasmic reticulum, where it accumulates, resulting in high levels of fluorescence when visualized under fluorescent light (395 nm excitation, 509 nm emission) (Haseloff *et al.*, 1997). Destructive sampling and lengthy staining protocols that limit other marker genes such as

the gene for the β -glucuronidase (GUS) enzyme (Jefferson, 1987) and luciferase (Van Leeuwen *et al.*, 2000) are not necessary.

The OXO gene was used as a reporter gene in onion using a staining technique described by Simmonds *et al.* (2004). Plant tissue expressing OXO was stained purple by the oxidation of oxalate into hydrogen peroxide and carbon dioxide, which yields a purple precipitate in a linked reaction by peroxidase in the presence of 4-chloronaphthol. Staining was observed after 5 min in tissue with high levels of OXO expression, such as germinating wheat grains, which were used as a positive control (Hunger, 2007). Although the stain intensity is directly proportional to the quantity of OXO present in the plant tissues, the quantity of OXO in plant tissues required for protection against invading pathogens has not yet been defined. To date, researchers have used *in vitro* spectrophotometric methods to measure the amount of OXO present in plant tissues and reported this value with the improvement in resistance to infection (Donaldson *et al.*, 2001).

2.7.4 Regeneration

High initial transformation rates in onion are often significantly reduced during abnormal plantlet regeneration *in vitro*. This is reflected in the onion transformation literature in which all onion transformation groups report the production of only a few plant lines from numerous transformation initials (Zheng *et al.*, 2001b; Bastar *et al.*, 2003; Aswath *et al.*, 2006). Many transgenic sectors of embryogenic tissue formed roots on shooting medium in our research. These structures seldom produce normal shoots under these circumstances. It is not clear why this occurs, although genotype and the duration of *in vitro* regeneration (as shown by the increasing difficulty of recovering phenotypically normal plants from older cultures) are also likely to be important variables in this process.

Several systems for the recovery of transgenic onion plants are reported in the literature (Eady *et al.*, 2000; Zheng *et al.*, 2001b; Bastar *et al.*, 2003; Aswath *et al.*, 2006). Zheng *et al.* (2001b) regenerated four onion lines showing sporadic transgene expression from approximately 1200 *A. tumefaciens*-treated cultures derived from

precultured mature zygotic embryos, and chimaeric transgenic onion plants regenerated from *A. tumefaciens*-treated organogenic structures formed on onion ovaries by Bastar *et al.* (2003). Aswath *et al.* (2006) reported the regeneration of somatic embryos from precultured seedling radicles, but did not report the number of phenotypically normal plants regenerated, and the constitutive nature of transgene expression in the putative transgenic onion plantlets developed by that group cannot be confirmed without the presence of a visual reporter gene.

To date, the most consistent method of regenerating transgenic onion plants showing constitutive transgene expression uses immature embryo-derived cultures (Eady *et al.*, 2000, 2003a; Eady, 2001a). As such, immature embryos from bulb onion cv. "CLK" extracted from seed with a recently blackened seed coat and liquid endosperm were selected for *A. tumefaciens*-mediated transformation in this research. At this stage of development, wounded, immature CLK embryos have proven to be receptive to genetic transformation and *in vitro* regeneration (Eady *et al.*, 2000). Wounding induces rapid cell division and DNA synthesis, and exposes receptive cells to *A. tumefaciens*. The addition of exogenous *vir* gene activators in the form of acetosyringone enhances the infection of nonhost onion tissue by *A. tumefaciens* (Dommissie *et al.*, 1990).

2.7.5 Ex-flasking

The transfer of onion plants from *in vitro* culture to soil in the glasshouse is highly successful. However, glasshouse conditions can significantly increase pressure from pests and diseases. Light and thermal units are also not optimal for all cultivars, making cultivation to maturity in the glasshouse, and the curing and storage of bulbs, particularly difficult. Losses due to pests and disease are difficult to prevent once such a problem has established. Problems such as these have not been resolved and are thought to be an integral part of transgenic onion cultivation in a glasshouse situation. Onions do not generally thrive in glasshouse conditions and the often compromised state of tissue culture material exacerbates the problem.

2.8 Analysis

2.8.1 Transgene detection

Initially, the presence of the transgene in putative transgenic onion tissue was screened using polymerase chain reaction (PCR) in order to amplify specific fragments of a particular transgene. For both the *bar* and *pmi* genes, we have been unable to specifically detect the presence of the transgene fragment without also obtaining very faint positive bands within nontransgenic onion samples. Despite a large difference in the degree of amplification (at least 10-fold), this uncertainty has led us to conclude that in onion, under our laboratory conditions, PCR cannot be used to conclusively demonstrate the presence of transgene fragments. However, it is routinely used to screen for CP4, *nptII*, and *m-gfpER* genes.

Currently, transformants are usually confirmed by Southern analysis (Southern, 1975) following the protocol of Eady *et al.* (2000). However, Southern analysis is expensive and time consuming. In cases of clonal propagation of a particular transgenic culture, it is necessary to confirm that all clones are derived from the same transformation event. In such a case, in which over 100 clones could easily be produced, Southern analysis would be inappropriate. For this scenario, and for the rapid easy fingerprinting of transgenic lines, our group has applied thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995) to enable the detection of transgenic lines (Eady *et al.*, 2005). TAIL-PCR consists of three sequential PCR reactions using nested primers from the known inserted DNA sequence directing amplification toward the unknown flanking region, and a particular arbitrary degenerate primer to amplify back from the unknown region. We have designed TAIL-PCR specific nested primers and identified an arbitrary degenerate primer that can efficiently amplify most integrations of a pBIN19-based T-DNA in an *Allium* genome. DNA from individuals of a population of 66 plants was used as template for a TAIL-PCR, using pBIN19 right border primers Apo4RB1, 2, 3, and the arbitrary degenerate primer AD2 (Liu and Whittier, 1995). From the 67 plants, 47 (70.1%) gave results distinctive of a TAIL-PCR step-down pattern.

Sixteen different classes of product signatures were found, and in many cases, multiple plants from the same transformation plate were found to be of clonal origin. In addition, plants from the same transformation plate with different TAIL-PCR signatures were found, indicating that different transformation events could also arise from the same transformation plate.

A further benefit of TAIL-PCR over Southern analysis is that it enables sequencing of genomic DNA that flanks T-DNA inserts. Sequences of TAIL-PCR products have thus far demonstrated very precise integration at the border of the T-DNA in *Allium*. Furthermore, these sequences may be useful in identifying PCR probes for two purposes.

First, onions take 18 months to reach sexual maturity; therefore segregation analysis causes a significant delay in the route to market. The identification of homozygous individuals at the seedling stage using PCR primers will prevent this delay. Second, PCR primers to detect sequences that flank T-DNA will provide a means of cultivar identification and (presumably) ownership.

Adaptor-ligated (AL)-PCR was developed for characterizing transgenic shallots (Zheng *et al.*, 2001a). This technique is similar to TAIL-PCR in that it also enables individual T-DNA integration events, including border sequences, to be defined. The AL-PCR protocol developed by Zheng *et al.* (2001b) successfully characterized transgenic lines with multiple integration events with higher sensitivity than southern hybridizations.

2.8.2 Transgene expression and stability

2.8.2.1 Visual reporter gene expression

Mature transgenic onion, leek, and garlic plants expressing *m-gfpER* reporter gene under the control of the CaMV 35S promoter have been developed (Eady *et al.*, 2000, 2005). Microscopy examination of these transformants during regeneration under selection criteria indicated that, occasionally a line may exhibit punctate gene expression, most plants that have regenerated do express the visual reporter gene constitutively (Eady *et al.*, 2000; Kondo *et al.*, 2000; Zheng *et al.*, 2001b). The profile of CaMV 35S expression in primary transformants and first filial generations

has been determined extensively using the *m-gfpER* gene (Eady *et al.*, 2003b). The conclusions from these studies indicate that transgenes in onion, despite its large genome, are expressed in the same general way as transgenes in other species.

Twelve independent onion *m-gfpER* transformants from initial studies have been selfed and F₁ plants produced. Initial results indicate that the transgene is usually inherited in a normal Mendelian fashion and that offspring are phenotypically normal (Eady *et al.*, 2003b).

2.8.2.2 Herbicide resistance gene expression

Onion plants containing a CaMV 35S-*bar* gene construct and the constitutively expressed CP-4-derived glyphosate resistance gene have been produced. All plants that have been confirmed by Southern analysis as containing the *bar* or CP-4-derived transgenes (one or two copies) have shown a strong tolerance to the herbicides Buster[®] or Roundup[®], respectively.

Initially, a 0.5% solution of the contact herbicide Buster was painted onto the leaves of the transformed onion plants. Plants that tolerated this treatment were then sprayed with commercially recommended concentrations of Buster for general-purpose weed control to confirm resistance (Eady *et al.*, 2003a). The level of resistance achieved indicated that the commercial production of transgenic onions containing a *bar* resistance gene is a feasible option for weed control in onions.

Glyphosate-tolerant plants produced to date, which were tested in a similar manner (Eady *et al.*, 2003a), have proven to be tolerant to twice the recommended field application rates required for general weed control. F₁ seed has recently been produced from these plants and they are currently being field tested for further assessment (www.ermanz.govt.nz). It has been estimated that the deployment of glyphosate-tolerant lines in New Zealand could substitute the application of up to 151 ha⁻¹ of mainly toxic and persistent herbicides per season for approximately 4.51 ha⁻¹ of low-toxicity short-lived glyphosate, which equates to an economic saving of about US\$250 per hectare (Eady, 2001b).

2.8.2.3 Antisense alliinase gene silencing

Three sets of transgenic onion plants containing antisense *alliinase* gene constructs have recently been produced. The presence of the construct in some transgenic plants has been determined by southern blot detection of flanking T-DNA sequences (Eady, 2002b). However, the precise number of independent transgenic lines has not yet been determined, because TAIL-PCR analysis has indicated that some material that was initially thought to be clonal is actually of independent transgenic origin or from nontransgenic escape regenerants. The three sets of plants contain a CaMV 35S driven antisense root *alliinase* gene (Lancaster *et al.*, 2000); a CaMV 35S driven antisense bulb *alliinase*, initially isolated by Clark (1993); and a bulb *alliinase* promoter (Gilpin *et al.*, 1995) driven antisense bulb *alliinase*. All constructs were developed by Pither-Joyce (M.D. Pither-Joyce, personal communication). Primary transgenic material has had root and bulb tissue analyzed for alliinase level (using Western analysis) and alliinase activity (by indirect analysis of pyruvate production; Randle and Bussard, 1993). Initial results are somewhat confusing due to several compounding factors. These include the precise nature of the transgenic line, variation in the size and physiological status of the primary transformants, sensitivity of the assays, and the inefficiency of the antisense approach, which only produces an effective silencing approach in approximately 10% of transgenic lines (Smith *et al.*, 2000). It appears that the antisense root *alliinase* constructs have little or no effect on reducing root alliinase levels. Several repeated assays have now indicated that none of the antisense root lines have significantly reduced root alliinase levels compared with control plants. If the antisense technique is working, then this would suggest that the root *alliinase* sequence described by Lancaster *et al.* (2000), with only ~50% homology with the other *alliinase* sequences (Randle and Lancaster, 2002), is not in fact the source of a rate-limiting root alliinase. Results from the antisense bulb *alliinase* lines have been much more encouraging and three lines were produced with barely detectable bulb alliinase levels and activity. However, progress with this research has been confounded by the poor survival of transgenic plants.

2.8.3 Development of homozygous transgenic lines

Transgenic hybrid onion seed from these transgenic lines has been developed by crossing a nontransgenic open-pollinated parental line with a transgenic parental plant carrying a single transgene in the hemizygous state. Some resulting seed produced by the nontransgenic parents will be hemizygous for the transgene and can be selected for to give F₁ heterozygous individuals containing the transgene. Self-fertilization of these individuals produces homozygous, hemizygous, and null F₂ progeny with respect to the transgene. These homozygous individuals can then be used to generate the bulk seed required for the production of commercial transgenic lines.

3. FUTURE ROAD MAP

Methods for the transformation of important *Allium* species are still very much in their infancy and transformation efficiencies of less than 1% of starting material are still the norm. The results obtained so far suggest that the difficulties faced in obtaining transgenic Alliums lie with the effective production and selection of regeneration-competent cells that are capable of taking DNA. DNA uptake via *Agrobacterium* or bombardment can be as efficient as that observed in many other plant transformation systems. DNA integration, when it occurs and is successfully maintained, also appears no different to that obtained in other plant transformation systems. Although the technology to create transgenic Allium plants has been slow to develop, scientists are now rapidly developing further gene systems that will enable biochemists to manipulate the key enzymes involved in sulfur and carbohydrate metabolism. This will further our understanding of the role of these pathways and enable us to identify those that are important for the plant's normal physiology, and those that comprise the nutraceutical elements of these crops.

In addition, the ability to transform elite Allium cultivars will enable the introgression of traits from outside the Allium gene pool that can confer useful agronomic traits. Pest and disease resistance traits, such as those being applied to many other crops, have the potential to reduce the levels of pesticides and fossil fuels required for this crop and pave the

way for the sustainable and efficient production of Allium crops.

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Asparagus

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1. INTRODUCTION

1.1 Botanical and Genome Aspects

The genus *Asparagus*, a member of the Liliaceae, has about 150–300 species, including herbaceous perennials, shrubs, vines, ornamentals, and food species, such as *Asparagus maritimus*, *Asparagus acutifolius*, *Asparagus Pseudoscaber*, and *Asparagus officinalis* (Lawrence, 1982; Garrison and Chin, 2005). The asparagus species are distributed widely throughout the temperate and tropical regions of the world. *A. officinalis* L. is native both to the Orient and to the eastern Mediterranean, where it was cultivated by the Greeks as a vegetable crop more than 2000 years ago. *A. officinalis* is dioecious; seedlings derived from female plants generally exhibit a sex ratio of 1:1. Male plants may have both staminate and hermaphroditic flowers (Reuther, 1984). *A. officinalis* is not sexually compatible with most other *Asparagus* species. Hybrids derived from interspecific crosses have no commercial value. Female plants are homogametic XX, male plants are heterogametic XY, and hermaphroditic flowers arise on male (XY) plants due to a rare mutation. Male plants with hermaphroditic flowers (andromonoecious) can be cross- or self-fertilized (Wircke, 1979; Reuther, 1984). Andromonoecious plants produce two genotypes: XY male and YY supermale after

self-fertilizing. Andromonoecious plants can comprise 10–20% of asparagus populations (Wircke, 1979). Crossing supermales with females produces all-male hybrids, which can be desirable for production because male plants are generally more productive than female plants. The occurrence in nature of these supermales has been reported to be less than 2% (Galli *et al.*, 1998).

European diploid ($2n = 2x = 20$) (Jessop, 1966) (Figure 1) *Asparagus* species possess approximately twice the nuclear DNA of diploid southern African species (Stajner *et al.*, 2002). *A. officinalis* has been reported to have 1308 Mbp (mega base pair) (Arumuganathan and Earle, 1991) whereas asparagus fern (*A. plumosus* Baker) has 695 Mbp of DNA per 1C nucleus (Stajner *et al.*, 2002). Due to the small genome size of *Asparagus* species among members of the core Asparagales, the species may provide a useful genomic model for Asparagales (Kuhl *et al.*, 2005).

1.2 Economic Importance and Major Problems of Asparagus Production

A. officinalis is an economically important vegetable crop in over 17 countries (Reuther, 1984). In 2004, the world's top four asparagus producers were: China at 587 500 tons, followed by Peru (186 000 tons), the United States (102 780 tons),

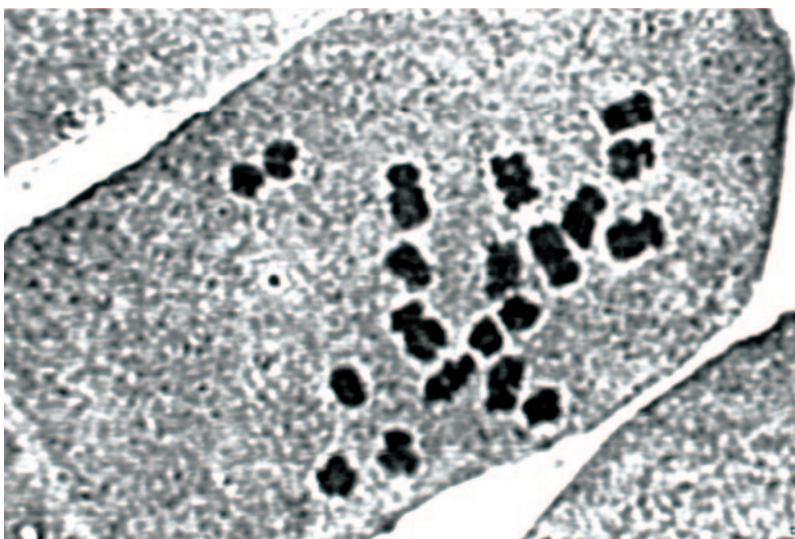


Figure 1 The chromosomes in *A. officinalis* cv. Lucullus 234 ($2n = 20$) [Reproduced from Dan (1994)]

and Mexico (67 247 tons) (FAO, 2005). The United States produces mainly green asparagus, with a total value of production of \$140 million in 2006. Within the United States, California ranks first in production with a value of \$70.8 million, followed by Washington at \$18.9 million and Michigan at \$14.9 million in 2006 (National Agricultural Statistics Service, 2006).

Asparagus is a perennial vegetable crop, and properly maintained asparagus fields should remain profitable for 10–15 years, if adequately managed. The marketable yield, however, declines as a result of crown deaths and a reduction in the quantity and size of the spears produced (Elmer, 2001). Furthermore, “early decline” or “replant disease” can occur, if old asparagus fields are replanted with asparagus. In such cases, the replanted fields usually do not reach a profitable state (Elmer, 2001). In the late 1970s and 1980s, asparagus fields were being removed from production after only 8–15 years due to sparse stands and small spear size, resulting in low yields (Takatori and Souther, 1978). This problem is due to a disease syndrome known as “asparagus decline”, which causes loss in longevity and productivity of established fields, difficulty in replanting asparagus where asparagus was previously grown, resulting in decreased annual yield of asparagus over time. The disease occurs throughout the world (Graham, 1955; Grogan

and Kimble, 1959; Van Bakel and Kerstens, 1970; Gindrat *et al.*, 1983; Lenna *et al.*, 1985; Maurer and Maddocks, 1985; Gordon-Lennox and Gindrat, 1987; Fantino, 1990; Lenna and Foletto, 1990; Schofield, 1991; Blok and Bollen, 1995; Schreuder *et al.*, 1995; Blok and Bollen, 1996; Brayford and Uk, 1996; Elmer *et al.*, 1996; Lori *et al.*, 1998; Doan and Carris, 1999; Guerrero *et al.*, 1999; Keulder, 1999; Pontaroli and Camadro, 2001; Fiume and Fiume, 2003; Quilambaqui-Jara *et al.*, 2004; Wong and Jeffries, 2006).

Factors that contribute to asparagus decline include abiotic factors, such as environmental stress (nutrient imbalance, low soil pH and soil moisture), physical stress (defoliation by asparagus beetle, cultural practices and allelopathic substances produced by asparagus tissue), and biotic factors such as asparagus virus I and II, and *Stemphylium vesicarium* (Kitahura *et al.*, 1972; Yang, 1982, 1985; Young, 1984; Shafer and Garrison, 1986). However, the most important factors associated with “asparagus decline” are four pathogenic fungi, including *Fusarium oxysporum* f. sp. *asparagi*, *F. proliferatum*, *F. redolens*, and *F. solani* (Graham, 1955; Grogan and Kimble, 1959; Van Bakel and Kerstens, 1970; Elmer, 2001; Quilambaqui-Jara *et al.*, 2004; Wong and Jeffries, 2006). *F. oxysporum* and *F. proliferatum* are important pathogens of major crops throughout the world. They are chitinous and facultative

parasites that colonize living and nonliving host tissue and may invade nonhost tissue (Hendrix and Nielson, 1958; Alexander, 1961). They are able to form chlamydospores or other resting structures that can survive in soil for many years. These characteristics make them especially persistent once they are established (Nelson *et al.*, 1981). *F. oxysporum* causes a vascular wilt that inhibits water uptake and carbohydrate transport within the plant. *F. proliferatum* is primarily a root-rotting organism. Asparagus plants infected by either pathogen show similar above-ground symptoms; the ferns are yellowed, stunted, wilted, and may die in different stages of development. *F. oxysporum* causes root rotting and the symptoms of vascular discoloration within the stem, root, and crown whereas *F. proliferatum* causes more extensive dry crown rot and brown stem pith discoloration but no vascular discoloration (LaMondia and Elmer, 1989; Sangalang *et al.*, 1995; Elmer *et al.*, 1996, 1997).

1.3 Traditional Breeding

Andromonoecious plants of asparagus produce two genotypes: XY male and YY supermale after self-fertilizing. Crossing supermales with females produces all-male hybrids. All-male hybrids produce greater yield, are more vigorous and have greater longevity (Ellison *et al.*, 1960). In addition, increased disease resistance and drought tolerance have been associated with male plants (Ellison, 1986). YY plants are highly valued for the production of hybrid seeds, as all the progeny from crosses with female XX plants will be of the male XY constitution. Therefore, asparagus breeding programs have focused on the development of all-male hybrid cultivars with high yield, good spear quality, and enhanced resistance to asparagus rust, crown, and root rot, stem blight, and purple spot.

Asparagus breeding faces several challenges. Genetic resources of commercial asparagus cultivars are narrow (Garrison and Chin, 2005). The dioecious nature of asparagus with random mating in production fields provides limited variation in plant characters that allow for selection of breeding material. Use of interspecific hybridization is limited due to sexual incompatibilities. In addition, asparagus has a long breeding cycle.

Garrison and Chin (2005) described advances in asparagus breeding methods in several areas including: (1) selection of seed from vigorous females in production fields, (2) selection of superior male and female plants for seed production in an isolation block from which Rutgers Beacon was developed (Ellison and Kinelski, 1985), (3) division of a single desirable female and male in an isolation block to reduce heterozygosity in progeny by which F₁ cvs. Mary Washington and Martha Washington have been developed, (4) development of inbred lines through sibling crosses for several generations leading to the Limbras hybrids, and (5) breeding of andromonoecious plants (uniform male hybrids produced by selfing andromonoecious plants) (Sneep, 1953).

Tissue culture plays an important role in modern asparagus breeding and commercial seed production. One of the most significant applications of tissue culture in asparagus breeding is anther culture, which has been pioneered by Falavigna (Falavigna *et al.*, 1982, 1984a, b, 1986, 1990, 1996; Falavigna and Soressi, 1983; Falavigna and Casali, 2002). Anther/microspore culture to produce doubled haploids is an especially efficient tool for asparagus breeding. Researchers have improved the efficiency of asparagus anther/microspore culture (Wolyn and Feng, 1993; Feng and Wolyn, 1994; Ziauddin *et al.*, 1996; Peng *et al.*, 1997; Chen *et al.*, 1998; Galli *et al.*, 1998; Peng and Wolyn, 1999; Shalaby *et al.*, 2003) and developed genetic markers to identify doubled haploids for asparagus breeding programs (Restivo *et al.*, 1995; Ozaki *et al.*, 1998; Eimert *et al.*, 2003). Several important cultivars including Eros, Marte, Itala, Ercole, Zeno, and Guelph Millennium have been developed using this technology (Falavigna and Casali, 2002; Garrison and Chin, 2005). Advantages of anther/microspore culture are as follows: homozygosity obtained when haploids are doubled with colchicine; saving years of inbreeding compared to sibling crosses or andromonoecious breeding techniques; and both homozygous males and females can be obtained from superior selected male plants. Some limitations of anther/microspore culture are that not all genotypes respond to standard techniques requiring time-consuming modifications to protocols in order to obtain doubled haploids from desirable breeding material (Garrison and Chin, 2005).

The productivity of asparagus at different ploidy levels has been considered for asparagus breeding (Garrison and Chin, 2005). The questions have been as follows: is the diploid level the most productive and useful for asparagus? How are various horticultural characteristics influenced by ploidy? Triploid asparagus from crosses between $2x$ and $4x$ or $4x$ and $2x$ have exhibited low fertility. However, Hiroshima Green is a successful triploid cultivar of asparagus developed in Japan. Tetraploid asparagus has several distinctive characters compared to diploids: longer cladophylls, larger flowers, larger stomata, and larger spear diameter, as observed in cvs. Violetto, de Albenga, Purple Passion, Dulce Verde, Pacific Purple, Seto Green, and Purple asparagus.

1.4 Conventional and Alternative Strategies to Control *Fusarium*

Conventional methods of controlling *Fusarium* spp. are limited. Chemical treatments, including crown dips and foliar sprays, have not been successful (Stephens *et al.*, 1991). Fumigation does not offer long-term effectiveness because of the perennial nature of the crop (Lacy, 1979). Furthermore, *Fusarium* spp. are ubiquitous and may re-infest the soil following fumigation and rapidly colonize young asparagus plants in the field (Damicone and Manning, 1985). Cultural control, such as incorporation of cruciferous residues, reduces the *Fusarium* population, but does not eliminate the disease (Stephens and Sink, 1992). To date, no *Fusarium* resistant cultivars have been developed (Takatori and Souther, 1978; Ellison, 1986).

Alternative strategies to control *Fusarium* include the following areas. The most successful strategy for *Fusarium* wilt control in other vegetable crops has been the development of resistant cultivars (Mace *et al.*, 1981). Possibilities exist for developing *Fusarium* resistant cultivars in asparagus. A cultivar of *A. officinalis*, Lucullus 234, had the highest resistance to virulent Michigan isolates FOA10 of *F. oxysporum* and FM12 of *F. proliferatum* among 90 cultivars and breeding lines of this species tested (Stephens *et al.*, 1989). Two ornamental cultivars (Sprengeri and Myersii) of *Asparagus densiflorus* (Kunth) Jessop were resistant to *F. oxysporum* and *F.*

proliferatum in greenhouse studies (Stephens *et al.*, 1989). Furthermore, Lewis and Shoemaker (1964) suggested that *A. densiflorus* cv. Sprengeri was immune to *F. oxysporum* f. *asparagi* in a laboratory study. However, sexual crosses of *A. officinalis* with the resistant species, *A. densiflorus* "Sprengeri" were unsuccessful, probably due to incompatibility barriers (Elmer *et al.*, 1989). In addition, by conventional methods, *A. officinalis* has low regenerative potential (Reuther, 1984) and the development of new cultivars requires many years because of perennial nature of the crop. It is likely that genetic diversity among the asparagus cultivars in North America is low (Gleason and Cronquist, 1963; Luzny, 1979; Ellison, 1986). Efficient plant regeneration systems have been developed from callus-derived protoplasts of different cultivars of *A. officinalis* L. (Figure 2) (Elmer *et al.*, 1989; Dan and Stephens, 1991). In this context, protoplast fusion technology can be an alternative to produce somatic hybrids between *A. officinalis* L. cv. Lucullus 234 and *A. densiflorus* cv. Sprengeri, which could be resistant to *F. oxysporum* f. *asparagi*.

Somaclonal variation has been used to recover genetic variability in several crops and offers an alternative to mutation breeding (Veilleux and Johnson, 1998). Two asparagus somaclonal lines with high levels of resistance to *Fusarium oxysporum* f. sp. *asparagi* have been developed from selection of plants regenerated from protoplasts of "Lucullus 234" in response to *F. oxysporum* and *F. proliferatum* (Dan and Stephens, 1995). In addition, no morphological or growth differences were observed between these lines and the parental cultivar. Later, Dan and Stephens (1997) reported that the two resistant somaclonal lines, which demonstrated higher levels of resistance to *Fusarium oxysporum* f. sp. *asparagi*, carried numerous DNA sequence variations different from the parental source, identified by random amplified polymorphic DNA (RAPD). The DNA sequence variation indicated possible mutations derived from tissue culture in the two lines. In a study of plants derived from embryogenic calli of asparagus, Kunitake *et al.* (1998) reported an increase in chromosomal variation among the regenerated plants with increasing duration of culture, particularly when haploid and diploid clones were the source of explants. In studies of molecular marker analysis

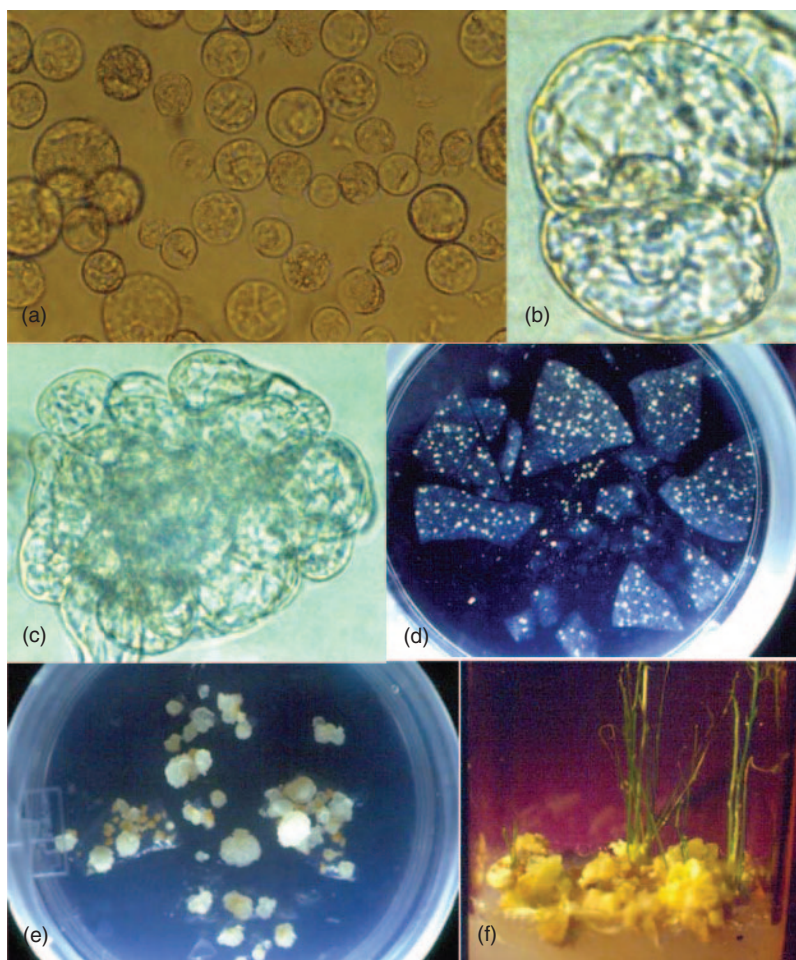


Figure 2 Development of *A. officinalis* cv. Lucullus 234 from callus-derived protoplasts to plants (a–f). (a) Asparagus protoplasts immediately after isolation from calli; (b) first division of protoplasts embedded in agarose of bead culture, 1 day after isolation; (c) a colony of about 10 cells derived from protoplasts embedded in agarose of bead culture 5 days after isolation; (d) microcalli derived from protoplasts embedded in agarose of bead culture 2 weeks after isolation; (e) calli derived from protoplasts on shoot-inducing medium, 1 week after initiation of bead culture; and (f) regeneration of shoots and plantlets from protoplasts-derived calli in hormone-free medium [Reproduced from Dan (1994)]

of regenerated asparagus plants, Raimondi *et al.* (2001) found that RAPD markers did not differentiate obviously different somaclones from controls; however, Pontaroli and Camadro (2005) reported differences in nearly 3% of amplified fragment length polymorphism (AFLP) markers between asparagus derived from long-term callus cultures and the control cultivars. The greater genome coverage of AFLPs and the longer duration of the callus cultures to accumulated genomic changes in the somaclones may underlie the differences between the studies.

The soil bacterium, *Agrobacterium tumefaciens*, can infect most dicotyledonous plants at wounding sites (Gelvin, 2003) and induce formation of tumors, called crown gall, via transfer of its Ti plasmid (Van Larebeke *et al.*, 1974; Zaenen *et al.*, 1974; Watson *et al.*, 1975). When the bacterium comes in contact with a wounded plant cell, the Ti-plasmid is transferred from the bacterium into the cell. A small segment of the plasmid, transfer DNA (T-DNA), is transferred from the plasmid to the nucleus of the plant cell, and becomes integrated into the plant nuclear genome

(Chilton *et al.*, 1977, 1978; Willmitzer *et al.*, 1980). *Agrobacterium*-mediated gene transfer has become a powerful tool for introducing foreign genes into many plant species to improve agricultural crops such as virus resistant potato and papaya (Gonsalves, 2004; Schubert, 2006) and increasing resistance to pathogenic viruses (Nelson *et al.*, 1988; Hoekema *et al.*, 1989; Fuentes *et al.*, 2006; Furutani *et al.*, 2006; Zhang *et al.*, 2006; Zhao and Zhao, 2006), fungi (Broglie *et al.*, 1991; Kanzaki *et al.*, 2004; Lin *et al.*, 2004; Pei *et al.*, 2005; Salehi *et al.*, 2005), and bacteria (Destefano-Beltran *et al.*, 1990; Datta, 2004; Lin *et al.*, 2004). Originally *Agrobacterium*-mediated transformation was expected to be limited to dicotyledonous plant species that the bacterium naturally infects; however, it was subsequently learned that *Agrobacterium* can transfer its T-DNA to cells of nonhost monocotyledonous plants such as asparagus (Hernalsteens *et al.*, 1984; Bytebier *et al.*, 1987; Delbreil *et al.*, 1993; Limanton-Grevet and Jullien, 2001), rice, wheat, corn, barley, sorghum and forage grasses (Graves and Goldman, 1986; Raineri *et al.*, 1990; Mooney *et al.*, 1991; Cheng *et al.*, 2004). Therefore, genes for resistance to *Fusarium* (Pei *et al.*, 2005) can now be introduced into *Asparagus* via *Agrobacterium*-mediated or biolistic transformation.

2. DEVELOPMENT OF TRANSGENICS

2.1 Asparagus Transformation

Asparagus was one of the first monocots for which regeneration of plants transformed by *A. tumefaciens* was reported (Bytebier *et al.*, 1987). In this study, spears of *in vivo* asparagus were surface sterilized, placed on half-strength MS (Murashige and Skoog, 1962) medium and inoculated with *A. tumefaciens* strain C58C1. Three calli, two of which regenerated plants, tested positive for the T-DNA that carried nopaline synthase and kanamycin resistance. Delbreil *et al.* (1993) improved upon the technique by inoculating embryogenic asparagus callus with *A. tumefaciens* carrying β -glucuronidase (*uidA*) and neomycin phosphotransferase II (*nptII*) genes and regenerated a plant that exhibited T-DNA integration. A more extensive analysis of T-DNA

integration into plants regenerated from embryogenic lines of asparagus that had been inoculated with *A. tumefaciens* strain AGL1Gin carrying a *uidA* gene and an *nptII* gene was conducted by Limanton-Grevet and Jullien (2001). Their transformation frequency ranged from 0.8 to 12.8 transformed plants per gram of inoculated somatic embryos. The T-DNA copy number per transformant ranged from one (in eight lines) to many among the 24 lines analyzed. The higher transformation rate was attributed to the more virulent strain of *Agrobacterium* used. Mendelian segregation of the transgene was observed after three of the six transformed plants were testcrossed to a nontransgenic to generate T₁.

An alternative to *Agrobacterium*-mediated transformation that has been used especially for monocots is the biolistic gun, also called the gene gun or particle bombardment. Three studies have been published on asparagus transformed using particle bombardment (Cabrera-Ponce *et al.*, 1997; Li and Wolyn, 1997; Shigemoto and Kohmura, 2002). Shigemoto and Kohmura (2002) reported that four transgenic asparagus plants were obtained from embryogenic cells by particle bombardment using a hygromycin phosphotransferase (*hpt*) gene and, in these plantlets, integration of *hpt* gene was confirmed by polymerase chain reaction (PCR) analysis. Li and Wolyn (1997) used tungsten particles coated with the pKGUS plasmid carrying the *nptII* and *uidA* genes. They obtained 10 transgenic plants derived from suspension culture of asparagus but they all revealed the same southern hybridization pattern, implying that they all derived from the same transformation event. The plasmid DNA appeared to have integrated into the asparagus genome as head-to-tail concatemers. The third particle bombardment report on asparagus was conducted with a plasmid carrying *hpt*, *gus*, and *pGPTV-Bar* (bialaphos resistance). Somatic embryo cultures of asparagus were bombarded followed by regeneration of transformed asparagus plants by secondary embryogenesis. The transformants varied for the number of integrated copies of the *nos-bar* and CaMV 35S-*hpt* genes present in the genome. However, five lines exhibited resistance to the herbicide phosphinothricin compared to the sensitive control plants.

In addition to *Agrobacterium*-mediated and biolistic transformation, there is a single report

of electroporation of asparagus protoplasts with plasmid DNA containing *nptII* and *gus* (Mukhopadhyay *et al.*, 2002). Callus-derived protoplasts were used as targets of transformation. The integration of foreign DNA into the genome of regenerated plants was confirmed by Southern blot analysis.

2.2 Selection of Transformed Tissue

As described above, three sources of antibiotic resistance genes, including *nptII* (Delbreil *et al.*, 1993; Li and Wolyn, 1997; Limanton-Grevet and Jullien, 2001), *hpt* (Cabrera-Ponce *et al.*, 1997; Shigemoto and Kohmura, 2002), and phosphinothricin-*N*-acetyltransferase (*pat*) (Cabrera-Ponce *et al.*, 1997) have been used for selection of transgenic asparagus. With *nptII*, selection for transformed plant material has been conducted using kanamycin in the range of 50–100 mg l⁻¹. For hygromycin selection after transformation with constructs carrying the *hpt* gene, 25–100 mg l⁻¹ were used during culture of embryogenic callus. In addition, herbicide resistance (*pat* gene) has been used to select transformed embryos after biolistic transformation.

2.3 Selection of Whole Plants

Despite the wealth of literature describing transformation of asparagus by any of the three different methods using a range of tissue types and strains of *A. tumefaciens*, there has been no attempt to introduce a gene of interest that would improve asparagus cultivation or quality. Only selectable markers and reporter genes have been introduced thus far into asparagus transformants. There is only a single mention of field tests of transformed asparagus, in New Zealand (Christey and Woodfield, 2001). In this study, the transgenic trait was listed as marker and reporter genes.

3. FUTURE ROAD MAP

3.1 Expected Products

Important traits such as herbicide tolerance, insect resistance including the *Bt* gene for control

of beetles and disease resistance genes for important asparagus diseases can be introduced transgenically into asparagus to enhance breeding programs. Breeding targets for increasing nutritional compounds in asparagus to improve its human health benefit include: the flavonoid rutin, an antioxidant that may reduce serum cholesterol; and anticarcinogens such as protodioscin (a steroid saponin) that inhibited cancer cell growth, decreased cholesterol in plasma and liver, and enhanced sexual activity (Garrison and Chin, 2005).

3.2 Expected Technologies

Plant transformation via *Agrobacterium* has significantly advanced from dicots to monocots in approximately last 20 years. Today agricultural biotechnology is heavily dependent on using *Agrobacterium* to produce transgenic plants. Technology to improve asparagus transformation efficiency can be advanced through studying: (1) transformation cell biology such as plant regeneration pathways, enhancement of *Agrobacterium* infection of explants and prevention of necrosis of transformed cells/tissues, (2) molecular biology, including identification of plant genes encoding proteins that interact with *Agrobacterium* virulence proteins, forward genetic screening to identify plant genes involved in *Agrobacterium*-mediated transformation, and reverse genetic screening for plant genes involved in *Agrobacterium*-mediated transformation (Gelvin, 2003), and (3) genomics approaches to identify plant genes that respond to *Agrobacterium* infection (Gelvin, 2003) and understand the mechanisms of *Agrobacterium*-induced necrosis and the role of antioxidants in plant transformation.

Identifying transformants that have successfully incorporated transgenes of interest, termed as transformation selection, is required for developing an efficient transformation method. Determining appropriate selective agents for asparagus under different culture conditions is critical to select and identify transformants. Despite approximately 50 selective marker genes that could be used for plant transformation (Miki and McHugh, 2004), only three selective marker genes, including *nptII*, *hpt*, and *pat*, have been used for *Asparagus* transformation. Many of the selective genes for plant transformation, which

have not been tested before, may be more effective in *Asparagus* transformation. Inserting introns into the coding region of *hpt* (Wang *et al.*, 1997) as its enhancing transgene expression in monocot species (Simpson and Filipowicz, 1996) can be an alternative strategy to improve selectable marker genes for asparagus transformation. The introduction of introns into *hpt* not only improved transformation frequency in *Agrobacterium*-mediated transformation of rice due to elevated *hpt* expression but also reduced copy numbers of the marker gene. In addition, inserting the introns into the marker gene also enabled better control of *Agrobacterium* growth during the transformation process (Wang *et al.*, 1997). This strategy also enhanced stable transformation with elite rice and barley cultivars as well (Upadhyaya *et al.*, 2000; Wang *et al.*, 2001). Promoters that have been identified in rice, corn, and wheat and showed increased transformation efficiency may be applied to asparagus transformation (Last *et al.*, 1991; Rasco-Gaunt *et al.*, 2003; Meyer *et al.*, 2004).

New cloning technology and vector design technology, recombinase-mediated auto-excision of transgenes directed by a tightly controlled microspore-specific promoter, allows efficient removal of either the selectable marker gene or of all introduced transgenes during microsporogenesis (Mlynárová *et al.*, 2006). This technology, which removes transgene via an integral part of the biology of pollen maturation, not requires any external stimulus such as chemical induction by spraying. This technology can open the door for advancing genetically modified (GM) crop development technology regarding public concern of the spread of antibiotic resistance.

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Leafy Vegetables

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1. INTRODUCTION

Leafy vegetables, consumed in the cooked or raw state, form an essential component of a well-balanced diet. Traditionally, lettuce has been an important leafy vegetable, being grown extensively as a salad crop worldwide and consumed primarily in the fresh state. Other leafy vegetables, such as chicory or spinach, are eaten both fresh and cooked. Currently, young leaves of leafy vegetables are becoming popular components of “baby salad” packs. Indeed, it is likely that the range of leafy vegetables will expand in the immediate future. This review focuses on lettuce, chicory, and spinach; other crops are mentioned briefly, as appropriate.

1.1 Origin, Distribution, and Economics of Production of Leafy Vegetables

1.1.1 Lettuce

Lettuce (*Lactuca sativa* L.), is a self-fertile, annual species of the family Asteraceae, with a chromosome complement of $2n = 2x = 18$. Seven morphological types of lettuce exist, namely (i) Crisphead, Iceberg or Cabbage, (ii) Butterhead, (iii) Cos, (iv) Leaf or Cutting, (v) Latin, (vi) Stem or Asparagus, and (vii) the Oilseed group (Ryder, 1999). Crisphead varieties have compact, large heads whilst Butterhead types have crumpled, soft-

textured leaves. Cos types are characterized by long, oval, dark upright green leaves, forming oblong heads or hearts. Leaf types, as the name implies, produce a rosette of loose leaves; Latin cultivars (cvs.) are intermediate between the Butterhead and Cos types in forming loose heads with oval leaves. In stem-type cultivars, the young fleshy stems are eaten after being cooked. Oil-seed-type lettuces bolt rapidly and are probably primitive forms of *L. sativa*. Lettuce is classified on its leaf shape and size, rather than pigmentation (Ryder, 1999).

The geographical origin of lettuce is uncertain, but it may be Egypt, the Mediterranean region, the Middle East, and South West Asia. Lettuce cultivation in Europe dates from the late 1400s, followed by introduction of the plant into America, with the selection of Crisphead cvs. (de Vries, 1997). Kesseli *et al.* (1991) suggested a polyphyletic origin for *L. sativa* based on variation from restriction fragment length polymorphism (RFLP) analysis of 67 accessions of *L. sativa* and 5 other *Lactuca* species. Frijters *et al.* (1997) in attempting to advance understanding of the lettuce genome, constructed a bacterial artificial chromosome (BAC) library containing large *Eco*R1 and *Hind*III genomic fragments, while Waycott *et al.* (1999) mapped genes for morphology to molecular markers. Subsequently, Johnson *et al.* (2000) reported a molecular comparison of cultivated lettuce with its wild progenitor, *Lactuca serriola*, while Dziechciarková

et al. (2004) exploited protein and molecular marker technologies to elucidate aspects of the taxonomy, biodiversity, genetics, and breeding in *Lactuca*.

The market for lettuce has expanded in the last decade, with more varieties being cultivated in line with consumer requirements for novel produce. The annual production of lettuce in the United States is about 4 million tons, valued at \$1.6 billion (United States Department of Agriculture, 1998), in excess of 2 billion heads being harvested annually. Seventy percent of US production is in the field in California (Pink and Keane, 1993). Other regions of cultivation are Canada, Northern Europe, and Mexico, South America, South Africa, the Middle East, Japan, China, and South-East Australia. Globally, 19 million tons were produced in 2002 (FAOSTAT, 2003). Lettuce ranks about 26th in comparison to other fruits and vegetables in terms of its contribution to the human diet, following tomato and orange with respect to bulk consumption in the United States.

1.1.2 Chicory

Chicory (*Chicorium intybus*) is also a member of the Asteraceae. Phenotypically, this biennial monoecious plant occurs as rosette, Witloof, and heading types. The former are open in habit with elongated and either divided or entire leaves; heading varieties produce green, variegated or red leaves, with heads of different morphology. Witloof chicory is produced by growing roots of 1-year-old plants of the rosette type in the dark in either sand or hydroponic culture to produce white buds (chicons). The latter are cooked as a luxury vegetable.

1.1.3 Spinach

Spinach (*Spinacia oleracea*), in the family Chenopodiaceae, is cultivated as an annual for its leaves and as a biennial for seed. Its growth habit is similar to that of lettuce and chicory with the fleshy, upright, wrinkled (savory), or smooth leaves forming a compact rosette. Flowering is initiated by temperature and photoperiod (Correll *et al.*, 1994). Spinach prefers cool climates and tolerates

frost better than most other leafy vegetables (Goto *et al.*, 1999a). Spinach is consumed both fresh and after processing (canned and frozen), with approximately 87 000 tons being processed during 1999 in the United States. The concentrations of dietary minerals and vitamins are typically high (Goto *et al.*, 1996) with about 93 mg of calcium and 51 mg of vitamin C in 100 g fresh weight of leaves, together with iron, phosphorus, and sodium (Ryder, 1999).

1.2 Genetic Improvement of Leafy Vegetables by Traditional Breeding

As in all crop plants, there is a continual requirement to genetically improve leafy vegetables to increase their resistance to insects, diseases, abiotic and biotic stresses. Yield and nutritional value are also important targets for genetic manipulation using conventional breeding and somatic cell technologies.

1.2.1 Lettuce

More effort has been invested in the genetic improvement of lettuce than other leafy vegetables by conventional breeding, probably reflecting consumer demands. Breeding objectives for cultivated lettuce include modifying leaf shape and color, manipulating head formation, delaying bolting, and the introduction of male sterility and resistance to insects, herbicides, and diseases. The latter include downy mildew and Lettuce Mosaic Virus (LMV) (Ryder, 2002), *Fusarium* wilt (Garibaldi *et al.*, 2004), root rot (Tsuchiya *et al.*, 2004), and corky root disease (Dufresne *et al.*, 2004; Mou and Bull, 2004). Resistance to dieback (Grube and Ryder, 2003; Grube *et al.*, 2005), *Sclerotinia minor* (Grube and Ryder, 2004), and *Bremia lactucae* (Jeuken and Lindhout, 2002; Lebeda and Petrželová, 2004) also represent key targets of conventional breeding. Improved leaf quality and succulence, with a decrease in latex and bitter taste, reduced nitrate accumulation under glass, and extended shelf life following harvest (de Vries, 1997), are relevant to this crop.

Self-fertilization is efficient in lettuce, although cross hybridization is possible. Pedigree breeding,

selection breeding, and back crossing are the principal methods of lettuce improvement (Ryder, 1999). *L. sativa* is sexually compatible with only a limited number of members of the genus, particularly *Lactuca saligna*, *L. serriola*, and *Lactuca virosa*. Important traits that have been introgressed from *L. virosa* into *L. sativa* include resistance to LMV, decreased susceptibility to tipburn, reduced bitterness, and a more extensive root system. Other useful traits include resistances to downy mildew and LMV from *L. serriola*, leaf aphid (*L. virosa*), and cabbage lopper (*L. saligna*; de Vries, 1990). An emphasis of breeding programs has been to identify and to introduce novel genes for resistance to viruses and fungi (Witsenboer *et al.*, 1995). The fungal pathogen *B. lactucae*, which is prevalent in the cool wet climate of Northern Europe, is the cause of downy mildew, the most destructive fungal disease of lettuce. Most lettuce breeding programs incorporate mildew resistance. LMV, transmitted by aphids, is the most destructive viral disease of lettuce (Revers *et al.*, 1997), with aphids and whitefly acting as viral vectors. Jeuken and Lindhout (2004) developed backcross inbred lines (BILs) in which chromosome segments from the wild species, *L. saligna*, were introgressed into *L. sativa*, through four to five backcrosses and one generation of selfing, with marker-assisted selection commencing in the fourth backcross generation. BIL association mapping enabled the location to be determined of 12 morphological traits and amplified fragment length polymorphism (AFLP) markers. Such BILs will be essential in future genetic studies in *Lactuca*.

Product quality is important, particularly uniformity of size, yield, postharvest shelf life, and nitrate content. Accumulation of nitrate in lettuce and other vegetables is undesirable, since in humans nitrate produces nitrite that hinders the binding of oxygen to hemoglobin. Nitrite also generates carcinogenic nitrosamines that may cause gastric cancers (Gunes *et al.*, 1995). As lettuce is generally consumed raw, there is a need to limit nitrate concentration in this crop (Gaudreau *et al.*, 1995; Santamaria, 1997). Year-round cropping, uniformity of size and time to maturity are primary commercial targets. Quality and appearance govern saleability; prolonged postharvest shelf life is equally important because of the rapid decay of the crop.

Molecular marker technologies will facilitate breeding programs with identification of genes and markers, resulting in the development of a genetic map for lettuce. Ninety or more genes in lettuce encode for leaf and floral morphologies and pigmentation, disease and insect resistances, seed and fertility and plant development (Ryder, 1999). Kesseli *et al.* (1994) constructed a genetic map using isozyme, RFLP, and random amplified polymorphic DNA (RAPD) analyses. Other morphological traits were mapped in lettuce relative to RAPD markers (Waycott *et al.*, 1999). Traits for morphological characteristics such as dwarf phenotype, white seed, brown seed, salmon flower color, pale yellow flower color, virescent juvenile leaf color, plump involucre, yellow seed, and anthocyanin spotting were linked to RAPD loci. Pigmentation and form is diverse in lettuce (Ryder, 1999; Ryder *et al.*, 1999) with several of the forms being characterized genetically. Ryder (1999) reviewed progress in identifying genes that govern disease resistance and morphological characteristics, while Waycott *et al.* (1995) studied the genetic control of plant stature in lettuce.

A BAC library was also constructed containing large *EcoRI* and *HindIII* genomic fragments of lettuce (Frijters *et al.*, 1997) and screened with markers linked to disease resistance genes. Additionally, quantitative trait loci (QTL) were identified for differences between wild and cultivated lettuce with respect to root architecture (Johnson *et al.*, 2000). Undoubtedly, continued expansion of the genetic map for lettuce will facilitate the identification of agronomically useful genes for introduction into this crop.

1.2.2 Chicory

Chicory is monoecious with sexual hybridization being hampered by the biennial life cycle, the sporophytic self-incompatibility of many cultivars (Ryder, 1999), and the difficulty in synchronizing bolting of different genotypes (Varotto *et al.*, 2000). Flowering occurs following vernalization at 8–18 °C when day length exceeds 13 h during the second season of growth. Several traits are targets for both conventional breeding and genetic manipulation technologies. Chicory roots contain up to 20% stored carbohydrates (Frulleux *et al.*, 1997), which, upon hydrolysis, yield 18% fructose

and 2% sucrose, which are of industrial interest. Genetic improvement of cultivars grown for their roots may result in plants with enhanced fructose production and increased commercial value. One of the stored carbohydrates, inulin, is a probiotic of bifidiobacteria present in the human gut, where it is thought that bacteria convert inulin to compounds that display anticarcinogenic activity (Vijn and Smeekens, 1999). Roots of chicory provide a coffee substitute (Bais *et al.*, 2001). Since seedling growth in the field is slow, resulting in weeds out-competing the crop, resistance to nonselective herbicides is a key target for crop improvement. The production of high quality chicons is also important commercially. Indeed, during forcing, both the taproot and chicons of Witloof chicory are susceptible to bacterial and fungal infections (Chupeau, 1989), making resistance to pathogens additional targets for genetic improvement. As in lettuce, nitrogen metabolism is a target for crop improvement (Abid *et al.*, 1995).

1.2.3 Spinach

Spinach is dioecious making traditional breeding difficult, although monoecious individuals do occur. Flowers of both male and female plants are formed in clusters of 6–12 and develop sequentially. Female flowers have a single ovary. Sex is influenced by X and Y chromosomes (Panday and Kalloo, 1986), as well as environmental factors including light, temperature, and growth regulators (Chaliakhyan and Khyranin, 1978). Application of gibberellic acid (GA₃) increased the percentage of male plants to 79%, while treatment with benzylaminopurine (BAP), indole acetic acid (IAA), and abscisic acid (ABA) increased the percentage of female plants. Targets for spinach breeders include nutritional quality, tolerance to elevated temperature and acid soils, resistance to viral and fungal diseases, and a reduction in oxalate concentrations, since the latter interferes with Ca²⁺ uptake in humans (Goto *et al.*, 1996).

2. TISSUE CULTURE OF LEAFY VEGETABLES AS A BASIS FOR GENETIC MANIPULATION

Tissue culture-based technologies involving micropropagation, *in vitro* pollination, anther and

ovule culture, embryo rescue, exposure of somaclonal variation, somatic hybridization and transformation, are approaches that complement conventional plant breeding. Reproducible tissue culture-based procedures for the regeneration of fertile plants from cultured explants, cells, and isolated protoplasts, are fundamental to the exploitation of these technologies. Importantly, several *Lactuca* species, including *L. sativa*, chicory, and spinach, are amenable to culture in the laboratory, with shoot regeneration from cultured cells. Additionally, protoplasts of lettuce are totipotent. In general, tissue culture of spinach is more difficult than that of lettuce or chicory.

2.1 Lettuce

2.1.1 Embryo rescue and organogenesis

Shoot tips were cultured in order to micropropagate F₁ hybrid lettuce plants (Takano *et al.*, 1988), while embryo rescue permitted the recovery of sexual hybrids between *L. sativa* and *L. virosa* (Maisonneuve *et al.*, 1995). The response of explants, such as seedling hypocotyls, to the components of growth media, particularly growth regulators, in terms of adventitious bud initiation, was discussed by Michelmore and Eash (1988) and Xinrun and Conner (1992). Ampomah-Dwamena *et al.* (1997) screened 22 lettuce genotypes of different morphological groups for their shoot regeneration response on Schenk and Hildebrandt (SH; 1972) medium containing 3% (w/v) sucrose, 0.1 mg l⁻¹ IAA, 0.5 mg l⁻¹ kinetin, and 0.05 mg l⁻¹ zeatin. Reproducible shoot regeneration was recorded from Crisphead-, Butterhead-, Leaf- and Cos-type lettuce varieties. In general, regeneration was achieved using a medium with the Murashige and Skoog (MS; 1962) formulation with 3% (w/v) sucrose, 0.04 mg l⁻¹ α -naphthalene acetic acid (NAA), 0.5 mg l⁻¹ BAP (benzyladenine, BA), and semi-solidified with 0.8% (w/v) agar at pH 5.8. An incubation temperature of 23 \pm 2 °C with a 16 h photoperiod (18 μ mol m⁻² s⁻¹, daylight fluorescent tubes) was satisfactory. Marked differences occur in callus initiation and shoot regeneration from different cultivars, while hyperhydricity may impair shoot regeneration in some cvs. Investigations of plant regeneration from suspension cultured cells exploited batch cultures, although Teng *et al.*

(1993) studied regeneration from cells cultured in 21 bioreactors. These authors focused on several parameters, including the effects of foaming, aeration, and “wall effects” of the culture vessels. Aeration through a screen column with 150 μm pores, prevented foaming, removed the wall effect, and maximized shoot regeneration.

Frequently, seedling cotyledons have been the source of explants because of their uniformity and their rapid shoot regeneration response (Webb *et al.*, 1984). Regeneration normally proceeds by organogenesis, although Zhou *et al.* (1992) reported somatic embryogenesis from cotyledons on MS-based medium with either 2.0 mg l^{-1} BA and 0.2 mg l^{-1} NAA, or 0.2 mg l^{-1} BA with 2.0 mg l^{-1} NAA. More recently, Hunter and Burritt (2004) studied the effects of light on organogenesis from cotyledons of the cvs. Bambino, Greenway, Red Coral, and Red Oak Leaf. These authors suggested that phytochrome, cryptochrome, and blue light influence shoot regeneration from lettuce cotyledons.

2.1.2 Somaclonal variation in lettuce

Somaclonal variation, usually observed as morphological differences between individuals, is relatively common in plants regenerated from cultured cells and tissues. However, the precise mechanisms involved are still not clear (Kaeppeler *et al.*, 2000). Changes in chromosome complement, structure, and DNA sequence have been observed (Choi *et al.*, 2000). Somaclonal variation is probably related to several factors, such as increased DNA methylation following the “shock” of introducing tissues into culture, chromosome breakage and rearrangement (Jain, 2001), interference with the normal cell cycle, and amplification of repetitive DNA sequences (Gyulai *et al.*, 2003). Activation of transposable elements (Jain, 2001) and changes induced by growth regulators, especially 2,4-dichlorophenoxyacetic acid (2,4-D) (Arun *et al.*, 2003) may influence such variation. Tissues from differentiated organs, such as stems, leaves, and roots, often produce plants with more variation than explants with pre-existing meristems, such as shoot tips and axillary buds (Sahijram *et al.*, 2003). In addition to phenotypic analyses, molecular techniques such as RFLP markers, polymerase chain reaction (PCR)-based RAPD and AFLP technologies (Polanco and Ruiz, 2002), have been

developed to study somaclonal variation in tissue culture-derived plants (Rahman and Rajora, 2001; Sahijram *et al.*, 2003).

Somaclonal variation in lettuce has been exposed following culture of tissues and isolated protoplasts (Brown *et al.*, 1986). Such variation included increased vigor and early flowering habit, increased chlorophyll, and reduced susceptibility to both LMV and *B. lactucae*. Somaclonal variants were also observed during the establishment of protoplast-to-plant systems for lettuce, as discussed later. Protoplast-derived variants also exhibited variation in pigmentation, with elongated and wrinkled leaves, dwarfism and increased vigor. However, most of the somaclonal variants of lettuce have shown some deleterious characteristics, such as reduced fertility. Despite these negative effects, somaclonal variation may expose genetically stable, agronomically useful characteristics and any variant plants arising from culture are worthy of evaluation.

2.2 Chicory

2.2.1 *In vitro* pollination, microspore culture, embryo rescue, and organogenesis

Six cultivars of chicory, including the cv. Chioggia, have been pollinated *in vitro* with pollen from *Lactuca tatarica* and *Cicerbita alpina* (Doré *et al.*, 1996). The most embryos resulted following pollination by *C. alpina*. However, only the cv. 449-2 produced embryos and plants when crossed with *L. tatarica*, two plants being obtained from 55 capitulae. The generation of haploid plants or hybrids was dependent on the cv., with the cv. BO9 producing hybrids and cv. FD1-12 producing haploid plants.

Ovule pollination has been employed in chicory to overcome self-incompatibility (Castaño and De Proft, 2000). Ovules isolated from flower heads and buds of the cvs. Flash and Carolus were cultured on media based on the MS or B5 (Gamborg *et al.*, 1968) formulations with 4–6% (w/v) sucrose. Culture of pollinated ovules at 22 °C with a 16 h photoperiod produced some seedlings. The optimum response was in the cv. Carolus on MS medium with 7.5 mg l^{-1} glycine, 0.5 mg l^{-1} IAA, 0.5 mg l^{-1} kinetin, and 4 mg l^{-1} GA₃.

Chromosome analyses showed the plants to be diploid, as expected.

Unfortunately, anther and ovule culture, together with pollen irradiation and ovule irradiation, have been unsuccessful in generating haploid plants in chicory. However, success has been obtained through microspore culture (Theilerhedtrich and Hunter, 1995). Uninucleate microspores were cultured on MS-based medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} IAA, and 2 mg l^{-1} zeatin for up to 6 months to induce callus. The latter was subcultured on medium containing 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} IAA; shoots were induced by decreasing the inorganic components in the presence of 0.4 mg l^{-1} kinetin and 0.2 mg l^{-1} IAA. Adventitious roots developed on the regenerated shoots in half-strength Quoirin and Lepoivre (1977) medium with 0.2 mg l^{-1} indole butyric acid (IBA). Forty-four of 450 regenerated plants were haploids.

Varotto *et al.* (2000) used embryo rescue to regenerate plants from zygotic embryos of the cv. Rosso di Chioggia. Plants were hand pollinated with pollen from five different genotypes in order to overcome self-incompatibility. Embryos at the heart or torpedo stages 72 h after pollination, cultured on semi-solid B5 medium with Morel and Wetmore's (1951) vitamins, 800 mg l^{-1} glutamine and 1% sucrose, with a 14 h photoperiod at 25°C , produced green plants within 2 weeks of culture. Younger embryos failed to develop or produced abnormal plants. Culture of zygotic embryos may reduce the duration of breeding programs, since it circumvents embryo abortion.

Cells of chicory, like those of lettuce, are totipotent (Frulleux *et al.*, 1997) enabling plants to be regenerated by organogenesis and somatic embryogenesis from cultured tissues. Mix (1985) regenerated fertile plants of spinach by culturing vein explants from unexpanded leaves on MS-based medium. The shoot regeneration response varied between cvs. In the same year, Bennici and Riepma (1985) demonstrated that storage roots of the cv. Cicoria Dolce de Soncino regenerated shoots on agar-solidified medium. Such shoots, when excised, developed roots on transfer to MS-based medium supplemented with IBA, permitting the recovery of intact, fertile plants.

Early in the 1990s, leaf explants of the cv. Witloof produced nodular tissues on the medium of Quoirin and Lepoivre (1977), with BAP and

IBA as the growth regulators (Pieron *et al.*, 1993). Tissues were transferred to medium lacking growth regulators to stimulate bud formation followed by shoot development. Tissues developed in contact with the vascular bundles of the leaves (Pieron *et al.*, 1998), vascular centers within these tissues being the sites of bud initiation. Adventitious buds were also initiated from leaf explants excised from the cv. 474, a sexual hybrid between *C. intybus* and *C. endivia* (Decout *et al.*, 1994). Shoot formation was optimum at 25°C , with wounding being essential for morphogenesis. An interesting observation of possible application to chicory breeding, was that addition to the culture medium of silver nitrate or putrescine, both at 40 mM, initiated flowering of regenerated shoots within 28 days of the introduction of explants into culture (Bais *et al.*, 2001). Later, Rehman *et al.* (2003) improved the efficiency of shoot regeneration from leaf explants of Witloof chicory using MS-based medium with IAA, kinetin, and casein hydrolysate, followed by rooting on medium with IBA. Plants regenerated from leaf explants accumulated more esculin than plants grown *in vivo*. The ability to regenerate plants by organogenesis, from cultured leaf explants, seedling cotyledons, and shoot buds provided a basis for the subsequent generation of transgenic plants in this leafy vegetable (Vermeulen *et al.*, 1992; Abid *et al.*, 1995; Frulleux *et al.*, 1997).

2.2.2 Somatic embryogenesis in chicory

Plants can also be regenerated by somatic embryogenesis in chicory, as in the cv. Witloof (Heirwegh *et al.*, 1985). Dubois *et al.* (1988), while investigating androgenesis in chicory, reported the formation of somatic embryos from the sporophytic regions of cultured anthers. Styles from the cv. 474 were cultured on semi-solid, half-strength, MS-based medium, in which KNO_3 of the original formulation was replaced with 10.1 mM KCl and 1.7 mM glutamine, with the inclusion of the microelements of Heller (1953), 5×10^{-3} Fe-EDTA, Morel and Wetmore's (1951) vitamins, 0.06 M sucrose, the growth regulators NAA at $0.1 \mu\text{M}$ and 2, isopentenyladenine (2,ip) at $2.5 \mu\text{M}$. Styles were most embryogenic at 35°C in the dark, with each explant giving 10–50 somatic embryos. Temperature was crucial in

somatic embryogenesis. Subsequently, Guedira *et al.* (1989) obtained somatic embryos from anthers of micropropagated plants, with anthers at the tetrad and microspore stages being the most responsive. As in the work of Dubois *et al.* (1988), darkness and a temperature of 35 °C were essential to induce somatic embryos on medium containing 0.02 mg l⁻¹ NAA and 0.5 mg l⁻¹ 2,ip. Glutamine and ammonium nitrate were most suitable as nitrogen sources. Some plants derived from somatic embryos showed phenotypic variation, such as anthocyanin-pigmented veins and narrow or indented leaves.

In addition to regenerating shoots by organogenesis as already discussed, the chicory cv. 474 produced somatic embryos. Robatche-Claive *et al.* (1992) investigated the process in leaf explants of 3-month-old plants, the material being incubated in the dark (35 °C) in agitated liquid medium. Histological studies confirmed the synchronous development of somatic embryos in explants cultured with 30 mM sucrose, the embryos increasing in number as the sucrose concentration increased to 360 mM.

In studies to further knowledge of the role of carbohydrates in somatic embryogenesis, Couillerot *et al.* (1993) used root segments, also from the cv. 474, to investigate the effects of cellobiose, fructose, galactose, glucose, lactose, maltose, melibiose, raffinose, sucrose, and trehalose (each at 120 mM) in the culture medium. Sucrose was most effective in maximizing the number of somatic embryos, although fructose, glucose, maltose, and raffinose resulted in satisfactory development of embryos. The embryogenic potential of explants was correlated with polyamines, which the authors detected in the induction medium after 5 days of culture. High concentrations were detected of putrescine, spermine, and spermidine in the medium that induced somatic embryos. In contrast, such compounds were not released from explants on a noninductive medium. However, it was not possible to synchronize the formation of somatic embryos from root explants of chicory, unlike the situation with leaf explants (Robatche-Claive *et al.*, 1992).

Following the report that temperature was a crucial parameter in somatic embryogenesis of spinach (Dubois *et al.*, 1988), Decout *et al.* (1994) investigated the effect of temperature on cultures of the chicory cv. 474. Wounding of

the leaf explants was essential to promote a response. After 5 days, cells near the wound sites commenced mitotic division, although the pattern of division was dependent on the incubation temperature. As in the work of Dubois *et al.* (1988), somatic embryogenesis was most pronounced at 35 °C, although organogenesis was poor at this temperature. Somatic embryos were infrequent at 25 °C; both somatic embryos and shoots developed at 30 °C. The authors suggested that temperature influences the cell cytoskeleton and microtubules, which subsequently affects the initiation of somatic embryogenesis.

Cell suspension cultures initiated from callus originating from the veins of fully developed leaves of the chicory cv. Witloof, also exhibited somatic embryogenesis (Mohamed-Yasseen and Splittstoesser, 1995). Callus was stimulated by placing leaf vein segments on semi-solidified MS-based medium containing 30 g l⁻¹ sucrose, 100 mg l⁻¹ casein hydrolysate, and 1.3 µM 2,4-D, and 1.3 µM kinetin. Cultures were incubated in the dark for 8–12 weeks and transferred to new medium every 4 weeks. Callus was maintained in liquid medium for 4–6 weeks. Transfer to liquid MS-based medium with 30 g l⁻¹ sucrose and 1.8 µM BAP, induced somatic embryos on all calli. Although the somatic embryos were hyperhydric (vitrified), they germinated on half-strength MS-based medium semi-solidified with 8 g l⁻¹ agar. Interestingly, glucanases were detected in the medium after incubating explants of the cv. 474 in the dark at 35 °C (Helleboid *et al.*, 1998), enzyme concentrations being three times more in the medium following the culture of embryogenic lines compared with nonembryogenic tissues. Subsequently, Helleboid *et al.* (2000) investigated the proteins released into the medium by culturing for 4 days leaf explants of embryogenic and nonembryogenic tissues of the cv. 474. Concentrations of proteins of 25, 32, and 38 kDa were eightfold greater in the embryogenic line, suggesting that they may be involved in somatic embryogenesis. As the concentration of carbohydrate in the culture medium is crucial in maintaining growth, Bellettre *et al.* (1999) investigated the influence on somatic embryogenesis of doubling the sucrose concentration from 30 mM to 60 mM with 330 mM glycerol, following the initial procedure of Dubois *et al.* (1988). Increasing the sucrose concentration not only reduced the

embryo induction period, but also stimulated the number of somatic embryos.

2.3 Spinach

2.3.1 Organogenesis in spinach

Spinach is less amenable to culture than lettuce or chicory, although cultured tissues are totipotent. Usually, seeds are germinated in the dark on half-strength MS-based medium lacking growth regulators at 20–25 °C. Removal of the pericarp may be essential to minimize contaminating micro-organisms (Sasaki, 1989; Molvig and Rose, 1994). Robust sterilization (e.g., immersion in 2–3% sodium hypochlorite solution for 2 h) may also be essential (Komai *et al.*, 1996a). Five to ten days old seedlings are normally used as a source of explants, although Al-Khayri *et al.* (1991) used leaves from 3-month-old plants as source material. Knoll *et al.* (1997) employed root explants from shoots that had been in culture for up to 24 months.

Several culture conditions have been reported to induce differentiation in spinach, including temperatures of 20 °C (Al-Khayri *et al.*, 1991) to 25 °C (Komai *et al.*, 1996b), and a 16 h photoperiod with a light intensity of 35–90 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. Callus has also been initiated in continuous light (Mii *et al.*, 1992) and in the dark (Al-Khayri *et al.*, 1991; Molvig and Rose, 1994). MS medium has been employed most extensively, although Knoll *et al.* (1997) used Nitsch and Nitsch (1969) medium. Zdravkovic-Korac and Neskovic (1999) used a basal medium containing extra vitamins. Sucrose (10–30 g l^{-1}) has been the main carbon source, with the pH of culture media ranging from 5.8 to 6.5.

Adventitious shoot formation was reported from callus of seedling apices on MS medium with 4.65 μM kinetin and 2.89 μM GA₃ (Neskovic and Radojevic, 1973). Later, Sasaki (1989) cultured hypocotyl explants of the cvs. Wakakusa, Nippon, Toko, Hoyo, and Tokai on MS medium containing 20 g l^{-1} sucrose with various concentrations and combinations of GA₃ (0, 10⁻⁷, 10⁻⁶, 10⁻⁵, and 10⁻⁴ M) and 15 mg l^{-1} IAA. Adventitious shoot buds were observed in all cultivars except Tokai; callus of Nippon developed the most shoots. Interestingly, there was an inverse correlation between callus initiation and regeneration, since cultivars such as Nippon, exhibited poor callus

development, but maximum shoot regeneration. Shoots excised after 5–6 weeks, rooted on MS-based medium containing 10 mg l^{-1} IBA, flowered and set seed.

Al-Khayri *et al.* (1991) optimized organogenesis from leaf explants of 3-month-old plants of the cvs. High Pack, Grandstand, Baker, and Kent on MS medium with 2 mg l^{-1} kinetin and 0.1, 0.5, or 1.0 mg l^{-1} 2,4-D, in the dark, or with a 10 h photoperiod. Shoots were induced with the same concentration of kinetin and 0.1 mg l^{-1} 2,4-D, 1.0 mg l^{-1} GA₃, and 100 mg l^{-1} ascorbic acid. As in earlier studies, shoots were rooted with 1 mg l^{-1} IBA, the latter being more effective than other auxins (NAA, IAA, IBA, and 2,4-D). Al-Khayri *et al.* (1991) also found that cultivars that readily produced callus exhibited poor shoot regeneration, as reported by Sasaki (1989). Callus induced in the dark regenerated more shoots than callus in the light. Al-Khayri *et al.* (1991) stressed that the system was highly cultivar dependent and was not a general protocol. Shoots remained vegetative under short days but flowered under long-day conditions. In their subsequent experiments, Al-Khayri *et al.* (1992a) targeted the cvs. High Pack and Baker, which had exhibited previously the most shoot regeneration. Supplementation of the medium with 15% (v/v) coconut milk increased callus production from explants, reduced the time for callus initiation by 7 days, stimulated shoot formation and, overall, reduced the culture period by 8–7 weeks. The cv. High Pack was more responsive than Baker in producing shoots. Al-Khayri *et al.* (1992b) also investigated the role of GA₃ in the culture medium, since GA₃ was essential for shoot regeneration. GA₃ at 7.2 μM resulted in organogenesis from 70% of the explant-derived calli. Reduction of GA₃ to 1.4 μM was recommended for shoot multiplication, since concentrations above this value unexpectedly reduced plant height.

Kondo *et al.* (1991) also induced the multiplication of shoot primordia of the cvs. Minsterland and Jiromaru in liquid MS medium with 0.02–1 mg l^{-1} IAA and 2 mg l^{-1} BAP. Roots formed on shoots exposed to IBA at 0.2 mg l^{-1} , or when maintained on medium lacking growth regulators. In agreement with Komai *et al.* (1996c) and Zdravkovic-Korac and Neskovic (1999), plant regeneration was observed only in cultures with red pigmentation.

Other workers employed hypocotyl explants from 7 days old seedlings of the cv. Sunlight to induce callus on MS medium with 7 mg l^{-1} NAA, at pH 5.8–6.5 (Sato *et al.*, 1992). Mii *et al.* (1992) cultured 7-day-old explants from 9 spinach cultivars on Nitsch (1969) medium containing 10 mg l^{-1} IAA. This work again reflected cultivar differences, a phenomenon common to the culture of spinach tissues. Preculture of explants on medium with 5,6-Cl₂-IAA for 20 days before transfer to medium lacking growth regulators, was beneficial in stimulating shoot regeneration from hypocotyl-derived calli.

The regeneration procedure reported originally by Neskovic and Radojevic (1973) was used as a basis, with modification, to regenerate plants from cotyledons, roots, and hypocotyls of the cv. Hybrid 102 (Molvig and Rose, 1994). Growth of callus initiated in the dark on MS medium with $4.6 \mu\text{M}$ kinetin and $4.5 \mu\text{M}$ 2,4-D, was enhanced by a 16 h photoperiod on medium with the same concentration of kinetin, but with $2.9 \mu\text{M}$ GA₃ replacing 2,4-D. Shoots and roots were observed on the explants after several culture cycles, each of 4–6 weeks duration. Shoots excised and transferred to hormone-free medium developed into plants.

Following the report of Molvig and Rose (1994), Xiao and Branchard (1995) cultured hypocotyl and root explants on MS medium containing $85.6 \mu\text{M}$ IAA and $100 \mu\text{M}$ GA₃. These elevated concentrations of growth regulators induced more than 90% of the root explants to produce adventitious shoots. In contrast, hypocotyl explants were less responsive, with only 75% of the explants regenerating shoots. Ninety-two percent of the shoots excised and cultured on medium with 2.9 – $5.7 \mu\text{M}$ IAA developed roots. Zhang and Zeevaart (1999) described a rapid and efficient procedure for regenerating plants from cotyledons of 5-day-old seedlings of the cv. Longstanding Bloomsdale Dark Green that was exploited later to generate transgenic plants of spinach.

2.3.2 Somatic embryogenesis in spinach

One of the first reports of somatic embryogenesis in spinach was that of Xiao and Branchard (1993) who cultured hypocotyl explants of the cv. Carpo on semi-solid MS-based medium supplemented

with 3% sucrose, 0.01 mg l^{-1} biotin, 250 mg l^{-1} glutamine, 15 mg l^{-1} IAA, and 34.6 mg l^{-1} GA₃. Callus was induced in the dark followed by a 10-h photoperiod preceding continuous light. Somatic embryos were induced after transfer of callus to liquid medium with 0.5 mg l^{-1} IAA and 3.5 mg l^{-1} GA₃ in continuous light; reduction in IAA concentration was essential for somatic embryogenesis. Somatic embryos developed into plants in liquid medium containing 0.5 mg l^{-1} IBA under continuous light. Subsequent studies focused on the cv. Jiromaru (Komai *et al.*, 1995), somatic embryos being initiated from root explants of 10-day-old seedlings on MS medium with 20 g l^{-1} sucrose combined with 10 or $30 \mu\text{M}$ NAA and 0– $100 \mu\text{M}$ GA₃. A nitrate:ammonium ratio of 2:1 in the medium stimulated callus induction. After 28 days exposure to a 16-h photoperiod, cells were cultured in liquid or on semi-solid MS medium lacking growth regulators to induce embryogenesis. Most plants were regenerated from cell masses in liquid medium, with somatic embryos being detected at all stages of development. As in organogenesis, GA₃ in the callus induction medium was essential for somatic embryogenesis.

In extending their work, Komai *et al.* (1996c) concentrated on the details of somatic embryogenesis. Explants of cotyledons, roots, hypocotyls, and leaves from eight cultivars were cultured on six different media with 10 g l^{-1} sucrose, $0.1 \mu\text{M}$ GA₃, and $10 \mu\text{M}$ NAA. Root explants of the cvs. Jiromaru, Hoyo and Nihon produced the most embryogenic tissues compared with the cvs. King of Denmark, Ujo, Minsterland, Viroflay, and Nobel; half-strength MS-based medium was the most suitable for callus induction and somatic embryo formation. Komai *et al.* (1996d) also targeted root explants of the cv. Jiromaru, their growth regulator studies including IAA, IBA, NAA, 2,4-D, 2-ip, kinetin, BAP, N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU), and GA₃, either individually or in combination. Callus was induced on medium with all of the auxins assessed, 30 or $100 \mu\text{M}$ IBA with 10 or $30 \mu\text{M}$ NAA inducing the optimum response. GA₃ was essential for the formation of embryogenic callus.

Komai *et al.* (1996b) also studied the effect of carbohydrate on somatic embryogenesis using the cv. Jiromaru and the culture conditions that they established earlier. They modified

the medium with 29, 87, or 145 mM glucose, galactose, mannose, fructose, sorbose, maltose, cellobiose, lactose, or raffinose replacing sucrose. Embryos developed on medium with most of the carbohydrates assessed, although somatic embryogenesis was optimal with 29 mM fructose, this possibly being related to fructose being metabolized in preference to other carbohydrates. In contrast to the results of Komai *et al.* (1996c), Zdravkovic-Korac and Neskovic (1999) found that GA₃ and IAA suppressed somatic embryogenesis, whereas ABA (4 μ M) stimulated this process. Disks from fully expanded young leaves of the cv. Matador were cultured on MS-based medium containing 20 g l⁻¹ sucrose, 100 mg l⁻¹ myo-inositol, 2.0 mg l⁻¹ thiamine hydrochloride, 2.0 mg l⁻¹ pyridoxine hydrochloride, 2.0 mg l⁻¹ adenine, 5.0 mg l⁻¹ nicotinic acid, 4.4 μ M 2,4-D, and 4.6 μ M kinetin. Embryo induction occurred on transfer of explants to medium lacking 2,4-D, but supplemented with ABA, GA₃, or IAA. In agreement with the earlier studies of Komai *et al.* (1996c), embryos formed only from red pigmented callus. Somatic embryos were easily distinguished from the parental callus and any developing buds, since tissues regenerated simultaneously by organogenesis. Ethylene in somatic embryogenesis was investigated using root explants of the cv. Nippon (Ishizaki *et al.*, 2000). Inclusion of 10 μ M ethephon in callus induction medium increased by 50% the initiation of embryogenic callus. The authors suggested that although ethylene may be critical during the early stages of callus initiation, it later inhibits somatic embryo development.

3. PROTOPLAST-TO-PLANT SYSTEMS FOR GENE TRANSFER IN LEAFY VEGETABLES

3.1 Protoplasts and Somatic Hybridization in Lettuce

Protoplast fusion circumvents pre- and postzygotic barriers often associated with sexual hybridization, enabling the introgression of useful genetic traits into cultivated species, as in *L. sativa* from sexually incompatible *Lactuca* species. In lettuce, protoplast-to-plant systems have been developed as a basis for gene transfer by somatic hybridization for *L. sativa* with wild *Lactuca*

species, including *L. serriola*, *L. saligna*, and *L. virosa* (Webb *et al.*, 1994). General procedures of protoplast isolation, chemical and/or electrical fusion, and the culture conditions to generate somatic hybrid plants, are summarized in reviews by Davey *et al.* (2000a, b, 2005a, b, c). Examples of somatic hybrid plants in lettuce include those between *L. sativa* and *L. perennis* or *L. tartarica* (Chupeau *et al.*, 1994; Maisonneuve *et al.*, 1995), *L. sativa* with *L. virosa* (Matsumoto, 1991), *L. sativa* with *L. debilis* or *L. indica* (Mizutani *et al.*, 1989), and *L. sativa* with *L. serriola* (Matsumoto, 1987). Somatic hybrids of *L. sativa* (+) *L. tartarica* were sensitive to climatic conditions (Maisonneuve *et al.*, 1995), and did not set seed after pollination by donor cultivars. Resistance to downy mildew was transferred from *L. tartarica* into *L. sativa*, but fertility of the resulting hybrids was low. Somatic hybrids of *L. sativa* (+) *L. virosa* were generated in order to transfer resistance to *Nasonovia ribisnigri* (leaf aphid), *Erysiphe cichoracearum* (powdery mildew), and *Pseudomonas cichorii* (bacterial rot) from the wild species into the cultivated crop. Subsequently, Mizutani and Tanaka (2003) analyzed isozymes in lettuce and the wild species *L. serriola*, *L. saligna*, *L. virosa*, and *L. indica*. Enzyme polymorphism revealed that the somatic hybrid between lettuce and *L. indica* was composed of 4 genomes of *L. sativa* and 2 genomes of *L. indica*, such polymorphisms being useful genetic markers in lettuce breeding. There are no reports of the detailed evaluation of somatic hybrids, especially under field conditions.

3.2 Protoplasts and Somatic Hybridization in Chicory

In chicory, protoplast-derived cell colonies often became necrotic with plant regeneration being difficult to stimulate, despite protoplast plating efficiencies of 60–80% (Crepigny *et al.*, 1982). Young leaves from glasshouse-grown plants of the cv. Red Witloof released protoplasts when incubated with a wall degrading enzyme mixture consisting of 0.02% (w/v) Macerozyme, 0.1% (w/v) Onozuka Cellulase R10, and 0.05% (w/v) Driselase. Cells were cultured on MS-based medium supplemented with Heller's (1953) microelements, Morel and

Wetmore's (1951) vitamins and 5 g l^{-1} sucrose, the survival of protoplast-derived colonies was stimulated with glutamine (10 mM) as the sole nitrogen source. Protoplast-derived callus developed buds, but only limited numbers of plants were regenerated on medium with 0.5 mg l^{-1} IAA and 1 mg l^{-1} BAP. Following these investigations, Saksi *et al.* (1986) prepared protoplasts from leaves of 21-day-old axenically grown seedlings of the Witloof cv. Flash, the medium of Crepy *et al.* (1982) being supplemented with 1.0 mg l^{-1} of both BAP and NAA. Again, glutamine was essential to division of protoplast-derived cells, while sucrose at 5.0 g l^{-1} was advantageous for protoplast growth. Shoots formed on protoplast-derived callus cultured on medium in which NH_4NO_3 was replaced by glutamine, and supplemented with 0.05 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. Plants were transferred to the glasshouse after 4–5 months. Generally, young leaves have been the preferred source material, in terms of protoplast yield, as tissues from roots, stems, bracts, and callus, gave poor yields (Chupeau, 1989). Optimization of plant growth conditions was crucial in maximizing the yields of viable protoplasts (Chupeau, 1989; Rambaud *et al.*, 1990).

The chicory cv. 474, known to be highly embryogenic from earlier studies, was exploited to develop protoplast-to-plant systems. Mesophyll protoplasts cultured on half-strength MS-based medium lacking NH_4NO_3 but containing Heller's (1953) microelements, Morel and Wetmore's (1951) vitamins, 375 mg l^{-1} glutamine, 150 mg l^{-1} myo-inositol, 5 g l^{-1} sucrose, and 90 g l^{-1} mannitol, together with $10.7 \text{ } \mu\text{M}$ NAA and $5.0 \text{ } \mu\text{M}$ 2,ip, developed into somatic embryos by 3 procedures (Sidikou-Seyni *et al.*, 1992). First, 7-day-old protoplast-derived callus were transferred to semi-solid medium with 500 mg l^{-1} glutamine, 50 g l^{-1} mannitol, and 20 g l^{-1} sucrose and supplemented with $0.5 \text{ } \mu\text{M}$ NAA and $2.5 \text{ } \mu\text{M}$ 2,ip. In the second procedure, protoplast-derived colonies were transferred to medium optimized for somatic embryo induction, with $0.1 \text{ } \mu\text{M}$ NAA and $2.5 \text{ } \mu\text{M}$ 2,ip (Dubois *et al.*, 1988). Thirdly, well-developed callus was transferred to the same somatic embryo induction medium. Embryos developed when transferred to agitated liquid Heller's (1953) medium containing $0.15 \text{ } \mu\text{M}$ GA_3 , followed by germination on semi-solid Heller medium lacking

growth regulators. A callus stage was essential in the development of single protoplasts to embryos. Regenerated plants were morphologically normal and fertile.

Plants have also been regenerated from protoplasts of Italian red chicory cultivars (Varotto *et al.*, 1997), the cv. 363 giving 10 plants per protoplast-derived callus. Subsequently, Nenz *et al.* (2000) compared the culture of leaf mesophyll protoplasts of the cv. Rosso di Chioggia in liquid medium, semi-solid medium, and after embedding in droplets of alginate-solidified medium. Protoplasts in droplets of calcium alginate produced plants within 2 months. The plating efficiency was maximal (56.2%) on medium with 8 g l^{-1} mannitol, 10 mg l^{-1} NAA, and 4.5 mg l^{-1} BAP. The same medium also resulted in optimum division frequency (35.9%) of protoplast-derived cells.

In contrast to lettuce, there is only one report of the transfer of genetic material into chicory by intergeneric protoplast fusion. Varotto *et al.* (2001) generated male sterile asymmetric hybrids (cybrids) by fusing iodoacetic acid inactivated mesophyll protoplasts from a red chicory genotype CH363 with γ -irradiated hypocotyl protoplasts of a male sterile line of sunflower (CMSHA89). Mixing protoplasts in a 3:1 or 1:1 ratio (chicory to sunflower), followed by fusion with polyethylene glycol (PEG), and culture in alginate droplets (Nenz *et al.*, 2000) resulted in plant regeneration, three plants of which were asymmetric hybrids. Such cybrids were morphologically similar to the chicory parent. However, their anthers contained less pollen at anthesis than plants of the genotype CH363 and failed to undergo self-pollination.

3.3 Protoplasts of Spinach

Otsuki and Takebe (1969) pioneered the isolation of spinach protoplasts to investigate chloroplast physiology. Later, Rose (1980) cultured protoplasts from leaves of 3-week-old seedlings on MS medium with 0.5% sucrose, 0.4 M sorbitol, and 0.4 M mannitol. Protoplasts prepared in the presence of Ca^{2+} and incubated under reduced light showed 50% survival after 8 days of culture. Cell wall regeneration was inhibited in the dark. Goto and Miyazaki (1992) were the first investigators to

regenerate plants from leaf protoplasts of the cv. Jiromaru, mitotic division of protoplast-derived cells leading to cell colony formation on half-strength MS medium containing 5 mg l^{-1} BAP and 1 mg l^{-1} 2,4-D. As reported by Rose (1980), protoplasts were cultured at $1 \times 10^5 \text{ ml}^{-1}$ at 25°C under reduced light for 7 days, before transfer to stronger light conditions. Adventitious shoot formation was induced on MS medium with 1.0 mg l^{-1} kinetin and 1.0 mg l^{-1} zeatin, or various combinations of BAP and NAA; shoots were rooted in liquid MS medium with 1 mg l^{-1} IBA or 1 mg l^{-1} IAA. Subsequently, Goto *et al.* (1996) stimulated organogenesis using the same approach, but with the addition of KM8P vitamins (Kao and Michayluk, 1975) and 0.5 M glucose as osmoticum in the culture medium to increase the protoplast plating efficiency. Adjustment of the pH of the shoot regeneration medium to 6.3 promoted rooting of regenerated shoots. In analyzing the regeneration of protoplast-derived tissues of seven spinach cultivars, Goto *et al.* (1998a) reported that the plating efficiencies and cell colony formation of the cvs. Ujo, Jiromaru, Hojo, and Minsterland exceeded those of Sapporo-aba, Viroflay, and Viking. Shoot regeneration from callus was not observed in the latter three cultivars, again emphasizing cultivar-specific responses. Smooth seeded spinach cultivars were less amenable to culture than prickly seeded varieties.

Concurrent with the studies of Goto *et al.* (1996), Komai *et al.* (1996a) described somatic embryogenesis from protoplasts of cotyledons, roots and hypocotyls from 10-day-old seedlings, and from leaves of 30-day-old plants of the cv. Jiromaru. These studies were based on their results of somatic embryogenesis from cultured root explants (Komai *et al.*, 1996b). Isolated protoplasts were cultured on a medium similar to that described by Goto and Miyazaki (1992), but with $1 \mu\text{M}$ 2,4-D or $10 \mu\text{M}$ NAA and $10 \mu\text{M}$ zeatin. An extended dark period of 21 days at 25°C was followed by transfer to reduced light for a further 21 days, during which time the cultures were diluted every 3 days with new medium. Protoplast-derived cells produced callus when the medium was supplemented with 30 mM sucrose, 30 mM glucose, 500 mg l^{-1} casein hydroxylate, $0.3 \mu\text{M}$ 2,4-D, and $3 \mu\text{M}$ zeatin, whilst maintaining the cultures under a 16 h photoperiod; somatic embryos formed on protoplast-derived

callus when the growth regulators were changed to $0.1 \mu\text{M}$ GA₃ with $10 \mu\text{M}$ NAA. Adventitious roots were also induced from protoplast-derived callus. Importantly, these roots could be stimulated to produce multiple somatic embryos on MS-based medium supplemented with 30 mM fructose.

In optimizing the efficiency of the protoplast-to-plant system, Goto *et al.* (1999a) evaluated the effect of organic acids on cell division and shoot multiplication. Citric acid at 0.1 M, and other organic acids, were beneficial in the presence of 0.5 M glucose, enhancing the division of protoplast-derived cells, callus formation, and shoot regeneration. In the presence of 0.1 M citric acid, cell division was recorded in protoplast cultures at plating densities of $20 \times 10^4 \text{ ml}^{-1}$. Leaves from 25-day-old seedlings were the preferred source material for the isolation of viable, totipotent protoplasts. There are no studies of the culture of spinach protoplasts after 1999 and no reports of the exploitation of isolated protoplasts in somatic hybridization in this leafy vegetable.

4. TRANSFORMATION OF LEAFY VEGETABLES

The labor-intensive procedures associated with gene transfer by protoplast fusion have encouraged workers to focus attention on genetic modification by transformation. Additionally, recent advancements in recombinant-DNA technology have facilitated the delivery of specific genes to target species without the transfer of other, often unwanted, genetic traits that occurs during sexual and somatic hybridization.

4.1 Transformation of Lettuce

4.1.1 Basic procedures

Initial investigations in lettuce established reliable transformation protocols for this crop, exploiting protoplast-to-plant systems then available. Leaf protoplasts were electroporated in the presence of either pCAMV CAT, carrying the chloramphenicol acetyltransferase (*cat*) gene driven by the cauliflower mosaic virus (CaMV) 35S RNA promoter, or pABD1 with the neomycin

phosphotransferase (*nptII*) gene (Chupeau, 1989). The *cat* gene was used to demonstrate transient gene expression. The selection of kanamycin resistant plants following transformation with pABD1 and subsequent analysis of their seed progeny, confirmed that antibiotic resistance was inherited as a dominant Mendelian trait with integration of foreign DNA into the genomic DNA of antibiotic resistant plants. Despite this initial progress, few lettuce genotypes were transformed by direct DNA uptake into isolated protoplasts because of the requirement to first establish a plant regeneration system. Consequently, protoplast transformation was superseded by *Agrobacterium*-based transformation systems. The molecular biology of *Agrobacterium*-mediated gene delivery has been summarized in several excellent reviews (Gelvin, 2003; Valentine, 2003; Lacroix *et al.*, 2006; Tzfira and Citovsky, 2006), together with recent developments in the construction of gene vectors (Cheng *et al.*, 2004; Chung *et al.*, 2005).

In early transformation experiments, Micheltore *et al.* (1987) used wild type octopine (ACH5) and nopaline (C58) strains of *Agrobacterium tumefaciens* to induce crown galls on explants of cotyledons from 4-day-old seedlings of the Butterhead cv. Cobham Green. The unusual amino acids octopine or nopaline, characteristic of tumors incited by *A. tumefaciens* strains ACH5 and C58, respectively, were synthesized by the resulting tumors. The same investigators also used the engineered strain, GV3111 of *A. tumefaciens* harboring the cointegrate vectors pTiB6S3, pMON120, or pMON200, or the binary vector pMON505. Plasmid MON200 carried the chimaeric *nptII* gene with a *nos* (nopaline synthase) promoter and terminator (*nos.nptII.nos*). Explants transformed by GV3111 carrying either pMON200 or pMON505, produced callus on medium with 50 mg l⁻¹ of kanamycin sulfate; shoots were regenerated following transformation experiments with the disarmed pTiB6S3. These initial studies provided evidence that the lettuce genome could be manipulated using *A. tumefaciens*-mediated gene delivery. Subsequently, the cv. Kayser was transformed by *A. tumefaciens* strain LBA4404 harboring the binary vector pTRA415 (Enomoto *et al.*, 1990). The vector carried the *nptII* gene as the selectable marker, and the β -glucuronidase (*gus*) reporter gene. The latter was driven by the CaMV 35S promoter, or

the stress or salicylic acid-inducible pathogenesis-related (PR) 1a protein-encoding gene promoter from tobacco. GUS activity increased 3- to 50-fold in PR-*gus* transformed plants, confirming that the transgene was expressed normally under the regulated control of the *PR 1a* promoter. Vectors constructed by the Monsanto company were used in early studies of lettuce transformation, as in Lake Nyah with *A. tumefaciens* carrying the cointegrate vector pMON200 (Webb, 1992). Transgenic plants of the cv. South Bay were also generated using *A. tumefaciens* strain A208 with the engineered Ti plasmid, pTiT37SE, or the binary vectors pMON9749 or pMON9793 (Torres *et al.*, 1993). Expression of the *gus* gene in transgenic plants was confirmed histochemically, while PCR and Southern hybridization analyses showed integration of the transgene into the genome of transgenic plants.

The protocols employed in these early investigations were often genotype dependent. Therefore, Curtis *et al.* (1994a, 1995) focused attention on developing a reliable, genotype-independent protocol for *A. tumefaciens*-mediated transformation of 13 lettuce cultivars, including Crisphead cultivars that previously had been difficult to transform. Cotyledons of 7-day-old seedlings were inoculated with *A. tumefaciens* strains 0065 or 1065 with the binary vector pMOG23 carrying the chimeric *nos.nptII.nos* gene and a CaMV 35S *gus*-intron reporter gene. Strain 1065 was supervirulent as it also carried pTOK47 with extra copies of the Ti plasmid virulence genes *virB*, *virC*, and *virG*. Supervirulence was essential for transforming some cultivars, such as Reflex (Curtis *et al.*, 1994a). The bacterial density of the inoculum was crucial with respect to transformation, as suggested earlier by Micheltore *et al.* (1987). Minor modifications of the transformation protocol were described by Davey *et al.* (2001). The ease of transforming lettuce with *Agrobacterium* was stated by Wroblewski *et al.* (2005), who used *L. sativa* cvs. Valmaine and Mariska and the wild lettuce *L. serriola* (LS102) in transient *gus* expression assays. The laboratory strain C58C1 of *A. tumefaciens* was reliable for delivering genes to lettuce.

Whilst gene insertion by *Agrobacterium* is the procedure of choice, transgene expression may be inconsistent in lettuce (Davey *et al.*, 2001). The choice of promoters is crucial for stable

gene expression. Gene copy number and DNA methylation also influence transgene expression in this leafy vegetable. In their evaluation of gene expression in the cv. Evola, McCabe *et al.* (1999b) showed that expression of the *bar* gene for resistance to the herbicide glufosinate ammonium, was more consistent in T₁, T₂, and T₃ seed generations when the gene was driven by the -784 plastocyanin (*petE*) promoter from pea, compared to the CaMV promoter. This difference in gene expression was probably related to the fact that the CaMV promoter has 59 possible methylation sites per kb of DNA compared to 18 per kb for the *petE* promoter. Other workers also used the CaMV 35S promoter in lettuce (Enomoto *et al.*, 1990; Curtis *et al.*, 1994a, b; Pang *et al.*, 1996; McCabe *et al.*, 1999a), and the *nos* (Michelmore *et al.*, 1987; Yang *et al.*, 1993; Curtis *et al.*, 1994a, 1996a, b; McCabe *et al.*, 1999a), *Mac* (Curtis *et al.*, 1994b), *petE* (McCabe *et al.*, 1999b), and *ACT1* (McCabe *et al.*, unpublished) promoters. The tobacco pathogenesis-related protein gene promoter *PR1a* resulted in *gus* gene expression following its induction by salicylic acid (Enomoto *et al.*, 1990), while the *Mas* (mannopine synthase) promoter gave in root-specific expression (Curtis *et al.*, 1994b); the tapetum-specific promoter, *A9*, was used to induce male sterility in lettuce (Curtis *et al.*, 1996b). Transformation of the cv. Evola with the *ipt* (isopentenyl phosphotransferase) gene from *A. tumefaciens* driven by the senescence-specific promoter, *SAG12*, from *Arabidopsis thaliana*, resulted in a 48-fold increase in zeatin riboside equivalents in leaves of 60-day-old plants.

As in many transgenic plants, transfer DNA (T-DNA) integration into lettuce may be complex (McCabe *et al.*, 1999a), with little or no correlation between gene copy number and transgene expression. Post-transcriptional gene silencing was correlated with transgene dosage in lettuce transformed with a tomato spotted wilt virus N (TSWV N) coat protein gene with the CaMV 35S promoter (Pang *et al.*, 1996). The demethylating agent 5-azacytidine was used to alleviate silencing of the CaMV 35S-*gus* gene in transgenic plants of the cv. Raisia (McCabe *et al.*, 1999a). Clearly, several factors may contribute to transgene silencing, including the complexity of the lettuce genome with its heterochromatic or repetitive DNA (Stam *et al.*, 1997) and methylation. Transgene insertion into highly

methylated regions of the genome also results in transgene inactivation (Pröls and Meyer, 1992). Exploiting supervirulent strains of *Agrobacterium* for lettuce transformation may result in multiple T-DNA insertions at different genomic loci, increasing the probability of transgene integration at sites that are not susceptible to methylation and gene silencing (McCabe *et al.*, 1999a).

4.1.2 Introduction of agronomically important genes into lettuce

Several genes of agronomic importance have been introduced into lettuce, with considerable advances being made during the last decade.

4.1.2.1 Herbicide tolerance

Resistance to herbicides and disease is a prime target for genetic manipulation since these agents cause severe crop losses. Herbicide resistance has been introduced into lettuce from both academic and commercial perspectives. Thus, Mohapatra *et al.* (1999) introduced the *bar* gene from the bacterium *Streptomyces hygroscopicus* into seedling cotyledons of the cv. Evola by *Agrobacterium*-mediated transformation with strains 0310 and 1310. The strain 1310 carried the hypervirulent pTOK47 in addition to the binary vector with the *nptII* and *bar* genes. Plasmid TOK47 in strain 1310 gave multiple insertions of T-DNA into some plants, whereas strain 0310 gave single gene inserts in all plants analyzed by DNA-DNA hybridization. Axenic seedlings grew on medium with glufosinate ammonium at 5 mg l⁻¹, while glasshouse-grown plants were resistant to the herbicide when the latter was sprayed on the plants at 300 mg l⁻¹. This investigation confirmed that herbicide resistance can be introduced into lettuce with stable expression in seed generations. Although sexual hybridization has been used to transfer sulfonylurea resistance into cultivated lettuce from the wild species, *L. serriola* (Mallory-Smith *et al.*, 1990), sexual incompatibility and lack of availability of herbicide resistance in the *Lactuca* gene pool limit further introgression of herbicide tolerance into the cultivated crop to transformation-based procedures.

4.1.2.2 Virus resistance

Experiments have been conducted to introduce virus resistance into lettuce using a coat protein gene-mediated approach, Pang *et al.* (1996) transferring the nucleocapsid (N) protein gene of the lettuce tospovirus, TSWV, into two lettuce breeding lines. Transgenic plants expressing the nucleocapsid (N) protein gene were protected against TSWV. In studies with lettuce infectious yellows virus (LIYV), Falk (1996) was unable to detect LIYV resistance in transgenic lettuce, although the same construct resulted in virus resistance in tobacco. Dinant *et al.* (1993) also introduced a LMV coat protein (LMV-CP) gene into LMV-susceptible lettuce, but the transgene conferred only poor resistance to this virus. Loss of virus resistance was more pronounced in lettuce during subsequent seed generations, an observation also made by Gilbertson (1996). Dinant *et al.* (1997) also introduced the coat protein gene from LMV strain O into the virus-susceptible cvs. Girelle, Jessy, and Cocarde. Several transgenic plants accumulated LMV-CP. As in other examples of potyvirus sequence-mediated protection, some plants were completely virus resistant, but in others this resistance was not sustained, with the development of viral infection symptoms. The efficiency of this strategy to induce LMV resistance was considered to be related to the developmental stage of the transgenic plants at the time of their inoculation with the virus.

Simple but robust procedures are useful in assessing viral resistance of plants. Mazier *et al.* (2004) used explant-derived shoots and *in vitro* grown seedlings to assess lettuce cultivars for their resistance to LMV. Excellent correlation was obtained in cultivars already known to be LMV resistant and their resistance after virus inoculation of *in vitro* grown material. This procedure, with its limited space requirements and environmental safety, should be especially useful in handling recombinant viruses.

4.1.2.3 Fungal and insect resistance

In attempts to understand the molecular basis for resistance to the fungus *B. lactucae*, Okubara *et al.* (1997) generated transformed plants of the cv. Diana using *A. tumefaciens* carrying

constructs with the maize *Ac* transposase and *Ds*. Their studies showed that several *Dm* genes for resistance to downy mildew were located in four clusters in the lettuce genome, with the largest cluster containing at least nine genes. More information, relating to *Dm* genes, has been discussed by Jeuken and Lindhout (2002). *Sclerotinia sclerotiorum* induces rot in crops, especially lettuce, pathogenesis being associated with the production of oxalic acid. Thus, in studies to decrease synthesis of this compound, Dias *et al.* (2006) introduced the decarboxylase (*oxdc*) gene from *Flammulina* sp. into lettuce, and showed that leaves from two transgenic plants inoculated with agar plugs of a 2-day-old culture of *S. sclerotiorum* failed to develop disease symptoms. Since verticillium wilt incited by *Verticillium dahliae* is also a major threat to lettuce cultivation, particularly in California, Vallad *et al.* (2006) inoculated the susceptible cvs. Saunas and Sniper and the resistant cvs. La Brillante and Little Gem with 29 isolates of *V. dahliae* and two isolates of *Verticillium albo-atrum*. Molecular evidence from the intergenic spacer region of the nuclear ribosomal RNA gene suggested that certain isolates form a phylogenetically distinct subgroup that differs in virulence toward specific lettuce cultivars. Such information may have relevance in directing the targeting of genes for *Verticillium* resistance to certain lettuce cultivars. Ferritin, an iron storage protein, sequesters iron in plants. Following earlier studies with tobacco in which overproduction of ferritin reduced oxidative stress and improved tolerance to pathogens by sequestering free-iron that would normally produce iron-mediated Fenton oxidants, such as hydroxyl radicals (OH•) (Deak *et al.*, 1998), Goto *et al.* (2000) transformed lettuce with the CaMV 35S promoter driving the soybean ferritin complementary DNA (cDNA), with the *nptII* gene for kanamycin selection of transgenic plants. Transgenic plants accumulated 1.2- to 1.7-fold more iron than wild-type plants and grew faster than the latter, possibly related to a reduction of iron-mediated Fenton oxidants, such as hydroxyl radicals.

Sesquiterpene lactones play a role in disease resistance through the phytoalexin response. Such compounds include lactucin and lettucenin A. Thus, Bennett *et al.* (2002) cloned genes for germacrene A synthases in order to change the

profile of these latex-expressed compounds and to enhance disease and insect resistance using sense and antisense approaches in lettuce.

Transformation of plants with proteinase inhibitors that act on proteolytic enzymes may confer resistance to pests and pathogens. In studies of the role of the proteinase inhibitor II, *SaPIN2a*, from *Solanum americanum*, Xu *et al.* (2004) transformed lettuce with the *SaPIN2a* gene driven by the CaMV 35S promoter and suggested that this approach may be exploited to counteract pest and pathogen attack. Subsequently, Fan and Wu (2005) also introduced a *PIN2* gene into lettuce and Chinese flowering cabbage (*Brassica campestris* ssp. *parachinensis*), while Chye *et al.* (2006) extended these investigations to show that expression of *SaPIN2a* in lettuce conferred resistance to cabbage looper (*Trichoplusia ni*) caterpillars.

4.1.2.4 Drought, cold, and salinity tolerance

Drought and cold restrict the performance of lettuce, especially under field conditions. Curtis *et al.* (1996a) transformed the lettuce cv. Lake Nyah with the *rolAB* genes from *Agrobacterium rhizogenes* to stimulate root formation, with the aim of increasing drought tolerance. Expression of the *rolAB* genes may also increase the endogenous concentration of auxin, reducing the incidence of leaf russet spotting. Pileggi *et al.* (2001) focused attention on the development of crops under conditions of drought, salinity, and cold by transforming the cv. Grand Rapids with a mutated *P5CS* gene for δ -1-pyrroline-5-carboxylate synthase, that catalyzes two steps of proline biosynthesis in plants. The mutated gene is insensitive to feedback inhibition by proline. Increased concentration of proline acts like an osmoprotectant that could confer resistance to drought, salinity, and cold on transgenic plants. Transgenic lettuce plants obtained in these studies were tolerant to freezing.

Abdel-Kader (2001) studied drought-induced stress in the cvs. Longifolia-baladi and Crispamignonette. The results showed that drought induced lipid peroxidation and catalase activity, but decreased protein content, peroxidase and esterase activities. GA treatment of plants alleviated the drought effects. Vanjildorj *et al.* (2005) also targeted drought and cold tolerance

in lettuce by overexpressing the *Arabidopsis ABF3* gene, encoding a transcription factor for the expression of ABA responsive genes, in the lettuce cv. Chongchima. Transgenic plants were phenotypically normal, produced seed, and were more tolerant than wild-type plants to drought and cold stresses.

Salinity limits crop production in many areas, particularly on marginal lands and where crops have been irrigated intensively. Consequently, increased salt tolerance is a key target for transformation. In this respect, Park *et al.* (2005) introduced a late embryogenesis abundant protein gene from *Brassica napus* into lettuce. The six transgenic plants generated exhibited enhanced growth compared to nontransformed plants under salt stress imposed by exposure to 100 mM sodium chloride.

4.1.2.5 Taste, nutritional value, and anticancer activity

Kisiel *et al.* (1995) used *A. rhizogenes* strain LBA9402 to transform leaf explants from axenic seedlings of *L. virosa*, this *Lactuca* species being a traditional medicine because of its analgesic and sedative properties. The latter properties result from sesquiterpene lactones, which accumulate mainly in the latex. Transformed roots were induced and cultured to maximize biomass production and to facilitate analysis of secondary products. Eight sesquiterpene lactones were isolated from transformed roots.

Cultivated lettuce often has a bitter taste. In attempts to reduce bitterness, Sun *et al.* (2006) cloned the gene for the sweet and taste-modifying protein, miraculin, from the pulp of berries of *Richadella dulcifica*, a West African shrub. This gene, with the CaMV 35S promoter, was introduced into the cv. Kaiser using *A. tumefaciens* GV2260. Expression of the gene in transgenic plants resulted in the accumulation of significant concentrations of the sweet-enhancing protein. Since miraculin is active at extremely low concentrations, it may be used by diabetics as a food sweetener. Genes for other taste-modifying proteins, such as thaumatin, monellin, mabinlin, pentadin, brazzein, and curculin, have been cloned and sequenced and are available for insertion and expression in lettuce and other crop plants (Faus, 2000).

In addition to enhancing disease resistance, manipulation of sesquiterpene lactones in lettuce may also be exploited to reduce bitterness (Bennett *et al.*, 2002). Vitamin E, that includes tocopherols, are lipid soluble antioxidants, with tocopherol existing in the α , β , γ , and δ isoforms. The relative vitamin E potencies of these isoforms are 100%, 50%, 10%, and 3%, respectively. Cho *et al.* (2005) expressed a cDNA encoding γ -tocopherol methyltransferase from *A. thaliana* in the lettuce cv. Chungchima in efforts to improve tocopherol composition. Transgene inheritance and expression in transformed plants increased enzyme activity and conversion of γ -tocopherol to the more potent α form. As Cho *et al.* (2005) emphasized, conversion of γ -tocopherol to α -tocopherol in major food crops could increase their value and importance in human health, since vitamin E reduces the risk of several serious disorders. The latter include cardiovascular disease and cancer, in addition to slowing ageing and enhancing the function of the immune system.

Stilbenes, especially resveratrol, show cancer chemopreventative activity and have a role in the prevention of coronary heart disease and arteriosclerosis. Stilbene synthase is the key enzyme in resveratrol biosynthesis, with several stilbene synthase genes being isolated recently. Although lettuce contains the substrates for stilbene synthase, resveratrol is not synthesized. Thus, in order to engineer lettuce for synthesis of this compound, Liu *et al.* (2006) fused a cDNA encoding stilbene synthase from *Parthenocissus henryana* to the CaMV 35S promoter with the *bar* gene as a selectable marker. The expression construct was flanked by matrix attachment regions to maximize expression of the gene of interest in transgenic plants. Quantitative analysis showed that resveratrol in transgenic plants was $56.0 \pm 5.52 \mu\text{g g}^{-1}$ leaf fresh weight, comparable to that in the skin of grape fruit. In addition, an anticancer assay with HeLa cells showed that apoptosis was induced by 200 μM resveratrol extracted from lettuce leaves.

4.1.2.6 Accumulation of trace elements and nitrate

Zinc is an essential element in human nutrition, as its deficiency severely impairs organ function. In experiments to fortify lettuce with this

element, Zuo *et al.* (2002) expressed a mouse metallothionein mutant β -cDNA in the cv. Salinas 88 following *Agrobacterium*-mediated gene delivery. The concentration of zinc in transgenic plants increased to $400 \mu\text{g g}^{-1}$ dry weight, this being considerably more than in wild-type plants.

Nitrate is the major source of nitrogen for higher plants, with nitrate reductase catalyzing the first step in its assimilation. However, lettuce and other leafy vegetables such as chicory, rocket, and spinach, provide most of the nitrogen intake in typical diets (De Martin and Restani, 2003). Lettuce often accumulates nitrate to concentrations that exceed the maximum permitted concentration. Indeed, nitrate concentration is one of several parameters that govern the marketability of the crop, especially in winter, especially when plants accumulate nitrate because of low light conditions. In experiments to reduce nitrate accumulation in lettuce, Curtis *et al.* (1999a) introduced the *nia2* cDNA for nitrate reductase from tobacco driven by the 35S promoter into the cv. Evola. Unfortunately, none of the transgenic plants exhibited a reduction in nitrate content compared to the wild-type plants at harvest, although plants with nitrate concentrations slightly less than those of wild-type plants were observed during cultivation. Subsequently, Dubois *et al.* (2005) investigated nitrate accumulation in the cv. Jessy using a similar 35S::*nia2* construct. They again concluded that none of the plants carrying the transgene exhibited a reduction in nitrate accumulation, although the transgene was expressed. Transgene-specific silencing extended to the homologous endogenous nitrate reductase mRNA (messenger RNA) resulting in chlorosis and eventual death of the transgenic plants. Clearly, this approach for reducing nitrate accumulation has difficulties.

4.1.2.7 Extending postharvest shelf life

Lettuce and other leafy vegetables deteriorate rapidly after harvest. Consequently, delaying leaf senescence is an important target for genetic manipulation. Curtis *et al.* (1999b) introduced the *T-cyt* gene (synonym *ipt*, *tmr* or gene 4) coding for isopentenyl phosphotransferase, which is involved in cytokinin biosynthesis, from the T-DNA of *A. tumefaciens* on the binary vector pMOG23 into the lettuce cv. Saladin. Transgenic plants were phenotypically normal following transfer to the

glasshouse, set viable seed, and had increased cytokinin and chlorophyll contents in their leaves compared to nontransformed plants. Such results indicated the possibility of delaying senescence in lettuce, possibly reducing the requirement for postharvest controlled environmental conditions to prolong the shelf life of harvested plants.

In subsequent, more elaborate investigations, the *ipt* gene driven by the senescence-specific promoter *SAG12* from *A. thaliana* (*pSAG12-IPT*) was introduced into the lettuce cv. Evola. Plants homozygous for the transgene exhibited significantly delayed postharvest leaf senescence (McCabe *et al.*, 2001). Importantly, the transgene was activated only during senescence, particularly when the latter process commenced in the outer (lower) leaves, initiating cytokinin biosynthesis. The latter then inhibited leaf senescence, simultaneously attenuating activity of the *pSAG12-IPT* gene, preventing cytokinin overproduction. Heads of transgenic plants retained chlorophyll for up to 7 days longer in their outer leaves after harvest compared to leaves of nontransformed plants. Mature plants were morphologically normal when harvested, with no significant differences in head diameter or fresh weight of leaves or roots compared to their nontransformed counterparts. In later experiments, the metabolites synthesized by *pSAG12-IPT* transformed plants were compared with those metabolites synthesized by nontransformed plants using atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) and linked gas chromatography/atmospheric pressure chemical ionization/electron ionization mass spectrometry (GC-APCI/EI-MS) (Garratt *et al.*, 2005). During storage, heads of transgenic plants showed a threefold increase in the concentrations of acetaldehyde, ethanol, and dimethyl sulfide, increase in the latter compound being paralleled by an accumulation of reactive oxygen species. Such investigations emphasize the importance of detailed metabolite profiling of plants following transgene insertion, as the integration of a gene to modify one or more traits may affect other biosynthetic pathways.

Attention has also been given to the effects of ethylene on leaf senescence, since it influences fruit ripening and senescence. Thus, in the study by Kim and Botella (2004), cotyledons from 6-day-old seedlings of the cvs. LE126 and Seagreen were transformed by *A. tumefaciens*

carrying a construct with the ethylene mutant receptor gene *etr1-1*, under the control of the same promoter, *SAG12*, as used by McCabe *et al.* (2001). Expression of *etr1-1* induced root formation from 34% of the explants with a delay in callus and shoot initiation. Only a limited number of explants regenerated shoots, indicating that ethylene insensitivity conferred by *etr1-1* modifies shoot regeneration. Additional studies are required to characterize fully the long-term effects of ethylene on leaf senescence in lettuce.

4.1.2.8 Synthesis of high value recombinant proteins

The synthesis of pharmaceutically important compounds in plants through recombinant-DNA technology has important medical and economic implications, with plant-made oral vaccines having the potential to overcome many of the limitations of traditional vaccines. For example, Webster *et al.* (2006) expressed a measles virus hemagglutinin protein in transgenic lettuce in efforts to produce a vaccine, while Kim *et al.* (2006a) also produced a cholera toxin B subunit in lettuce following *Agrobacterium*-mediated transformation of cotyledons. Western blot analysis and enzyme-linked immunosorbent assay quantification provided an estimated 48 ng of toxin B protein per 20 µg of total soluble protein, amounting to 0.24% of the latter. The generation of transgenic plants has also been directed to the oral delivery of recombinant proteins to animals. Thus, Legocki *et al.* (2005) showed that transgenic lettuce expressing the E2 glycoprotein of Classical Swine Fever Virus or cysteine protease from *Fasciola hepatica*, induced immunoprotective properties in laboratory mice. Such experiments provide a basis for the transformation of plants exploited more routinely in the raw state for animal feeds, such as grasses, root crops, and members of the Brassicaceae.

Negrrouk *et al.* (2005) developed a simple system to produce recombinant antibodies in lettuce and, since the procedure negates the requirement for plant growth facilities, it should be applicable to any cultivar. Mature harvested heads were dipped in a suspension of *A. tumefaciens* strain C58C1 carrying the appropriate construct, before vacuum infiltration. Subsequently, the heads were

rinsed in water and incubated on wet paper in closed transparent boxes at 20–26 °C, with a 16 h photoperiod, for 3–4 days. Leaves were homogenized and protein extracted to yield 20–80 mg kg⁻¹ fresh weight of leaf tissue of humanized IgG1 k antitissue factor antibody (hOAT) in less than 7 days. This simple procedure should be amenable to scale-up for a range of products. Joh and VanderGheynst (2006) also emphasized the merits of agroinfiltration of plant tissues for transient gene expression as an alternative to stable transformation of target plants. In fact, the correct combination of *Agrobacterium* and plant tissues can result in the synthesis of more recombinant protein compared to that produced in stably transformed plants, as reported for lettuce by Wroblewski *et al.* (2005). In studies from the same group, Joh *et al.* (2005) also noted that different leaf tissues had different GUS activities, gene expression also being influenced by leaf pigmentation.

4.1.2.9 Male sterility

Male-sterile plants are important as pollen recipients for hybrid plant production by sexual hybridization. In experiments to induce male sterility in lettuce, a pathogenesis-related β -1,3-glucanase gene linked to a tapetum-specific promoter, *A9*, was cloned into the binary vector pBIN19 and the latter introduced into *A. tumefaciens* carrying pGV2260, prior to transformation of the cv. Lake Nyah (Curtis *et al.*, 1996b). Transgene expression resulted in dissolution of the callose wall of developing microspores, inhibiting pollen grain development, resulting in male sterility. This, or a similar approach, may eliminate the need to remove pollen from the stigmatic surface of recipient plants to avoid self-pollination, prior to application of donor pollen.

4.2 Introduction of Genes into Chicory

4.2.1 Modification of plant morphology and physiology

Currently, there are few reports of the introduction and expression of genes in chicory. An early report of genetic manipulation involved the

transformation of root and pedicel explants of the cv. Hybrid Flash by *A. rhizogenes* strains A4RSII and 8169. Plants regenerated from roots transformed by strain A4SRII changed from biennial to annual flowering, and exhibited stunted growth, wrinkled leaves, reduced development of taproots, but proliferic development of lateral roots (Sun *et al.*, 1991). Although the plants still had a requirement for an inductive period to promote flowering, a vernalization period was unnecessary. The phenotypic alteration of growth habit in these transgenic plants was at least partially associated with expression of the *rol A-C* genes on the Ri T-DNA, these genes being shown to have a similar effect on flowering in other biennial plants, such as carrot.

Following the report by Sun *et al.* (1991), Limami *et al.* (1998) investigated the switch from biennial to annual flowering. Backcrossing of transformed with nontransformed plants produced a line that retained annual flowering in the absence of other phenotypic abnormalities. Molecular analyses indicated that the annual habit was associated with a truncated T-DNA inserted into the plant genome. When transgenic plants were harvested at the end of a 5-month period, the inulin concentration in their roots was found to be the same as that in nontransformed plants, ranging from 71% to 75% of the root dry weight. However, at the end of the vegetative period, the free fructose content increased 8- to 10-fold in the roots of transformed plants because of increased inulin hydrolysis in the transgenic plants at this time. In contrast to the situation in nontransformed plants, chicon induction in transgenic plants did not require a vernalization period of 0–2 °C for 14 days.

Vijn *et al.* (1997) exploited the transformation protocol of Vermeulen *et al.* (1992) to introduce a fructan:fructan 6G-fructosyltransferase (6G-FFT) from onion into chicory. The results of these experiments showed that it was possible to modify the type of fructan synthesized in a fructan-producing plant. Transgenic plants were found to accumulate the fructan encoded by the transgene, together with endogenous fructans. Genes, such as *gus* and *gfp* (green fluorescent protein), are useful as reporters as their expression can be monitored at all stages of cellular differentiation. For example, in studying the origin of shoots from cultured leaf explants, Pieron and Watillon (2001)

transformed chicory with a 23 kb fragment of an apple calmodulin promoter fused to the coding sequence of the *uidA* (*gus*) gene. The origin and development of shoots were readily detected by the indigo staining of cells expressing the *gus* gene.

4.2.2 Herbicide tolerance

The introduction of herbicide tolerance into chicory was addressed using a mutant acetolactate synthase gene (*csr1-1*) from *Arabidopsis*, which conferred resistance to the herbicide chlorsulfuron (Vermeulen *et al.*, 1992). Leaf discs from 8-week-old plants were inoculated with an overnight culture of *A. tumefaciens* strain LBA4404 harboring pGH6, the latter carrying the gene of interest and the *neo* gene (from pBIN19) conferring kanamycin resistance on transformed plant cells. Leaf discs were transferred after co-cultivation with bacteria to semi-solid MS-based shoot induction medium, shoot regeneration occurring after 12–15 days in the presence of 0.1 mg l^{-1} IAA and 1 mg l^{-1} BAP. Transformed shoots were selected, micropropagated, and acclimatized to glasshouse conditions; flowering was induced by vernalizing the plants for 2 months at 6°C prior to self-pollination. Seeds germinated *in vitro* and seedlings were resistant to both kanamycin and chlorsulfuron.

4.2.3 Assessments of the response of explants to *Agrobacterium* inoculation

In studies to increase transgenic plant regeneration, taproots, leaf discs, and cotyledons, the latter from etiolated seedlings of the Witloof cv. Flash, were transformed using *A. tumefaciens* strains carrying either pGSGLUC1 or pTDE4, encoding *nptII* and *uidA* (*gus*) genes under different promoters (Abid *et al.*, 1995). Genes on pGSGLUC1 were driven by the TR1' and TR2' promoters, while on pTDE4 the *uidA* and *nptII* genes were controlled by the CaMV 35S and *nos* promoters, respectively. The suspension of *Agrobacterium* for inoculating the explants was cultured in medium containing $100 \mu\text{M}$ acetosyringone. The culture medium used was crucial

in these experiments. Taproots were incubated on Heller's (1953) medium containing 75 mg l^{-1} kanamycin. Leaves and cotyledons were wounded manually prior to bacterial inoculation and before culture on half-strength MS-based medium in the presence of 0.1 mg l^{-1} BAP for 2 days followed by selection with $75 \mu\text{g ml}^{-1}$ kanamycin. After 2–3 weeks, shoots were transferred to agar-solidified Heller's medium containing 10 g l^{-1} sucrose and $75 \mu\text{g ml}^{-1}$ kanamycin to induce roots. Cotyledon explants were the most responsive to *Agrobacterium* inoculation. As shoots developed rapidly from taproots, it was necessary to maintain root explants on a medium that was relatively poor in nutrients to prevent the production of chimaeric shoots. Leaf and cotyledon tissues required a more nutrient rich medium for shoot initiation. In these experiments, kanamycin selection did not inhibit shoot regeneration from culture explants, although in earlier work, Vermeulen *et al.* (1992) demonstrated that kanamycin inhibited shoot regeneration.

Buds have also been used in transformation studies of chicory (Frulleux *et al.*, 1997). Explants of the cvs. Hicor, Inula, Tilda, VBF, VBG, and VAX were cultured on semi-solid B5-based medium containing 0.9 mM BAP. *A. tumefaciens* strain GV2260:35SGUSINT carrying the *gus* gene was used in transient expression studies. *Agrobacterium* strain EHA101 carrying pGV1531 with *nos-nptII* and CaMV 35S-*uidA* genes was used for stable transformation. Shoot buds (each 2–3 mm in size) were inoculated with an overnight bacterial suspension, before co-cultivation with the bacteria for 2 days. Explants were washed with a solution of carbenicillin at 500 mg l^{-1} before culture with the same concentration of the antibiotic to eliminate bacteria. Kanamycin at 100 mg l^{-1} was used for initial selection the concentration being increased to 200 mg l^{-1} after 4 weeks. Shoots were exposed to this selection pressure for 4 months, before being rooted on 0.98 mM IBA. Leaf discs excised from putatively transformed plants regenerated shoots on kanamycin-containing medium, while Southern analysis confirmed the presence of the *nptII* gene in kanamycin resistant plants. Transformed plants were self-pollinated after vernalization treatment (6°C); segregation analysis showed a 3:1 ratio of kanamycin resistant to sensitive seedlings.

More recent investigations have used *A. rhizogenes* for gene delivery. Bais *et al.* (2001) inoculated hypocotyls from 2- to 10-day-old seedlings of the chicory cv. Lucknow Local with strains LMG-150, A20/83, and AZ/83 of mannopine-type bacteria. After co-cultivation on MS-based medium for 3 days, explants were exposed to 500 mg l⁻¹ of carbenicillin, with subculture to new medium every 3 days. Roots that developed were transferred to liquid MS-based medium in the dark on a rotary shaker to obtain axenic cultures of transformed “hairy” roots. A bacterial concentration of 10⁸ cells ml⁻¹ was optimal for inoculation, with most explants producing transformed roots following inoculation with strain LMG-150. However, this response declined with explants taken from seedlings more than 2 days old. Shoots were induced from transformed roots on MS-based medium with 4.0 mg l⁻¹ BAP and 1.0 mg l⁻¹ NAA, and were micropropagated on medium with 0.5 mg l⁻¹ of GA₃, 2.0 mg l⁻¹ 2,ip, and 0–50 mM putrescine. The latter at 40 mM was optimal for shoot induction and stimulated flowering *in vitro* of regenerated shoots. Putrescine could be replaced by silver nitrate at 40 μM to stimulate shoot multiplication. In their subsequent investigation, Bais and Ravishankar (2003) showed IAA and IAA oxidase to be more in roots induced by strain LMG-150, with IAA, IAA oxidase, coumarin, and biomass being stimulated by treating cultured roots with putrescine at 1.5 mM.

4.3 Spinach

4.3.1 Transformation by *Agrobacterium*

The transformation of spinach by *Agrobacterium* is considerably more difficult to achieve compared to lettuce and chicory and is highly cultivar dependent. Al-Khayri (1995) transformed spinach using an established culture system (Al-Khayri *et al.*, 1991). They inoculated leaf discs from 3-month-old plants, hypocotyl segments from 14-day-old seedlings and cell suspensions from leaf-derived callus of the cv. High Pack, with *A. tumefaciens* strain A208 carrying the disarmed binary vector pMON9749, the latter with the CaMV 35S-*gus* and *nos-nptII* genes, and a gene for spectinomycin

resistance. Leaf explants and hypocotyl segments were co-cultivated with *Agrobacterium* on MS-based medium supplemented with 2.0 mg l⁻¹ kinetin and 0.5 mg l⁻¹ 2,4-D to induce callus. Later, the explants were transferred to medium with 100 mg l⁻¹ cefotaxime, 100 mg l⁻¹ carbenicillin, and 50 mg l⁻¹ kanamycin to select transformed callus and transgenic shoots. Callus formed in the dark on 3–8% of the leaf explants and on 4–10% of the hypocotyl segments. Cell suspensions were inoculated with 50–200 μl aliquots of an overnight bacterial suspension, washed 2 days later with liquid MS-based medium to remove the bacteria and incubated in liquid medium with antibiotics, including kanamycin, for 21 days in the light. Cells were plated onto kanamycin-containing selection medium for 21 days in the dark, prior to transfer to semi-solid shoot regeneration medium. Most cells in the suspension were GUS positive after incubation with agrobacteria. Consequently, it was argued that cell suspensions provided the optimum experimental system for spinach transformation. Kanamycin resistant plants transferred to the glasshouse were morphologically normal.

Three publications appeared in 1997. Knoll *et al.* (1997) described shoot regeneration using root explants as the starting material. Roots that were the source of the explants were induced on cultured seedling hypocotyl segments of the cvs. Longstanding Round and RS No. E, this regeneration system being used for transformation. *A. tumefaciens* strain 0065 and the supervirulent strain 1065, carrying the binary vector pMOG23 with *nptII* and *gus* genes, were used for inoculation. Explants were immersed in a 1:10 (v:v) dilution of an overnight culture for 2 s or in a 1:1 dilution of the bacterium for 10 min. Inoculated explants were cocultivated for 2 days with agrobacteria followed by transfer to Nitsch and Nitsch (1969) medium supplemented with 100 mg l⁻¹ cefotaxime, 100 mg l⁻¹ carbenicillin, and 50 mg l⁻¹ kanamycin sulfate for selection. Although transgenic plants were not obtained, histochemical studies showed shoot buds exhibiting GUS activity 56 days after bacterial inoculation on explants taken from roots that had been cultured for 20 months prior to inoculation with strain 1065.

Daniels *et al.* (1997) targeted leaf and cotyledon explants using *A. tumefaciens* strain AGLO

carrying a binary vector with the *nptII* gene and the *gfp* gene for green fluorescent protein. The *gfp* gene with the CaMV 35S promoter, was designed to target GFP to the endoplasmic reticulum. Explants were inoculated with overnight bacterial cultures prior to culture in the dark at 24 °C for 84 days on callus induction medium (Al-Khayri *et al.*, 1991). Stable gene expression was reported in cultured plant tissues 21 weeks from the initiation of the experiment. A bacterial suspension of optical density 1.0 resulted in a higher percentage of calli expressing *gfp* than a more dilute bacterial suspension with an optical density of 0.3. Although transformed callus was obtained from leaf explants, cotyledon explants could not be transformed using the same approach.

The spinach cvs. Fall Green and High Pack were targeted by Yang *et al.* (1997) in attempts to introduce virus resistance into the latter cultivar. *A. tumefaciens* strain LBA4404 was used carrying the binary vector pB1121, with the coat protein gene of the CMV isolates, SP103, and SP104, replacing the *gus* gene of the original construct. Leaf strips and hypocotyl segments from 6-week-old plants were inoculated with overnight cultures of *Agrobacterium* to which 0.05 mM acetosyringone had been added 4 h before explant inoculation. Explants were transferred to callus induction medium (Al-Khayri *et al.*, 1991) after 2 days of co-cultivation with bacteria on semi-solid MS-based medium. The latter was supplemented with 237 µM carbenicillin, 209 µM cefotaxime, and 86 µM kanamycin, with transfer of cultures to new medium every 4 weeks over a period of 12 weeks. Calli were then transferred to shoot regeneration and root induction media (Al-Khayri *et al.*, 1991), both supplemented with cefotaxime and carbenicillin, in the light. Shoots were induced on both leaf and hypocotyl-derived calli of the cv. High Pack. Shoots were not obtained from the cv. Fall Green. In contrast, 12% of the regenerated plants of the cv. High Pack carried the CMV coat protein gene, the latter being detected using the PCR, with Southern and Northern blots confirming the presence of the gene and its protein product in transgenic plants.

Zhang and Zeevaart (1999) reported a rapid and efficient procedure for generating transgenic spinach plants by inoculating cotyledons excised from 5-day-old seedlings of the cv. Longstanding Bloomsdale Dark Green. As reported by Yang

et al. (1997), *A. tumefaciens* strain LBA4404 pB1121 was used, in which the CaMV 35S-*gus* region of the original construct was replaced by 35S-*smgfp*, a gene encoding a more soluble version of a codon-modified green fluorescent protein. The rationale for using the *gfp* gene as a marker was based on the earlier studies of Yang *et al.* (1997) and Daniels *et al.* (1997). *Agrobacterium* was cultured overnight with 0.2 mM acetosyringone, before being diluted 1:10 by volume with new medium and culture for a further 48 h prior to inoculating explants. Co-culture with *agrobacteria* was on MS-based medium with 1.0 mg l⁻¹ BAP and 0.4 mg l⁻¹ NAA, prior to transfer to selection medium containing 100 mg l⁻¹ cefotaxime, 200 mg l⁻¹ carbenicillin, and 50 mg l⁻¹ kanamycin. Eighty-six percent of the inoculated explants cultured in the presence of kanamycin regenerated shoots. The latter were excised from the parental explants and were immersed in liquid MS-based medium supplemented with 20 mg l⁻¹ IBA for 2 h, before transfer to semi-solid MS-based medium containing 1.0 mg l⁻¹ IBA for rooting. Kanamycin resistant plants were hybridized with wild-type plants, as the dioecious nature of spinach precluded self-fertilization. Seeds were germinated on selection medium containing kanamycin sulfate at 50 mg l⁻¹ with a 1:1 segregation of resistant to susceptible T₁ generation seedling; seedlings of the T₂ generation were found to segregate 3:1 (resistant to susceptible), suggesting that the *nptII* gene was inherited in a Mendelian fashion. Northern and Southern analyses established the transgenic nature of the T₂ generation plants. This investigation by Zhang and Zeevaart (1999) represented a major advance in spinach transformation.

The limited reports on spinach transformation probably reflect the difficulty of inserting genes into this leafy vegetable. The age and source of explants that are inoculated with *Agrobacterium* are critical in maximizing transformation. In attempts to stimulate transformation, Daniels *et al.* (1997) supplemented the medium for bacterial inoculation with the surfactant Pluronic F-68, this increasing transformation from 5.1% to 19.7%. Dilution of the *Agrobacterium* suspension and the time of inoculation are important, Knoll *et al.* (1997) reporting that a 1:10 (v:v) dilution of an overnight *Agrobacterium* culture stimulated transformation compared with an

undiluted culture, or a bacterial suspension diluted 1:1 by volume as the inoculum. *Agrobacteria* used for inoculation of plant tissues are also important, supervirulent strains, such as 1065, giving the highest rates of transformation.

4.3.2 Viral vectors for gene introduction into spinach

As in lettuce, investigations have been initiated to induce spinach to synthesize pharmaceutically important products. In circumventing the requirement for *Agrobacterium*-mediated gene delivery, Karasev *et al.* (2005) cloned a HIV-1 *tat* gene into a tobacco mosaic virus based vector in studies to develop a preventative AIDS vaccine. The HIV-1 *tat* protein has been exploited as a possible vaccine candidate because of its broad nonspecific action, with the potential of delivery through the mucosal route following *tat* expression in an edible plant. Spinach plants inoculated with *tat*-producing constructs synthesized up to 300 µg of *tat* per gram of leaf tissue. The attraction of such a system is its rapidity, since plants can be harvested for use within 7–14 days of inoculation with the viral constructs.

5. PRESENT AND FUTURE STRATEGIES FOR LEAFY VEGETABLES

5.1 Plastid Transformation—The Way Ahead?

Whilst genetic manipulation studies have focused, in general, on the introduction of genes into the nucleus, interest in plastid transformation has gained momentum, since it offers advantages compared to nuclear transformation. Such advantages of plastid transformation include lack of gene silencing because of the ability to integrate genes into specific intergenic regions of the plastome, and the potential for high foreign gene expression. Transgene containment is also an important issue because of the maternal inheritance of cytoplasmic genomes (Daniell *et al.*, 2002; Maliga, 2003; Skarjinskaia *et al.*, 2003). In the first report of plastid transformation in lettuce, Lelivelt *et al.* (2005) used PEG-induced DNA uptake into isolated leaf protoplasts of the

lettuce cv. Flora to generate fertile, homoplasmic plants. The vector used for transformation targeted genes to the *trnA-trnI* intergenic region of the lettuce plastome, with the *aadA* gene for resistance to spectinomycin as a selectable marker. Spectinomycin resistance and heterologous gene transcription were demonstrated in first seed generation transgenic (T₁) plants, following self-pollination of primary regenerants. Crossing of transgenic plants with male sterile wild-type lettuce confirmed that antibiotic resistance was not transmitted by pollen. Using a similar strategy, Kanamoto *et al.* (2006) reported transplastomic plants of the lettuce cv. Cisco, the *aadA* gene being inserted between the *rbcL* and *accD* genes of the lettuce genome. Both Lelivelt *et al.* (2005) and Kanamoto *et al.* (2006) discussed the potential of plastid transformation for the production and delivery of edible human therapeutic proteins. Plastid transformation is also an important option for chicory and spinach and it seems likely that this technology will be developed for these leafy vegetables in due course.

5.2 Risks and Consumer Concerns

Maintaining adequate supplies of leafy vegetables is an ongoing procedure in order to satisfy consumer demands. For example, the market for lettuce has increased in the last two decades, accompanied in some cases, by a change in the range of cultivars being grown, especially for processed mixed salad. Since the viability of lettuce seed is limited when stored at room temperature, experiments have been conducted on the longevity of cryogenically stored seeds (Walters *et al.*, 2004). Cryogenic storage prolonged seed viability giving estimated half-lives in excess of 500 and 3400 years for freshly harvested seed stored in the vapor and liquid phases, respectively, of liquid nitrogen. This approach may be important in the future long-term conservation of elite lines not only of lettuce but also of other leafy vegetables.

All leafy vegetables deteriorate rapidly following harvest, with a considerable investment of effort to maintain quality and shelf life of cut material. Harvesting increases respiration, stimulating deterioration, with increase in the synthesis of phenylalanine ammonia lyase and phenolic compounds, such as chlorogenic acid that

cause tissue browning (Kang and Saltveit, 2003). Exposing cut heads and leaves of iceberg lettuce to 1-methylcyclopropene decreased the accumulation of phenolic compounds and tissue discoloration (Saltveit, 2004), while wound-induced accumulation of phenolic compounds and browning in the Romaine lettuce cv. Longifolia was reduced by exposure to n-alcohols (Choi *et al.*, 2005). Ragaert *et al.* (2006) reported the volatile metabolites generated by bacteria (*Pantoea agglomerans* and *Rahnella aquatilis*) and spoilage yeasts (*Pichia fermentans* and *Cryptococcus laurentii*) in lettuce.

Although leafy vegetables, as consumed in the raw state, make a significant contribution to a healthy diet, there is a need to reduce health risks from such uncooked vegetables. Consequently, attempts have been made to reduce microbial activity on the surface of harvested lettuce leaves, using, for example, ozone in combination with chlorine, and ozonated water to reduce surface microbes (Garcia *et al.*, 2003; Beltrán *et al.*, 2005) whilst retaining sensorial qualities. Washing with calcium lactate (Martin-Diana *et al.*, 2005; Rico *et al.*, 2006), exposure to chlorine dioxide gas (Lee *et al.*, 2004), and chlorine and peroxyacetic acid, as sanitizers (Beuchat *et al.*, 2004) have been investigated in destroying surface contaminants such as *Listeria monocytogenes*, *Escherichia coli* 0157:H7, *Salmonella typhimurium*, and *Enterobacter sakazakii* (Kim *et al.*, 2006b). Calcium lactate maintained quality better than treatment with ozone or ozone combined with calcium lactate (Rico *et al.*, 2006). Similar investigations have been performed on the effect of different sanitizers on the microbial, nutritional, and sensory qualities of leaves of rocket (*Diplotaxis tenuifolia*; Martinez-Sanchez *et al.*, 2006).

Exposure of lettuce to ultraviolet radiation could reduce microbial loading without affecting quality, as in the cv. Lollo Rosso (Allende and Artés, 2003; Yaun *et al.*, 2004; Gómez-López *et al.*, 2005). The atmosphere within the packaging of harvested material, the time of packaging following cutting, and the type of packaging, also influence the shelf life of the harvested produce (Kim *et al.*, 2005a, b; Del Nobile *et al.*, 2006). An interesting paper by Song *et al.* (2006) demonstrated that the method of irrigation of plants before harvest influences the microbial population on their leaves, subsurface drip irrigation having the potential to reduce

health risks of lettuce. Park *et al.* (2006) developed multiplex PCR assays to detect pathogens in leafy vegetables, since robust procedures are fundamental in detecting food-based pathogens.

6. CONCLUDING REMARKS

Globally, leafy vegetables are of considerable nutritional and economic importance, with lettuce and spinach being major sources of carotenoids, particularly lutein and zeaxanthin (Lucarini *et al.*, 2006). Consequently, it is not surprising that considerable effort will be focused on maximizing yield and durability of these crops through both conventional breeding and genetic manipulation approaches. Molecular procedures will facilitate the selection of germplasm for incorporation into breeding programs. Shoot regeneration from explants, tissues, and isolated protoplasts, facilitates the application of *in vitro*-based technologies to underpin conventional breeding. Whilst gene manipulation procedures have been based mainly upon transformation, somatic hybridization by protoplast fusion should not be ignored, since this provides a means of mobilizing genes without the need for sophisticated recombinant-DNA technology. Where transformation is exploited for cultivar improvement, long-term transgene expression may be compromised in certain lettuce cultivars. This issue needs to be addressed; the use of appropriate constructs and transformation protocols should reduce the incidence of this problem. Some cultivars of chicory are amenable to transformation. However, in general, transformation is not readily applicable to spinach at the present time. In both spinach and chicory, lack of protoplast-to-plant systems precludes the use of somatic hybridization for gene transfer, at least in the immediate future. Conventional breeding will continue to play a central role in the improvement of existing cultivars for traits, such as resistance to tipburn (Hayes, 2006) and in the development of new cultivars of leafy vegetables. In addition, it is likely that producers will become more long-term dependent upon somatic cell approaches in order to meet consumer demands in terms of the availability of new cultivars with improved nutritional quality, enhanced shelf life, and maximum food safety.

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Sugarcane

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Sugarcane is a large grass of the genus *Saccharum*, tribe Andropogoneae, family Poaceae. Modern sugarcane (*Saccharum* spp.) cultivars are interspecific hybrids derived from a hybridization process involving *Saccharum officinarum* (or “noble cane”) and *Saccharum spontaneum* (wild cane), followed by a series of backcrosses to the noble parent (Daniels and Roach, 1987).

The earliest known historical record of sugarcane and sugar is from Indian writings from 3000 to 3400 years ago. The generic name for sugarcane, *Saccharum*, originated from the Indian Sanskrit term “*sharkara*” for the crude sugary product obtained from the honey reeds. Dispersal of Indian sugarcane westward seems to have occurred during the first millennium BC. Soldiers of Alexander the Great are known to have carried it to Europe from India about 325 BC. Later, Greek and Roman

writers were familiar with the concept of the Indian honey reed and its “honey” (sugar) product. The early history of sugarcane is covered by a number of authors, including Deer (1949) and Barnes (1964).

The origin of sugarcane is a complex question that is best discussed in relation to its taxonomy and distribution in Southeast Asia, the Indonesian Archipelago, and New Guinea. Different species likely originated in various locations with *S. officinarum* and *Saccharum robustum* in New Guinea, *Saccharum barberi* in India, and *Saccharum sinense* in China. Dispersal of *S. officinarum* over a period of thousands of years is believed to have occurred both into the Pacific Ocean area, and along the island chain into Asia, whilst the thinner Indian canes were developed and cultivated in the North India/South China region.

Initially, pieces of cane stalk would have been chewed to express the sweet juice, and chewing canes still provide a conveniently packaged form of energy food in many cultures. Juice extraction

from the stalk, and concentrating it by drying or heating to produce a crude sugary product, must have been developed in a rudimentary form at least 3000 years ago. The art of sugar manufacture took longer to develop, probably in India and perhaps less than 2000 years ago. Deer (1949) considered that Nestorian Christian monks at the mouth of the Euphrates river were the first to refine the crude raw product into a form of “white” sugar about 450 AD.

The Mediterranean sugar industry was the first major one in Europe, and began about the time of the Arabian conquest of Egypt in 640 AD. They spread it across North Africa and into Spain by 750 AD, where it was important for many years, with 30 000 ha under cane by 1150 AD. By the early 1500s AD, cane was carried by the Spanish to the Caribbean and the Americas, and by the Portuguese to West Africa and Brazil. And so the worldwide sugarcane industry was born.

Prior to the 20th century, the world sugarcane industry was dependent on the noble canes (*S. officinarum*) and the cane from India and China (*S. barberi* and *S. sinense*, respectively) (Sreenivasan *et al.*, 1987). These canes were characterized by high sucrose levels and low fiber contents, but were susceptible to several pests and diseases, notably sereh disease (Arceneaux, 1965). Sugarcane breeding and selection became a directed, ongoing process following the observation in 1858 that sugarcane panicles produce viable seed (Stevenson, 1965). The Dutch established a breeding and selection program in 1888 in Java to incorporate the disease resistance, hardiness, and tillering capacity of *S. spontaneum* into *S. officinarum* germplasm. Interspecific crosses were made between *S. officinarum* and the wild *S. spontaneum* (which was resistant to sereh) (Stevenson, 1965). The resultant hybrids were continually backcrossed to *S. officinarum* in a process called nobilization (Stevenson, 1965). This effort resulted in the release of the first of the nobilized hybrid cane cultivars, POJ2725 and POJ2878 in 1921. These two early cultivars served as the foundation in the pedigree of nearly all locally developed and adapted modern sugarcane cultivars worldwide (Moore, 2005). Nobilization became established as a method of retaining the desirable qualities of *S. officinarum*, retaining the hardiness and disease resistance of *S. spontaneum*, while diluting the negative effects of wild germplasm (Berding and Roach, 1987).

Currently, sugarcane is widely grown for sugar production in many tropical and subtropical countries (with a minimum of 600 mm of annual rainfall) in South, Central, and North America, the Caribbean, Africa and adjacent islands, Southern Asia and Australasia.

1.2 Evolution, Taxonomy, Cytological Features, and Genome Size

Mukherjee (1957) coined the term “*Saccharum* complex” to describe a large, closely related genera (*Erianthus* sect *Rhipidium*, *Miscanthus*, *Sclerostachya*, *Narenga*), which are considered to be involved in the evolution of cultivated species of *Saccharum*. The phylogenetic relationships within the *Saccharum* complex have been debated for many years (Daniels and Roach, 1987; Irvine, 1999). A long discourse on how the various species may have evolved is provided by Daniels and Roach (1987).

Sugarcane technologists consider six species to be important as progenitor species in the origin of modern commercial hybrids (*Saccharum* spp.). These six species (listed below) are distinguishable on the basis of sugar content, thickness of stalk, floral characteristics, chromosome number and epidermal hairs. The first four in the list below are in cultivation, while the last two (*S. spontaneum* and *S. robustum*) are wild species growing in southern Asia and New Guinea.

- *S. officinarum* L.: sweet, juicy, thick stalk garden cane, initially in New Guinea
- *S. barberi* Jesw.: sweet, thin stalk Indian canes
- *S. sinense* Roxb.: sweet, thin stalk Chinese canes
- *S. edule* Hassk.: edible inflorescence garden cane, New Guinea, Melanesia
- *S. spontaneum* L.: very thin, hardy wild canes, low sugar, New Guinea and southern Asia
- *S. robustum* Brandes & Jeswiet ex Grassl: tall, harder, thick stalk wild canes, a little juice and sugar, New Guinea and eastern Indonesia

Modern commercial sugarcane cultivars are highly heterozygous, complex polyploid, and aneuploid hybrids, often with four of the above species of *Saccharum* in their ancestry. Cytological studies showed that nobilization is characterized by asymmetric chromosome transmission (Bremer, 1961). In a cross between *S. officinarum* ($2n = 80$) as the female parent and

S. spontaneum ($2n = 40\text{--}128$) as the male parent, *S. officinarum* generally transmits two haploid chromosome sets while *S. spontaneum* transmits one. This $2n + n$ transmission is continued up to the second backcross. A consequence of this is that modern cultivars have chromosome numbers ranging from $2n = 99\text{--}130$ (Bremer, 1961). In addition, commercial sugarcane cultivars have complex polyploid (10–12 copies of the genome) and aneuploid (100–120 chromosomes) genomes. In polyploids such as sugarcane, the haploid chromosome number (1C value = n) is not the same as the monoploid number (= x) (Butterfield *et al.*, 2001). The monoploid genome size for *S. officinarum* ($x = 10$) is approximately 926 Mbp (mega base pair), and for *S. spontaneum* ($x = 8$) 760 Mbp (Butterfield *et al.*, 2001). This base genome number is roughly double the monoploid genome size of rice (415 Mbp) and similar to that of *Sorghum bicolor* Moench (760 Mbp) (Butterfield *et al.*, 2001).

1.3 Economic Importance: Production, Utilities, Economic Attributes, and Industrial Uses

Sugarcane is cultivated for its high rate of sucrose accumulation, ease of propagation via vegetative stem cuttings and multiple harvests from a single planting. It is a principal crop in tropical and subtropical regions, with a production estimate of over 1.3 million metric tons of sucrose per annum. It provides approximately 70% of the world's sugar (FAO, 2006).

Sugarcane has long been recognized as one of the most efficient crops in terms of converting solar energy into biomass (Alexander, 1973). It is one of the most effective photosynthesizers in the plant kingdom, able to convert 2% of incident solar radiation into plant biomass. It is the second largest contributor (10–12%) of dietary carbohydrate to humans after the cereals. Sugar processing meets the needs of both high-income consumers (e.g., refined white and specialty sugars) and low-income domestic consumers (e.g., the production of jaggery in India or panela in Colombia, where cane juice is boiled to make cakes of brown sugar). By-products of sugar milling such as bagasse, molasses, furfural, furfuryl alcohol, dextran, and diacetyl (O'Reilly, 1998) have several uses. For example, bagasse (a fibrous residue after sugar

extraction) can be used to fuel boilers in the sugar mills, to generate electricity for the local power grid, to manufacture paper, and as an animal feed. Molasses are used in syrups and animal feed and as a substrate for ethanol production.

Sugarcane is considered as a critical component of our bioenergy future as: (i) sugarcane is already used in the production of ethanol, produced by fermentation and distillation of sugars. Currently, Brazil is the world's largest producer of sugarcane ethanol (Moreira, 2000). Ethanol can be blended with gasoline (gasohol) or diesel (biodiesel or dieselhol); (ii) production of energy, such as ethanol, from sugars is more efficient than production from grains, in both cost for joule produced and energy input/output efficiency. For each unit of fossil energy input to the sugarcane agro-industrial system, nine units of renewable energy output (ethanol plus surplus bagasse) result, compared to less than 2 units resulting from grains, such as maize; and (iii) sugarcane is ranked first among all other crops for biomass production (FAO: <http://www.fao.org>; Moreira, 2006). As a perennial crop, it has more advantages for biofuels production than annual crops. It is more efficient at solar energy conversion, and it can be harvested annually for a number of years without replanting.

1.4 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

The objectives of sugarcane breeding programs around the world are to produce cultivars with improved characteristics such as increased cane yield, higher sucrose content, pest and disease resistance, tolerance to abiotic stress, and improved ratooning ability. Focused breeding programs have led to significant contributions to characters such as those listed above (Hogarth, 1976; Nuss, 2001).

Crossing of two parents is the first step in producing and selecting a new cultivar. If pollen and seed production do not occur naturally, as is the case in sugarcane growing areas with latitudes above 15° north and south, crossing is conducted in photoperiod glasshouse facilities where day length and temperature can be manipulated (Brett, 1974). Extensive evaluation of the progeny from the crosses is undertaken. The selection process

consists of several different stages and usually takes 10–14 years to release a variety. This prolonged period before a new sugarcane variety can be commercially released is largely due to the reliance of the selection process on phenotypic characters. At each stage, clones with unsuitable characters are discarded and the performances of selected clones are evaluated in larger plots (Parfitt, 2005).

Due to the narrow genetic base of modern varieties (Hogarth, 1987), sugarcane breeders have tried to exploit the genetic variation within the *Saccharum* complex, which shows great variation for a range of traits of interest to the breeders, such as sugar content, tolerance to drought and cold, pest and disease resistance, uprightiness, fiber content, tiller number, stalk size and strength, low suckering, easiness to detrash (suitability for mechanical, green harvest), and ratooning ability. Breeders have used *S. spontaneum*, *Miscanthus sinensis*, *Erianthus arundinaceus*, and *Erianthus rockii* in introgression initiatives.

S. spontaneum. In the development of modern sugarcane cultivars, an average of only 15–25% of chromatin is derived from *S. spontaneum* (D'Hont *et al.*, 1995), due primarily to the limited number of *S. spontaneum* genotypes used (Berding and Roach, 1987). Only two genotypes of *S. spontaneum* were used in the initial crosses made in the late 19th and early 20th centuries in India and Java (Martin, 1996). *S. spontaneum* has been considered as a source of positive alleles for traits involved in adaptation to different climatic conditions and for disease and insect resistance. Dunkelman and Breaux (1972) studied the agronomic habits of 32 apparently mosaic-resistant *S. spontaneum* genotypes to ascertain their potential utilization as breeding material, and found a genotype (US 56-15-8) particularly sweet, with a juice Brix reading of 11.5%. The characterization of sugar composition of *S. spontaneum* genotypes from the World Collection of *Saccharum* (Miami, Florida) also indicated that this species is a potential source of positive alleles for sugar content (Tai and Miller, 2001). These results were confirmed by da Silva *et al.* (2007) in a study involving molecular markers to test if alleles with positive effects for sucrose content could be found in *S. spontaneum*. Expressed sequence tags (ESTs) involved in sucrose accumulation from the metabolism of complex carbohydrate pathway (da Silva *et al.*, 2007) were

used to develop molecular markers. By targeting four functionally characterized sugar metabolism candidate genes to a set of 50 *S. spontaneum* genotypes showing variation in sugar content, *S. spontaneum*-specific polymorphic markers were identified. These markers are not present in commercial sugarcane genotypes and may therefore be used for tagging positive *S. spontaneum* alleles for introgression into commercial sugarcane genotypes. Efforts to introgress these alleles were made in 2005 at the US Department of Agriculture (USDA) Sugarcane Unit in Houma, Louisiana, with a cross involving the commercial cultivar HoCP00-950 and the *S. spontaneum* genotype MPTH97-216, from Thailand.

M. sinensis. A difficulty in breeding sugarcane for stress tolerance is the trade-off between stress tolerance and yield (Ming *et al.*, 2006). Developing varieties adapted to a wider range of climatic regimes could improve sugarcane production in water-restricted and/or colder regions. Sugarcane with increased water use efficiency and tolerance to drought or cold temperatures are critical selection criteria for that goal. Another way to overcome this difficulty would be to identify alternative alleles contributing to stress tolerance in the *Saccharum* complex and introgress these into commercial germplasm. Even though the water use efficiency of sugarcane is high, substantial amounts of water are required to maintain maximal growth and productivity. Since irrigation of sugarcane fields is limited, and irrigation of biomass crops is unlikely to be economic, it is important to identify genotypes that are tolerant to water stress. Screening with Paraquat (methyl viologen) for drought tolerance in sugarcane (Ming *et al.*, 2001b), wheat, and barley (Altinkut *et al.*, 2001) has proved to be a rapid and practical screening method, in conjunction with chlorophyll fluorescence measurements, for identifying and characterizing genetic variation in sugarcane water stress tolerance (Ming *et al.*, 2006).

Miscanthus species are exceptionally tolerant to low temperature and drought and are among the few plants in temperate climates that use the C4 photosynthetic pathway (Naidu *et al.*, 2003). In a study comparing drought tolerance of different *Miscanthus* species (*M. sacchariflorus*, *M. giganteus*, and *M. sinensis*), *M. sinensis* was the only one that did not show senescence caused by water deficit (Clifton-Brown *et al.*, 2002).

M. sinensis retained all of its green expanded leaf area irrespective of water supply, showing its complete resistance to senescence. Similarly, when commercial varieties, breeding lines and seedlings of sugarcane and *Miscanthus* were exposed to freezing temperatures for at least 2 h in the Rio Grande Valley of Texas in 2004, leaf damage was observed on all sugarcane plants, but not on *Miscanthus* × *Saccharum* sp. hybrid seedlings (Figure 1). *Miscanthus* species also have high cellulose fiber content and are considered a potential energy crop by the European Union (EU) (Clifton-Brown *et al.*, 2004).

If the superior drought and cold tolerance and high fiber of *Miscanthus* could be combined with the photosynthetic capacity of commercial sugarcane, it would be possible to produce a low-input, high-biomass, drought- and cold-tolerant energy crop. As *M. sinensis* ($2n = 2x = 38$) is closely related to sugarcane, it produces viable hybrids when crossed with sugarcane (Grassl, 1967; Lo *et al.*, 1986), and can be seen as a source of stress

tolerance genes for introgression purposes. Atienza *et al.* (2003) have shown that it is feasible to develop a marker-assisted selection program for biomass production in *Miscanthus* using quantitative trait loci (QTL) to detect markers for traits such as diameter, height, and panicle size. These QTLs could assist in introgression work.

Erianthus species. The *Erianthus* genus contains eight species (Aitken *et al.*, 2007) and is also a potential donor for stress-resistant genes. *E. arundinaceus* has several traits desirable to sugarcane breeders such as tolerance to drought and waterlogging, high resistance to *Pachymetra* root rot, vigor, and good ratooning performance (Berding and Roach, 1987). Many attempts have been made to generate sugarcane × *E. arundinaceus* hybrids. *In situ* hybridization analysis of progeny from some of these crosses indicated that the introgressions were successful (D'Hont *et al.*, 1995; Figure 2). However, only one appears to have been successful in producing fertile offspring. Cai *et al.* (2005) describe the



Figure 1 *Miscanthus sinensis* × *Saccharum* sp. hybrid seedlings showing cold tolerance under subfreezing field conditions

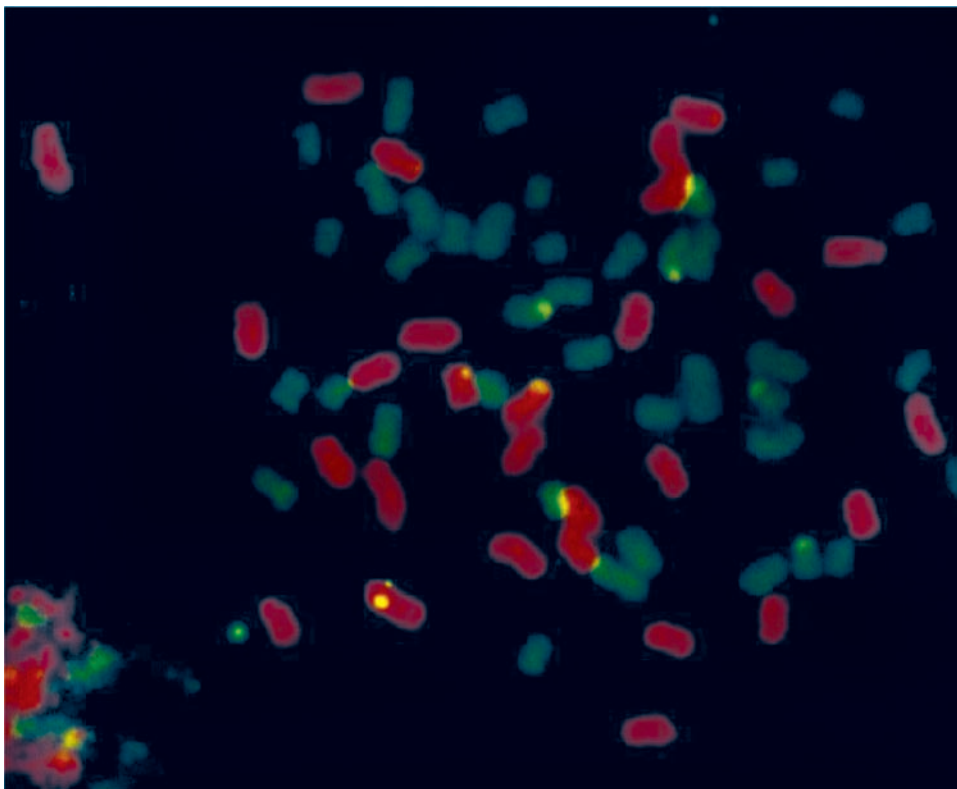


Figure 2 Chromosomes from a root-tip cell of an intergeneric hybrid fluorescing after genomic *in situ* hybridization. *Saccharum officinarum* chromosomes are green and *Erianthus arundinaceus* chromosomes are red [Reproduced with permission from George Piperidis, BSES Limited]

intergeneric hybridization of two populations using the *S. officinarum* Badilla as the female parent in both crosses with pollen from three different *E. arundinaceus* clones from Hainan, China. They also describe the backcrossing of the F₁ hybrid, YC96-40 with sugarcane cultivar CP84-1198 to generate the backcross 1 (BC₁) population, demonstrating the fertility of the intergeneric hybrid. Molecular markers were used to confirm both the introgression into the F₁ and BC₁ populations and the parentage of the BC₁s.

E. rockii. A species originating in the Yunnan, Sichuan, and the Tibetan regions of China, has good vigor, cold and drought tolerance, and good ratooning ability. *E. rockii* was recently reported by Aitken *et al.* (2007) to have been successfully used as the male parent for intergeneric hybridization with (1) Vietnam-niuzhe (*S. officinarum*) and (2) interspecific hybrid Fiji (*S. officinarum* × *S. spontaneum*). Using amplified fragment length

polymorphisms (AFLP), Aitken *et al.* (2007) showed that all of 10 screened *E. rockii* × *S. officinarum* crosses were true intergeneric hybrids, but that 9 of 10 of the *E. rockii* × Fiji crosses were selves of the *E. rockii* female parent and only one was a true introgression. Further analysis showed that there was a ($n + n$) transmission of gametes.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Approaches

Conventional breeding in sugarcane has several limitations that can be overcome by transgenic approaches. Listed below are a few such examples.

1. Commercial sugarcane cultivars possess different proportions of chromosomes, complex recombinational events, and varying chromosome sets (aneuploidy) (Sreenivasan

et al., 1987). This genomic complexity brings difficulties in applying conventional plant breeding for cultivar improvement. In addition, conventional breeding is a multistage, laborious, and time-consuming process requiring 10–14 years to develop a new cultivar. A single fault, such as disease susceptibility in an otherwise elite cultivar, could cause the cultivar to be abandoned. Conventional breeding approaches to correct such faults in an existing cultivar are impractical in sugarcane, due to the genetic complexity of cultivars (Birch and Maretzki, 1993). The capacity to introduce specific genes by transgenic approach, without major genetic reassortment following crossing, could be used to rescue flawed cultivars (Birch and Maretzki, 1993). For example, the successful production of sugarcane plants resistant to leaf scald disease was achieved by transgenic approach (Zhang *et al.*, 1999).

2. Although breeding efforts in sugarcane have been successful in increasing cane production, only limited success has been achieved recently in increasing sugar content. For example, there has been no increase in sugar content over the last 40 years in Australian sugarcane (Bonnett *et al.*, 2004b). In the USA, Legendre (1995) reported that the average sucrose content of new candidate varieties decreased 3.5% on the fifth cycle of recurrent selection, as compared to the previous cycle, indicating that a limit has been reached for this trait. The QTL analysis of interspecific F₁ populations also indicated that modern sugarcane cultivars have a limited (biased subset) population of genes controlling sugar content (Ming *et al.*, 2001b). In contrast, metabolic engineering of sugarcane through transgenic approaches could improve sugar content. For example, transgenic sugarcane with doubled sugar content was achieved when attempting to produce isomaltulose in sugarcane (Wu and Birch, 2007).
3. Production of novel products in sugarcane is not possible by conventional breeding. In contrast, metabolic engineering through transgenic approaches could produce new products, such as alternative sugars, biopolymers, pharmaceuticals, and high-value proteins. For example, successful production of sorbitol (Chong *et al.*, 2007), isomaltulose (Wu and

Birch, 2007), *p*-hydroxybenzoic acid (pHBA) and biodegradable polymer (McQualter *et al.*, 2005; Petrasovits *et al.*, 2007) has been achieved in transgenic sugarcane, which cannot be achieved through conventional breeding.

4. Conventional breeding allows transfer of traits and genes only between sexually compatible species. Hence, transfer of traits from noncompatible species is impossible. In contrast, transgenic approaches allow insertion of novel genes from sexually noncompatible plants/organisms, enable expression of native genes at different levels in specific tissues or under novel developmental patterns of expression.
5. The number of traits to be considered when selecting for variety development is determined by the degree of genetic linkage among those traits. If linkages are rare, several traits can be selected simultaneously. In the case of sugarcane the extent of those linkages is still uncertain (Ming *et al.*, 2006). Recent advances in molecular marker-assisted selection and transformation technologies can alleviate the problem. Thus, genetic transformation by modern molecular techniques (see in Section 2) has the potential to enhance a host of traits including sugar, pest and disease resistance, tolerance to drought and cold, vigor, plant architecture and fiber, and to produce alternative products such as biopolymers and isomaltulose in sugarcane.

2. DEVELOPMENT OF TRANSGENIC SUGARCANE

Sugarcane is a prime candidate for the application of genetic engineering, as single characters can be introduced into the complex genetic background of elite commercial clones to correct negative factors, such as disease susceptibility. Successful genetic engineering requires a reliable tissue culture system and efficient transformation methods. Sugarcane was one of the first monocotyledonous crop plants used successfully for establishment of tissue cultures (Barba and Nickell, 1969; Nickell and Maretzki, 1969), regeneration of plants (Heinz and Mee, 1969), and isolation of protoplasts (Maretzki and Nickell, 1973; Nickell and Heinz, 1973).

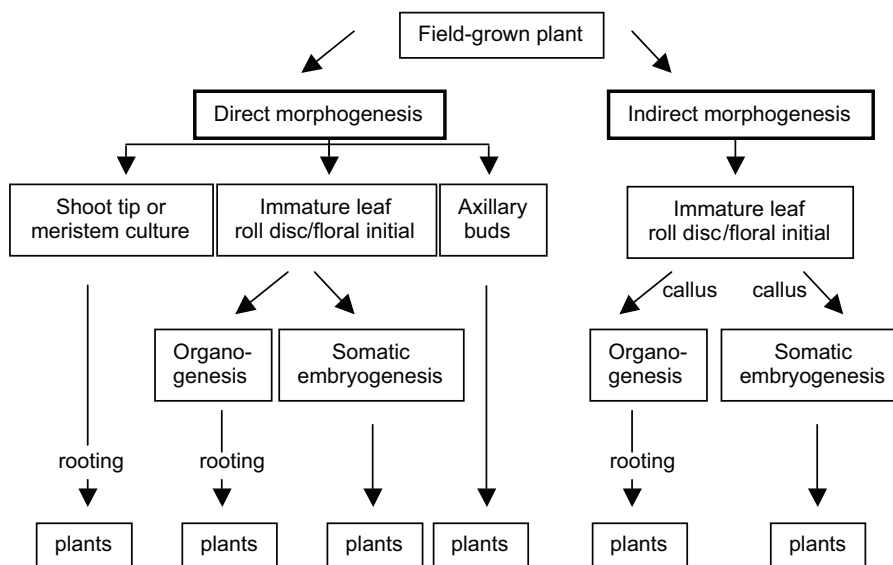


Figure 3 Summary of direct and indirect morphogenic pathways for sugarcane regeneration. In the routes where the rooting event is noted, external application of growth hormones is usually required for root production

2.1 Tissue Culture and Transformation

The early-established robust tissue culture/regeneration system of sugarcane became a foundation for efficient genetic transformation of this crop. The explant most frequently used for transformation experiments in sugarcane is embryogenic callus. This callus is produced from the culture of immature leaf whorls immediately above the apical meristem. The whorls are sliced into thin (2–3 mm) transverse sections and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with an auxin (usually 2,4-dichlorophenoxyacetic acid) and sucrose, and subcultured every 3–4 weeks. Prolific compact embryogenic callus is produced within 2 months. However, this varies with different clones and needs to be optimized for the genotype being used.

2.1.1 Regeneration of plants

Sugarcane has a well-established history of *in vitro* regeneration that began in the late 1960s (Heinz and Mee, 1969). Regeneration of sugarcane can occur via several different pathways (Figure 3) (reviewed by Snyman, 2004; Laksh-

manan, 2006; Lakshmanan *et al.*, 2005). The pathways of regeneration by somatic embryogenesis or organogenesis have been well characterized and documented (Ho and Vasil, 1983; Guiderdoni and Demarly, 1988; Taylor *et al.*, 1992). For the regeneration of transgenic plants *in vitro*, the route of morphogenesis is dependent on the explant targeted for DNA delivery. Criteria for explant choice are: (i) a large number of regenerable cells and (ii) maintenance of regenerative capacity during the selection procedure.

Where embryogenic callus is used as the recipient material for foreign DNA, regeneration is via somatic embryogenesis (Bower *et al.*, 1996; Falco *et al.*, 2000) or organogenesis (Gallo-Meagher and Irvine, 1996). Although transgenic plants have been successfully generated via indirect morphogenesis, limitations include the amount of time taken to regenerate a transgenic plant (36 weeks from DNA delivery to glasshouse transfer) (Bower *et al.*, 1996; Snyman *et al.*, 2000) and the incidence of somaclonal variation as evidenced in agronomic variability when plants are evaluated in the field (Grof and Campbell, 2001; Vickers *et al.*, 2005b).

Alternative tissue targets for transgene delivery have been sought, such as exposed apical meristems from axillary buds followed by shoot

morphogenesis (Gambley *et al.*, 1993, 1994; Manickavasagam *et al.*, 2004), leaf roll discs (Snyman *et al.*, 2000, 2001) and pre-emergent inflorescences (Snyman *et al.*, 2006) followed by direct somatic embryogenesis. Although these methods have resulted in transformed plants in a reduced time frame compared to protocols employing indirect embryogenesis, determination of phenotypic fidelity in the field has yet to be published.

2.1.2 Transformation methods

Initial work on sugarcane transformation targeted protoplasts as recipient cells and DNA delivery was via polyethylene glycol (PEG) (Chen *et al.*, 1987) or electroporation (Rathus and Birch, 1992). However, neither technique resulted in the production of transgenic plants, as sugarcane regeneration from protoplasts is notoriously difficult. A significant milestone in sugarcane transformation was achieved when particle bombardment of embryogenic callus was reported and a protocol for recovery of transgenic plants was described (Bower and Birch, 1992). Embryogenic callus proved to be a reliable source of tissue for further refinement of microprojectile bombardment protocols and for the production of transgenic plants in several parts of the world (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Snyman *et al.*, 1996; Joyce *et al.*, 1998; Ingelbrecht *et al.*, 1999; Falco *et al.*, 2000). In addition, *Agrobacterium*-mediated DNA delivery was also developed using embryogenic callus as recipient cells (Arencibia *et al.*, 1998; Elliott *et al.*, 1998; Enriquez-Obregon *et al.*, 1998; Liu *et al.*, 2003).

Microprojectile bombardment using a gene gun has been achieved using either the particle inflow gun (PIG) (Finer *et al.*, 1992) or a BioRad device (Heiser, 1993). The principle of this transformation method is to coat either tungsten or gold particles with plasmid DNA containing the gene(s) of interest and to bombard these particles into embryogenic sugarcane callus using pressurized helium combined with a partial vacuum chamber. The first report of successful transformation of sugarcane suspension culture cells using the PIG gun was by Birch and Franks (1991) using the *GUS* (β -glucuronidase) reporter gene and kanamycin selectable marker gene, although no plants were

regenerated. This approach was further refined by Bower and Birch (1992) and is now widely adopted for the production of transgenic sugarcane (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1993, 1996; Franks and Birch, 1992; Snyman *et al.*, 1996; Joyce *et al.*, 1998; Ingelbrecht *et al.*, 1999; Falco *et al.*, 2000). Transformation efficiencies vary depending on genotype, quality of embryogenic callus, length of time spent *in vitro*, and selection regime employed, but most laboratories have developed a protocol that suits their requirements.

The desire to minimize transgene copy number and integration complexity, coupled with advances reported in *Agrobacterium*-mediated transformation in other monocotyledonous crops (reviews by Cheng *et al.*, 2004; Shrawat and Lorz, 2006; Wang and Ge, 2006), made this an appealing system to develop in sugarcane. Although not widely applied yet, *Agrobacterium*-mediated transformation using embryogenic callus and four different strains of *Agrobacterium* (Arencibia *et al.*, 1998; Elliott *et al.*, 1998; Enriquez-Obregon *et al.*, 1998; Liu *et al.*, 2003) and axillary buds with two strains (Manickavasagam *et al.*, 2004) has been reported. Although it is difficult to compare transformation efficiencies between the above papers, it appears that pretreatment of the callus prior to *Agrobacterium* co-cultivation improves transformation efficiency (e.g., a 30 min dehydration period). In addition, selection of a particular size of callus (1000 μm) (Arencibia *et al.*, 1998), and use of antinecrotic compounds (2 mg l^{-1} silver nitrate; 15 mg l^{-1} ascorbic acid (Enriquez-Obregon *et al.*, 1998) were reported to be advantageous. In both papers, Arencibia *et al.* (1998) and Enriquez-Obregon *et al.* (1998), the transgene copy number was 1–3. Elliott *et al.* (1998) applied no selection pressure for up to 6 weeks after co-cultivation. After this period, GFP (green fluorescent protein)-positive calli were visually selected and transferred to bialaphos at 1 mg l^{-1} . Two transgenic plants were regenerated that contained three and seven copies of the *GFP* gene, respectively.

More recently, transgenic plants were generated using axillary meristems of sugarcane (Manickavasagam *et al.*, 2004). This novel approach resulted in a transformation efficiency of 49.6%. They used two strains of *Agrobacterium*, LBA4404 and EHA105. The plasmid pGA492 contained the *nptII* (neomycin phosphotransferase) gene

driven by the *nos* (nopaline synthase) promoter and the *bar* and *gus* genes driven by cauliflower mosaic virus (CaMV) 35S promoter. Selection was on 5 mg l⁻¹ bialaphos, applied immediately after the co-cultivation period. Transformation efficiency was greatest in both strains when co-cultivation period was for 3 days in the presence of 50 µM acetosyringone. Thousands of plants were produced within 5 months. Although chimeric plants were reported, the incidence was eliminated in secondary shoots after five rounds of selection on Basta[®]-containing medium. When these plants were transferred to the greenhouse and sprayed with herbicide, a total of 336 plants (clones of 10 independent transformation events) representing 50% of total plants screened, displayed herbicide resistance. Southern blot analysis on a small subset of these plants further confirmed the presence of 1–2 copies of the transgene in each plant.

2.1.3 Selection of transformed tissues

Studies on transgenic sugarcane for technology development have involved marker and reporter genes. The reporter genes used include *GUS* (Jefferson *et al.*, 1987; Gnanasambandam and Birch, 2004; Braithwaite *et al.*, 2004), luciferase (*luc*) (Mudge *et al.*, 1996b; Gnanasambandam and Birch, 2004), maize anthocyanin regulatory elements (R and C1) (*ANT*) (Ludwig *et al.*, 1990; Bower *et al.*, 1996; Gnanasambandam and Birch, 2004), and *GFP* (Elliott *et al.*, 1998; Gnanasambandam and Birch, 2004; Gnanasambandam *et al.*, 2007; Petrasovits *et al.*, 2007). Comparative studies by Bower *et al.* (1996) on the suitability of *GUS*, *luc*, and *ANT* as reporter genes for transient assays in sugarcane transformation indicated that

the *ANT* system is the most suitable reporter system. *ANT* expression is visible within 8 h after bombardment and steadily increases in intensity up to 48 h after bombardment. The expressing cells remained visible in the target tissue for 2–3 weeks, before fading or being overgrown. In addition, the results were not confounded by background *ANT* activity. In contrast, the main disadvantage of using the *GUS* reporter gene is that the conditions for detection of gene activity are lethal to plant cells and therefore the transformed event is subsequently lost. Detection of both *luc* and *GFP* genes require specialized camera and/or detection systems. In addition, GFP detection is confounded by autofluorescence of the callus and chlorophyll in green plant tissues. However, the power of confocal laser scanning microscopy allows precise visualization of fluorescent signals within a narrow plane of focus, and the reconstruction of three-dimensional structures from serial optical sections (Haseloff *et al.*, 1997). This is an advantage of GFP over both GUS and *luc* (Gnanasambandam and Birch, 2004).

The most widely adopted stable-integration antibiotic marker used for sugarcane transformation is the *aphA2* *Escherichia coli* Tn5-derived *nptII*. The first successful application of the *nptII*-based selection system was reported as a step-wise incremental procedure using geneticin (Bower and Birch, 1992). This formed the foundation for subsequent protocols utilizing geneticin (also known as G418) (Bower *et al.*, 1996; Falco *et al.*, 2000; Snyman, 2004) or paromomycin (Joyce *et al.*, 2006a) as the selective agent (Table 1). Less widely used is the hygromycin phosphotransferase (*hph*) gene with selection on 20 mg l⁻¹ hygromycin (Arencibia *et al.*, 1998; Carmona *et al.*, 2005).

The first paper reporting herbicide resistance in sugarcane also described a selection procedure

Table 1 Selectable marker genes used in sugarcane transformation

Explant type	Transformation method	Gene	Selective agent (mg l ⁻¹)	Escapes	References
Callus	Biolistic	<i>NptII</i>	Geneticin (45)	Nil	Bower <i>et al.</i> , 1996; Joyce <i>et al.</i> , 1998
Callus	Biolistic	<i>Bar</i>	BASTA (1-3)	Yes	Gallo-Meagher and Irvine, 1996
Callus	<i>Agrobacterium</i>	<i>Hph</i>	Hygromycin (25)	Yes	Arencibia <i>et al.</i> , 1998
Callus	<i>Agrobacterium</i>	<i>Bar</i>	Bialaphos (1)	Yes	Elliott <i>et al.</i> , 1998
Immature leaf whorls	<i>Agrobacterium</i>	<i>Bar</i>	PPT (4)	Yes	Enriquez-Obregon <i>et al.</i> , 1998
Axillary meristem	<i>Agrobacterium</i>	<i>Bar</i>	BASTA (5)	Yes	Manickavasagam <i>et al.</i> , 2004
Callus	<i>Agrobacterium</i>	<i>NptII</i>	Paromomycin (150)	Nil	Joyce <i>et al.</i> , 2006a

incorporating a herbicidal agent, bialaphos (Gallo-Meagher and Irvine, 1996). The *bar* and *pat* genes from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*, respectively, encode the phosphinothricin acetyltransferase enzyme that leads to detoxification of phosphinothricin and its derivatives that are ingredients in some commercial herbicides. Selection using herbicides has been used widely, although each laboratory has to determine empirical regimes for selection of different explants and genotypes and formulations of active ingredients differ (Gallo-Meagher and Irvine, 1996; Enriquez-Obregon *et al.*, 1998; Manickavasagam *et al.*, 2004) (Table 1).

The use of the above genes, in addition to selection of transformed cells and plants, has been beneficial in generating information about transgene expression and stability in transgenic sugarcane (Gallo-Meagher and Irvine, 1996; Enriquez-Obregon *et al.*, 1998; Leibbrandt and Snyman, 2003).

There is limited published work on the use of positive selection systems, such as the *E. coli manA* phosphomannose isomerase (PMI) gene, in sugarcane. The ubiquitous plant enzyme hexokinase converts mannose to mannose-6-phosphate (Man-6-P). Man-6-P is toxic to plants, but most plants lack PMI and are inhibited by the accumulation of Man-6-P. PMI catalyzes the reversible interconversion of Man-6-P and fructose-6-phosphate, thereby releasing the Man-6-P inhibition and making mannose available as a carbon source for the plant. This system was first demonstrated for transformation of potato, sugar beet, and maize (Bojsen *et al.*, 1998, 1999) and since then it has been used successfully in a variety of other plant species. PMI has no adverse effects in acute mouse oral-toxicity tests, generates no detectable biochemical changes in mannose-associated pathways (Privalle *et al.*, 1999), lacks many attributes known to be associated with allergens (Privalle, 2002), and may thus be considered as an ideal selection protein for plant transformation. For sugarcane transformation, calli were selected on media containing 3 g l^{-1} mannose, in addition to the 20 g l^{-1} sucrose present in the media (Jain *et al.*, 2006). Plant regeneration was performed under the same level of selection. An increase of mannose from 1.5 to 3 g l^{-1} for rooting improved the overall transformation efficiency. The PMI encoding gene,

manA, was stably integrated and expressed in almost all of the transgenic lines.

Of the other nonantibiotic systems tested in sugarcane, glutamate-1-semialdehyde aminotransferase was unsuitable, while selection incorporating arabinol showed up to 100-fold lower transformation efficiency. In addition, arabinol is expensive, making it a less desirable selection agent.

2.1.4 Promoters and termination sequences

The two main sources for promoters are from microorganisms (viral or bacterial) or from plants. In addition, there are a few synthetic promoters that have been constructed in the laboratory by combining regulatory and/or enhancer elements from different viral or plant promoters. What is lacking in sugarcane, and in monocots in general, is a promoter that functions as strongly as the CaMV 35S promoter does in dicots. Schledzewski and Mendel (1994) compared transient reporter gene expression in transgenic cells of barley, maize, and tobacco driven by maize polyubiquitin1 (*Ubi-1*), rice *actin1*, *Emu*, or CaMV 35S promoter. CaMV 35S promoter had the highest GUS activity in tobacco ($316.71 \text{ nmol h}^{-1}$) compared to maize (9.88 nmol h^{-1}) and barley (1.22 nmol h^{-1}). In contrast, of the four promoters tested, the *Ubi-1* promoter showed highest expression in both maize and barley cells, but not in tobacco. However, CaMV 35S in tobacco showed a promoter strength equivalent to 2.5- to 5-fold greater than *Ubi-1* in maize (Table 2).

2.1.4.1 Viral promoters

The viral promoter most frequently used in plant transformation is the CaMV 35S promoter. However, this promoter is not highly expressed in sugarcane (Table 2). Schenk *et al.* (2001) reported the isolation of two novel promoters from a DNA virus that infects banana (banana streak virus, BSV). The region of two BSV isolates (2105 bp and 1322 bp) upstream of the open reading frame (ORF) were labeled as Mys and Cav, respectively, and assessed for promoter activity in stably transformed sugarcane callus using GFP (*sGFPs65T*) as a reporter gene.

Table 2 Reporter gene expression analysis in sugarcane

Promoter	Reporter gene	Transient expression ^(a)	Stable expression ^(a)	References
Maize Ubi-1 Rice Act1 Emu CaMV 35S	GUS	Transient in leaf (after 48 h) (nmol 4-MU min ⁻¹ mg ⁻¹ protein) 50.0 (1.00) 10.0 (0.20) 11.0 (0.20) 0.5 (0.01)		Gallo-Meagher and Irvine, 1993
Maize Ubi-1 ScRbcS	GUS	Percentage of total foci Total BS Meso Epi 771 2% 3% 95% 259 23% 8% 69%		Tang <i>et al.</i> , 1996
Maize Ubi1 Act1 Osa	Luciferase		(Fg luc µg ⁻¹ protein) 5–8-month-old plants Mean 84000 (1.00) 1000 (0.12) 3000 (0.04) Winter 96 Summer 96 Winter 97 175 (1.00) 125 (1.00) 4500 (1.00) 25 (0.14) 10 (0.10) 70 (0.05) 5 (0.03) 2 (0.02) 200 (0.40)	Hansom <i>et al.</i> , 1999
Maize Ubi-1 Osa Emu				
Maize Ubi-1 BSVCv	GFP		Mature Leaf (ug mg ⁻¹ protein) Mean 1.5 (1) 4.5 (3)	Schenk <i>et al.</i> , 2001

GUS	Maize Ubi-1	Transient (after 48 h) (nmol 4-MU min ⁻¹ mg ⁻¹ protein) Callus Leaf	Stable (after 4 months) (nmol 4-MU min ⁻¹ mg ⁻¹ protein) Callus Leaf	Liu <i>et al.</i> , 2003
	CaMV 35S			
	RiceUbiQ2			
GUS	Maize Ubi-1	Transient in callus (nmol 4-MU min ⁻¹ mg ⁻¹ protein) 0.6 0.8 0.9 0.5		Yang <i>et al.</i> , 2003
	SEF1			
	SPRP SPRP2.4			
NPTII	Maize Ubi-1	<i>In vitro</i> tissue culture plants (ng NptII mg ⁻¹ protein) Callus Leaf Root	Glasshouse plants (ng NptII mg ⁻¹ protein) Meristem Leaf Root	Braithwaite <i>et al.</i> , 2004
	SCBV (IMPs)			
	SCBV (IMHS)			
GUS	Maize Ubi-1	Stable callus (ng GUS mg ⁻¹ protein) Mean 1500 (1.0) 600 (0.4) 2000 (1.3)		Wei <i>et al.</i> , 2003
	ScUbi4 ^(b)			
	ScUbi9 ^(b)			

^(a)Numbers in brackets are expression relative to that with the Maize Ubi-1 promoter

^(b)Sc—Sugarcane

Results of the promoter analysis showed that after 19 months growth, the plants transformed with the BSV-Cav construct showed greater than threefold higher activities than the maize *Ubi-1* promoter ($10.62 \mu\text{g GFP mg}^{-1}$ total protein and $3.2 \mu\text{g GFP mg}^{-1}$ total protein, respectively). In addition, this promoter appeared to be expressing in a constitutive manner. GFP accumulation was observed in vascular tissue, bundle sheath cells as well as leaf parenchyma and epidermal cells of sugarcane. Expression, however, was strongest in the parenchyma cells with all three promoters.

Promoters from a sugarcane-specific badnavirus (sugarcane bacilliform virus, SCBV) were cloned and tested using two marker genes (*GUS* or *nptII*) (Braithwaite *et al.*, 2004). Three of the four promoters tested were amplified by polymerase chain reaction (PCR) from sugarcane plants containing the virus while the fourth promoter was subcloned from an almost genome-length clone of SCBV. All four promoters were active in sugarcane, with the highest GUS expression present when the subcloned region of SCBV was used for promoter construction. When different parts of the plantlets were assessed for GUS expression (Table 2), the meristems of young plants had the highest levels, there was some in young leaves, but no expression in roots. When *nptII* expression was assessed in young *in vitro* plantlets, however, transgene activity was present in callus, leaves, and roots to a similar level ($<0.01\%$ of total soluble protein). Thus, the lack of GUS activity in the roots may be a consequence of poor penetration of the GUS substrates to the root region. Interestingly, when the *nptII* transgenics were grown to maturity in the glasshouse, the lines driven by the SCBV promoter showed a five-fold higher activity than the *Ubi-1*-driven plants (Table 2).

2.1.4.2 Plant-derived promoters

The maize *Ubi-1* promoter (Christensen and Quail, 1996) is the most frequently used constitutive promoter for sugarcane transformation (Table 2) and its expression is usually higher than other plant-derived promoters (sugarcane *Ubi* and rice *actin1*). Expression of *luc* activity in sugarcane plants by maize *Ubi-1* promoter ranged between 200 and 300 000 relative light units (RLU) mg^{-1} protein compared to 200–2000 RLU mg^{-1} protein

in *actin1* lines (Hansom *et al.*, 1999; Table 2). In addition, this expression was independent of copy numbers.

Using particle bombardment of sugarcane callus, Liu *et al.* (2003) showed that there was a 1.6-fold increase in GUS expression by the rice *Ubi-2* promoter (mean = $78.6 \text{ nmol 4-MU min}^{-1} \text{ mg}^{-1}$ protein) over the maize *Ubi-1* promoter (mean = $47.6 \text{ nmol 4-MU min}^{-1} \text{ mg}^{-1}$ protein). The *GUS* gene driven by CaMV 35S showed no expression in regenerated plants, and was $<10\%$ of that of ubiquitin promoters in transient expression analysis (Table 2).

Tang *et al.* (1996) compared the expression patterns of GUS, driven by promoters of two sugarcane ribulose-1,5-bisphosphate small subunit (Rubisco) genes (*scrbc-1* and *-2*), in transient assays. Both promoters directed expression in leaf tissues, but not in calli. Although the overall expression levels were lower when compared to the maize *Ubi-1* promoter, *scrbc* promoters drove higher expression in the photosynthetic cells, especially in the bundle sheath cells (Table 2). Stable expression in transgenic callus lines and regenerated plants also showed that *scrbc-1* promoter directed GUS expression in leaves, but not in calli.

Two sugarcane polyubiquitin gene promoters (sugarcane *ubi4* and *ubi9*) were found to direct high levels of transient GUS expression in the following monocots: sugarcane, maize, sorghum, banana, pineapple, garlic, and rice cells (Wei *et al.*, 1999, 2003). Both of these monocot promoters were also sufficient to drive GUS expression in cells of tobacco. In these transient assays, the activities of the two sugarcane promoters were comparable to the strong monocot promoter, maize *Ubi-1* (Christensen and Quail, 1996). Similar to *Ubi-1*, sugarcane *ubi4* was heat shock inducible in stably transformed sugarcane callus lines, but sugarcane *ubi9* was not (Wei *et al.*, 2003). The physiological difference between the two sugarcane ubiquitin promoters corresponded to a MITE (miniature inverted-repeat transposable element) insertion that is present in the putative heat shock elements of sugarcane *ubi9* but not present in sugarcane *ubi4*. In transgenic sugarcane plants produced by particle gun bombardment, GUS expression from sugarcane *ubi4* and *ubi9* dropped to very low or nondetectable levels after plant regeneration. This drop in expression also occurred in *Ubi-1*

sugarcane lines. Nuclear run-on experiments showed the down-regulated transgenes continued to be transcribed at high levels, indicating that the lack of transgene expression was due to post-transcriptional gene silencing (PTGS). In contrast, sugarcane *ubi9* drove high levels of expression in transgenic rice plants produced via *Agrobacterium*-mediated transformation. This high level of expression continued after plant regeneration and was inherited in the T₁ generation (Wei *et al.*, 2003).

2.1.4.3 Synthetic promoters

The promoter *Emu* was synthesized by Last *et al.* (1991) and contains a truncated maize alcohol dehydrogenase (*Adh*) promoter as well as several copies of the maize anaerobic responsive elements from the *Adh* gene. In addition, it contains the *ocs*-element of the octopine synthase gene from *Agrobacterium*. Comparative studies of GUS expression in sugarcane protoplasts using constructs with either the *Emu* or the CaMV 35S promoter showed a 50–100-fold increase in *Emu*-driven GUS expression over the 35S promoter (Rathus and Birch, 1992). However, Joyce *et al.* (1998) and Bower *et al.* (1996) found that the *Emu* promoter could not drive strong expression in mature sugarcane plants (Table 2).

Osa is another synthetic promoter, developed by CSIRO Plant Industry Australia, which consists of multiple octopine synthase (OCS) enhancer elements, the core region from the CaMV 35S promoter and untranslated leader sequence from maize transposable element *Ac* (Bower *et al.*, 1996). Experiments in sugarcane using this promoter also showed much lower levels of gene expression than maize *Ubi-1* (Table 2).

2.1.4.4 Terminator sequences

The most commonly used terminator sequences for transformation of sugarcane callus is the *Nos* terminator sequence from *Agrobacterium*. There are a few reports where the *Agrobacterium* octopine synthase gene (*Ocs*) terminator sequence has been used (Elliott *et al.*, 1998). The studies have not compared the effect of terminator sequences on transgene expression in sugarcane; this is an

area of research that has been neglected and warrants further investigation.

2.1.5 Activity, stability of inheritance, and silencing of transgenes

The high level of ploidy found in all sugarcane may have some important implications for transformation, which are not normally encountered when working with diploid species. For instance, increase in ploidy by genome duplication, which has been suggested to play an important role in angiosperm evolution (Stephens, 1951; Ohno, 1970; Blanc *et al.*, 2000; Initiative, the Arabidopsis Genome, 2000; Paterson *et al.*, 2000, 2003, 2004, 2005; Bowers *et al.*, 2003; Vandepoele *et al.*, 2003; Wang *et al.*, 2005b), is associated with a host of rapid responses, including loss and restructuring of low-copy DNA sequences (Song *et al.*, 1995; Feldman *et al.*, 1997; Ozkan *et al.*, 2001; Shaked *et al.*, 2001; Kashkush *et al.*, 2002), activation of genes and retrotransposons (O'Neill *et al.*, 1998; Kashkush *et al.*, 2003), gene silencing (Chen and Pikaard, 1997a, b; Comai, 2000; Comai *et al.*, 2000; Lee and Chen, 2001), and organ-specific subfunctionalization of gene expression patterns (Adams *et al.*, 2003, 2004). Sugarcane appears to have undergone two such genome duplications in about the past 5 million years and these mechanisms may be especially important in providing raw material for evolutionary change. It is important to understand how stable the sugarcane genome is with respect to: (i) transposon activity; (ii) genome expansion or contraction; (iii) measuring the extent of gene silencing and alterations in gene expression; (iv) assessing *in vitro* regeneration systems for epigenetic variation in regenerated plantlets; and (v) designing transformation technologies to overcome the above constraints.

Molecular techniques for detection and characterization of transgenes with respect to integration pattern, copy number, and protein concentrations have been conducted and published (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996). However, these analyses were carried out on sugarcane plants maintained in glasshouses. Field analysis to determine expression and stability of transgenes is important because of sugarcane's multiple vegetative crop cycles and reports of PTGS.

The transgenesis approach in sugarcane has targeted elite commercial cultivars. It would be advantageous to be able to use transgenic plants as parents in breeding programs especially when the desired trait is not present in the sugarcane gene pool. Transgene inheritance and segregation of the *bar* herbicide resistance gene and the *hut* Sorghum mosaic virus (SrMV) coat protein gene was tracked in progeny arising from conventional crosses made between transgenic and nontransgenic parents (Butterfield *et al.*, 2002). The results demonstrated that transgenic plants can be used as parents in a sugarcane breeding program but screening progeny for a characteristic such as virus resistance that relies on PTGS mechanisms may have to be carried out after one vegetative cycle of growth after crossing.

PTGS is known to occur in sugarcane (Ingelbrecht *et al.*, 1999) and is considered to be one of the factors limiting accumulation of recombinant proteins (Wei *et al.*, 2003). It was thought that it could be reversed by retransformation with a gene encoding a viral suppressor of PTGS, such as HcPro from SrMV (Ingelbrecht *et al.*, 2000) and P0 from sugarcane yellow leaf virus (ScYLV) (Wang *et al.*, 2006, 2007). Despite the potential application in transgene silencing control, there are possible negative effects on endogenous gene regulation and virus susceptibility. Initial results indicate that there is no consistent correlation between the RNA expression levels of P0 or HcPro and the expression of a transgene and several miRNA-regulated endogenous genes. Further research is required to characterize the effect of these viral suppressors in transgenic sugarcane (Wang *et al.*, 2006, 2007).

Sugarcane plants transformed via particle bombardment with GM-CSF (human cytokine granulocyte macrophage colony stimulating factor), contain numerous copies of transgenes with complex integration patterns as revealed by Southern blot hybridization (Albert *et al.*, 2003; Wang *et al.*, 2003). Multiple approaches have been evaluated for the introduction of single- or low-copy transgenes, to determine if these methods can reduce transgene silencing (Albert *et al.*, 2004). These methods include the use of insert-only DNA for bombardment (Fu *et al.*, 2000), Cre/*lox* site-specific recombination to resolve multiple transgene copies (Srivastava and Ow, 2001), and the *Ac/Ds* transposon system to direct

transgene integration by transposition (Koprek *et al.*, 2000, 2001). The use of linear expression cassette-only DNA was reported to produce a high frequency of low-copy transgene insertions in rice, with no evidence of silencing through the R₄ generation (Fu *et al.*, 2000). In contrast, most transgenic sugarcane lines produced by this method contained multiple transgene copies, with only three of 27 selected lines containing three or fewer copies (Wang *et al.*, 2003). Cre/*lox* lines did contain fewer copies of the transgene as estimated by quantitative PCR (qPCR). However, accumulation of GM-CSF in the low-copy lines was not higher than in multicopy lines (Albert *et al.*, 2004). In addition, a vector system was developed to allow direct plasmid to chromosome transposition using *Ac/Ds* in monocot cells. This should allow single-copy transposition lines to be produced in a single generation without sexual crosses (Albert *et al.*, 2003). However, no increase in GM-CSF protein level was observed in the *Ac/Ds* lines.

2.1.6 Adverse effects on growth, yield, and quality

Most evaluation of transgene stability and expression patterns and performance of transgenic sugarcane plants in the field has been limited to a few lines (Gallo-Meagher and Irvine, 1996; Arencibia *et al.*, 1999; Leibbrandt and Snyman, 2003). However, results of recent studies where a larger number of lines were tested (Gilbert *et al.*, 2005; Vickers *et al.*, 2005b) may influence future transgenic approaches.

Initial field trials demonstrated stable expression of a herbicide-resistant transgene over three rounds of vegetative propagation in a single transformant (Gallo-Meagher and Irvine, 1996; Leibbrandt and Snyman, 2003), but no agronomic measurements were taken in the first study. In the latter trial, no differences were found between the transgenic line and wild-type control in phenotypic characters such as stalk height and diameter, agronomic performance indicators such as sucrose yield and fiber content, and disease susceptibility ratings to smut and rust. However, in a field trial where 100 transgenic lines were compared for Sugarcane mosaic virus (SCMV) resistance and yield characteristics, a

considerable amount of variability for measured parameters was reported, which the authors attributed largely to the effects of the cultivar used and the tissue culture process (Gilbert *et al.*, 2005; Vickers *et al.*, 2005b). Similarly, preliminary data from metabolomic analysis, comparing leaves from nontransformed sugarcane with leaves from transgenic sugarcane lines producing polyhydroxybutyrate (PHB), found that the vast majority of the variation was a tissue culture effect and was not from the insertion of the PHB metabolic pathway and the selectable marker genes (Purnell *et al.*, 2007).

2.1.7 Subcellular targeting

Most transgene expression constructs used for sugarcane transformation result in production of foreign gene products in the cytosol, but important metabolic activities ranging from photosynthesis to sugar storage are carried out in other compartments. Hence, targeting proteins to subcellular compartments may be necessary for effective resistance to pest and diseases and efficient metabolic engineering in sugarcane. For example, for increased resistance to sugarcane leaf-scald disease, resistance gene products may need to be targeted to the plastids, as the albicidin toxin from the pathogen *Xanthomonas albilineans* blocks plastid DNA replication and chloroplast development. Similarly, for efficient conversion of sucrose to alternative carbohydrates such as starch and fructans, foreign proteins need to be targeted to the sugarcane vacuoles in mature stem parenchyma cells, where most of the sucrose is stored.

The ability to target recombinant proteins to the correct subcellular location using efficient and appropriate targeting signals is one of the most important requirements for sugarcane metabolic engineering. Various targeting signals have been successfully tested to target heterologous proteins to different subcellular compartments in sugarcane. The availability of visible reporters such as GFP in combination with a transient assay system in sugarcane leaves allowed the testing of the efficiency of various targeting signals with less time and resources (Gnanasambandam *et al.*, 2007). While most of the tested signal sequences are from dicotyledons species, they were effective

in the monocotyledon sugarcane. So far, targeting signals for vacuoles, endoplasmic reticulum (ER), plastids, mitochondria, and peroxisomes have been tested in sugarcane (McQualter *et al.*, 2005; Petrasovits *et al.*, 2007; Brumbley *et al.*, 2006b; Gnanasambandam *et al.*, 2007). In future, signals for other compartments and efficiency of signals will be determined.

2.1.7.1 Protein targeting to vacuoles

Sugar storage vacuoles occupy about 80% of the total tissue space in mature sugarcane stem and accumulate sucrose up to 500 $\mu\text{mol g}^{-1}$ fresh weight (Moore, 1995). Due to the potential to engineer synthesis of valuable compounds other than sucrose in sugarcane (e.g., alternative carbohydrates such as starch and fructans), the sugarcane vacuole is one of the important target compartments for metabolic engineering. However, targeting proteins to the sugarcane vacuoles remains a challenge and differs in several aspects compared to targeting to other compartments: (i) the sugarcane vacuolar compartment is highly dynamic in function, and is acidic as indicated by neutral red accumulation in most protoplasts isolated from sugarcane suspension cells and stem storage parenchyma cells (Gnanasambandam and Birch, 2004). It is not known whether several vacuole types coexist in sugarcane cells with different pH and/or proteolytic activities. As a result, there may be several independent targeting mechanisms to different vacuole types in sugarcane; (ii) targeted proteins should be stable and functional in the vacuolar environment, engineered for the relevant pH and proteases. For example, when the N-terminal vacuolar targeting signal (NTPP) of potato patatin was used to target yeast invertase to the sugarcane vacuole, neither detectable amounts of invertase protein nor increased soluble acid invertase activity was observed (Ma *et al.*, 2000). Similarly, fusion of NTPP from sweet potato sporamin to various reporter proteins resulted in substantial reduction or loss of enzymatic activity in transient expression assays and in transformed sugarcane cells (Gnanasambandam and Birch, 2004); and (iii) protein targeting mechanisms to many compartments (e.g., peroxisomes, mitochondria, and ER) are highly conserved in

eukaryotes. In contrast, protein targeting to the plant vacuole through the endomembrane system is different from the lysosomal and vacuolar targeting mechanisms of animals and yeast. While mammalian cells use mannose-6-phosphate mediated lysosomal targeting, yeast cells use N-terminal propeptides for vacuolar targeting. In contrast, plants employ three different types of targeting signals that are found either at the N-terminus or the C-terminus or within the mature polypeptide (Matsuoka and Neuhaus, 1999).

Two signals, the NTPP of sweet potato sporamin and sugarcane legumain, were reported to be efficient in targeting reporter proteins to the sugarcane vacuole (Gnanasambandam and Birch, 2004; Rae *et al.*, 2006). In contrast, the C-terminal signal from tobacco chitinase was inefficient in targeting a reporter protein to the sugarcane vacuole (Gnanasambandam and Birch, 2004). Using the NTPP of sporamin, metabolic engineering of vacuolar compartment to produce high-value isomaltulose has been demonstrated successfully (Wu and Birch, 2007). Vacuolar targeting of the sucrose isomerase using the NTPP of sporamin allowed high isomaltulose yields (up to 440 $\mu\text{mol g}^{-1}$ fresh weight) in sugarcane stems. In contrast, expression of a cytosolic form of the same enzyme caused stunting with reduced sugar accumulation. Interestingly, isomaltulose accumulated in storage tissues without any decrease in stored sucrose concentration, resulting in doubled total sugar concentrations in harvested juice (Wu and Birch, 2007) in vacuolar targeted lines. The transgenic lines with enhanced sugar accumulation also showed increased photosynthesis, sucrose transport and sink strength (Wu and Birch, 2007).

2.1.7.2 Protein targeting to plastids

The N-terminal plastid transit peptides of RbcS (rubisco small subunit) genes from maize and pea were used to target heterologous enzymes to sugarcane plastids to successfully produce *p*-hydrobenzoic acid (pHBA; McQualter *et al.*, 2005) and polyhydroxybutyric acid (PHB biopolymer; Petrasovits *et al.*, 2007). The transit peptides from tomato DCL (defective chloroplast and leaves) and tobacco RbcS genes were shown to target GFP to the sugarcane leaf proplastids (Gnanasambandam *et al.*, 2007; Figure 4).

2.1.7.3 Protein targeting to mitochondria

Mitochondria are important organelles involved in ATP synthesis, photorespiration, and programmed cell death. The N-terminal mitochondrial presequence from F₁-ATPase β -subunit (ATPase- β) of *Nicotiana glauca* was shown to be effective in targeting GFP to the mitochondria (Petrasovits *et al.*, 2007; Figure 4). Although this signal was used to target bacterial enzymes to sugarcane mitochondria, no PHB polymer accumulation was observed in transgenic sugarcane (Petrasovits *et al.*, 2007).

2.1.7.4 Protein targeting to peroxisomes

Plant peroxisomes are involved in fatty acid β -oxidation, the glyoxylate cycle and photorespiration. The six amino acid C-terminal peroxisomal signal from *Spinacia oleracea* L. (spinach) glycolate oxidase was shown to be effective in targeting GFP to the peroxisomes in sugarcane leaves and callus (Gnanasambandam *et al.*, 2008; Figure 4).

2.1.7.5 Protein targeting to ER

Almost all of the proteins that will be secreted to the cell exterior, as well as those destined for the lumen of the ER, Golgi apparatus, or vacuoles, are initially delivered to the ER lumen (Gal, 1998). The N-terminal ER signal peptide from *Arabidopsis* basic chitinase and a C-terminal HDEL signal for protein retention in the ER was efficient in targeting and retention of GFP in the ER in sugarcane leaves (Gnanasambandam *et al.*, 2007; Figure 4). This HDEL signal in combination with the ER signal peptide of *PinII* was required for higher accumulation of GM-CSF in transgenic sugarcane (Wang *et al.*, 2005a).

2.2 Target Traits and Products

For sugarcane genetic engineering, specific targets fall into two broad areas: (i) input traits that improve crop performance and productivity such as pest and disease resistance, tolerance to abiotic stress, herbicide tolerance, and alterations to plant

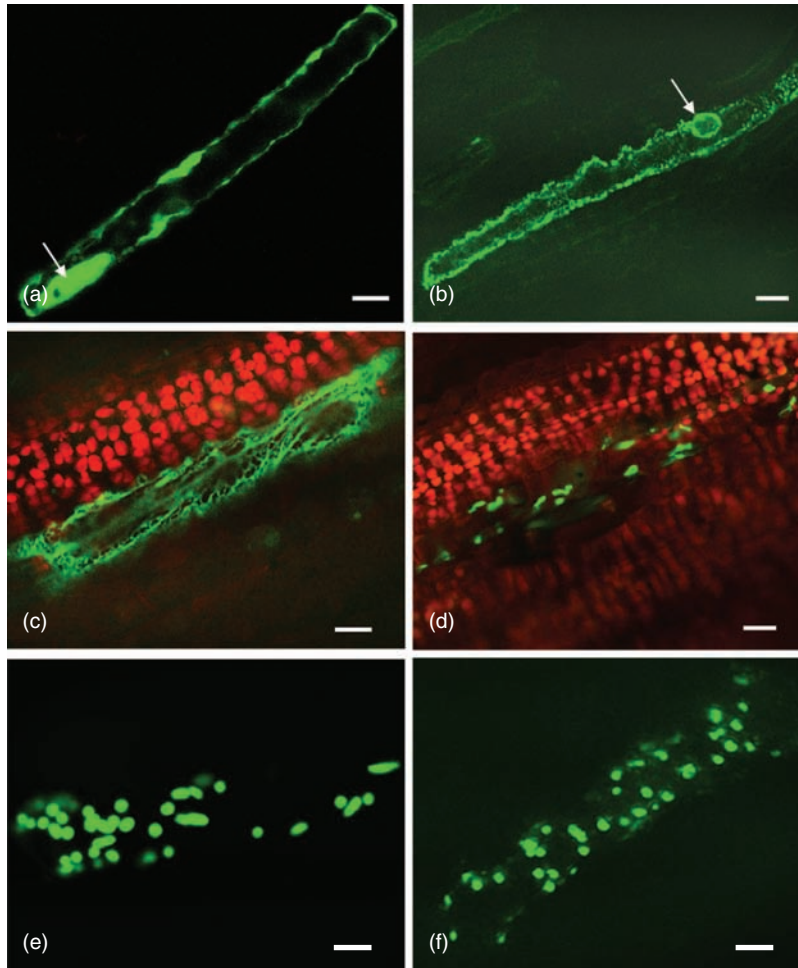


Figure 4 Confocal images of sugarcane leaf epidermal cells showing fluorescence of green fluorescent protein (GFP) in different subcellular compartments. Nontargeted GFP in the cytosol (a) and nucleus (arrow in a). ER-targeted GFP fluorescence in the ER (b, c; arrow in b shows perinuclear distribution). Plastid-, mitochondrial-, and peroxisomal-targeted GFP in the proplastids (d), mitochondria (e), and peroxisomes (f), respectively. All images show green channel GFP fluorescence except (c) and (d) that show merged images of green (GFP fluorescence) and red (chlorophyll autofluorescence) channels. Bar = 10 μ m [Reproduced with permission from Annathurai Gnanasambandam, BSES Limited, Australia]

architecture and (ii) output traits that modify quality and yield, compositions and use, such as production of more sucrose, biomass or novel compounds.

2.2.1 Disease resistance

Reports of pathogen-derived resistance to virus diseases include resistance to SCMV in otherwise susceptible sugarcane clones (Joyce *et al.*, 1998; Ingelbrecht *et al.*, 1999). Both groups used

the coat protein gene of the virus driven by the *Ubi-1* promoter with the *nos* terminator. Plants transformed with the coat protein gene of the SrMV strain SCH displayed a range of phenotypes, including immune, resistant, recovery, and susceptible plants, when challenged with virus. These observations enabled insights to the RNA-mediated PTGS resistance mechanism (Ingelbrecht *et al.*, 1999). Virus induced gene silencing (VIGS) has been suggested as the mechanism of resistance against viral infections. Interestingly, transgenic plants having the same

transgene integration pattern as determined by Southern blot analysis (i.e., clones) displayed a different response to mechanical SCMV infection. The reason for this peculiar result is still not clear (Ingelbrecht *et al.*, 1999).

Sugarcane yellow leaf disease, which is characterized by yellowing of the leaf midribs followed by tissue necrosis, is caused by ScYLV (Borth *et al.*, 1994; Schenck *et al.*, 1997; Vega *et al.*, 1997; Comstock *et al.*, 1998). Economic losses from sugarcane yellow leaf disease of up to 50% have been reported (Vega *et al.*, 1997). The ScYLV coat protein gene in the sense orientation driven by *Ubi-1* was used to generate virus-resistant transgenic sugarcane. Resistance levels were evaluated with inoculation of viruliferous aphids. Virus titers were determined using tissue blotting and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Two resistant transgenic sugarcane lines were identified based on the tissue blot analyses. However, virus RNA can still be detected using qRT-PCR in these lines. Greenhouse tests are currently being conducted to compare the yield difference between nontransformed and transgenic lines.

The transgenic approach for providing resistance to the more devastating viral disease in sugarcane, Fiji leaf gall has been tested (McQualter *et al.*, 2004). Resistance to Fiji leaf gall was produced by microprojectile-mediated transformation with a transgene encoding a translatable version of Fiji disease virus (FDV) segment 9 ORF 1 under the control of the maize *Ubi-1* promoter. The molecular phenotypes of the transgenic plants at both the DNA and RNA levels were not entirely consistent with a resistance mechanism based on PTGS. Transgenic plants showed very low steady state messenger-RNA (mRNA) levels under normal conditions, but many of these plants failed to show resistance upon challenge with the FDV virus, suggesting that the virus possessed a mechanism for overriding the post-transcriptional silencing mechanism. Further research required to achieve complete immunity to FDV includes: (i) additional characterization of the FDV genome, since only a superficial knowledge of the virus life cycle and replication strategy is currently known; (ii) a shotgun approach where all 12 ORFs contained within the FDV genome are used as transgenes, either singly or in various combinations (RNAi

(RNA interference) silencing constructs should be employed in this case); and (iii) determining if FDV possesses a dsRNA (double-stranded RNA) binding protein with the ability to suppress gene silencing. If so, then a more effective approach to achieve pathogen derived resistance to FDV in sugarcane might require deactivation of this protein.

Leaf scald is a serious disease of sugarcane caused by the bacterium *Xanthomonas albilineans*. The bacterium produces a toxin (albicidin) that blocks plastid DNA replication of the sugarcane plant. Zhang *et al.* (1999) identified a bacterium that could survive in the presence of albicidin because it carried an albicidin detoxification gene (*albD*). This gene was cloned from the bacterium and introduced into sugarcane. Some of the resulting transgenic plants were resistant to leaf scald disease (Hansom *et al.*, 1999). It is interesting to note that another bacterial pathogen of sugarcane *Leifsonia xyli* subsp. *xyli* (Lxx) like *Xanthomonas albilineans* colonizes the xylem vessels, was recently shown to have a gene homologous to the one in *Xanthomonas albilineans* encoding an enzyme to pump albicidin out of its cells (Monteiro-Vitorello *et al.*, 2004).

2.2.2 Insect resistance

The potential for using a modified *Bt* gene encoding the δ -endotoxin CryIA(c) from *Bacillus thuringiensis* strain *kurstaki* to combat damage by the lesser corn-stalk borer (LCB) was demonstrated by Fitch *et al.* (1996). The *Bt* gene and the selectable marker gene *nptII*, were under the control of the CaMV 35S promoter. Bombarded sugarcane calli were selected stepwise on 50–200 mg l⁻¹ of G418, an aminoglycoside antibiotic similar in structure to gentamicin B1. Insect bioassays indicated that LCB larvae fed on some lines of calli or leaves from the regenerated plants weighed less and showed higher mortality than those fed on nontransgenic tissues. Mortality was higher when LCB were fed transgenic calli as opposed to transgenic leaves, possibly due to different levels of the CryIA(c) protein in the two different tissues, although the protein levels were not reported. Leaves from other putatively transformed lines had no detectable effect on larvae survival. Southern hybridization indicated

that there were 1–5 copies of the *Bt* genes in the two most resistant lines.

Resistance to the other lepidopteran sugarcane stalk borers has also been reported using the δ -endotoxin gene from *B. thuringiensis*. Larval mortality and reduced levels of damage from *Diatraea saccharalis* in the field and *Proceras venosatus* in the glasshouse have been reported (Arencibia *et al.*, 1997; Weng *et al.*, 2006). In the short term, it is possible that intellectual property restrictions may limit widespread use of this technology in sugarcane. Consequently, the use of other antimetabolic compounds for improving plant resistance, such as proteinase inhibitors (Allsopp *et al.*, 1996; Falco and Silva-Filho, 2003) and lectins (Sétamou *et al.*, 2002a, b, c), has been tested with promising results from laboratory-based insect bioassays. These products impact on a wider range of insect/pests and studies on the effects on the Australian coleopteran whitegrub have been underway for several years (Allsopp *et al.*, 1996).

Canegrubs are a major pest in the Australian sugar industry causing yield losses up to AU \$80 million. The *PinII* and the snowdrop lectin (*Gna*) genes have both been used to generate transgenic sugarcane in attempts to control these pests (Allsopp *et al.*, 1996; Nutt *et al.*, 1999). Plants containing the *pinII* gene grew more slowly than the nontransformed control plants. This may have been due to metabolic disruption within the plants, as no *pinII* was found in the cell vacuole. Earlier work with artificial feeding trials had shown that avidin could reduce larval growth and increase larval mortality of *Antitroglus parvulus* (Childers canegrub) (Allsopp and McGhie, 1996). More recently, the gene for avidin, a biotin binding protein from chicken egg white, has been introduced into sugarcane for the control of canegrubs (Nutt *et al.*, 2006). Transgenic sugarcane plants have been regenerated, which contain avidin concentrations of up to 0.06% of total protein.

2.2.3 Sucrose metabolism

A suite of physiological processes and enzymes involved in sucrose accumulation have been identified and characterized over the last 50 years. These processes include: leaf reactions,

such as photosynthetic reactions, sucrose synthesis, metabolism and carbon partitioning across various membranes into different pools; phloem reactions, such as phloem loading in leaf, translocation to and unloading in various sink tissues (including primary storage in parenchyma cells of the stalk); stalk reactions, such as membrane transport, sucrose metabolism, carbon partitioning, and remobilization of stored sucrose; genetic and developmental controls, such as timing of maturation; and environmental perception and signal transduction pathways to coordinate plant development (Moore, 2005). Some of the genes encoding these enzymes have been cloned and used to transform sugarcane with the goal of altering sucrose accumulation (reviewed by Grof and Campbell, 2001). However, this reductionist approach has fallen short of expectation in almost all of the cases because of the complexity among the multitude of simultaneous processes and parallel pathways.

As tissue culture methods became established, they were exploited to elucidate the physiology and biochemistry of sugarcane carbohydrate accumulation, with particular emphasis on sugars (Komor *et al.*, 1981; Thom and Komor, 1984). Transgenic sugarcane cell lines were also used to study the effect of invertase expression in different cellular compartments on sucrose accumulation (Ma *et al.*, 2000). Overexpression of a yeast invertase gene (*SUC2*) in the apoplast led to rapid hydrolysis of sucrose and accumulation of hexoses, both in the medium and the cells, suggesting that hexose uptake, not hexose availability, was the limiting factor for sucrose accumulation. Cells transformed for overexpression of invertase in the cytoplasm did not show a significant change in the sugar composition in the medium, but did significantly reduce the sucrose content in the cells. Partial inhibition of the soluble invertase activity was achieved by transforming with a sugarcane soluble acid invertase complementary DNA (cDNA) (*SCINVm*) in the antisense orientation to result in increased sucrose accumulation. Intra- and extra-cellular sugar composition was very sensitive to changes in invertase activities in this tissue culture system (Ma *et al.*, 2000).

Pyrophosphate-dependent phosphofructokinase (PFK) activity in sugarcane is inversely correlated to sucrose concentration in maturing internodal tissues, but no clear physiological role

in sucrose metabolism has emerged (Whittaker and Botha, 1999). If endogenous PFP activity were to be down-regulated by antisense or co-suppression technologies, then sucrose concentration could be increased. In sugarcane transformed with the catalytic subunit, PFP- β , endogenous PFP gene expression was reduced by up to 40% and 80% in leaf roll and internodal tissue, respectively (Groenewald and Botha, 2001, 2008). Sucrose concentrations in these lines were significantly increased in immature internodes. This finding could make a valuable contribution to the productivity of sugarcane cultivars and elucidate the role of PFP in sucrose accumulation.

Vickers *et al.* (2005a) attempted to modify the endogenous polyphenol oxidase (PPO) activity in sugarcane by introducing sense and antisense constructs of the endogenous sugarcane PPO gene driven by the *Ubi-1* promoter. The rationale behind this approach was that the inhibition of PPO activity in juice by chemical inhibitors, elevated pH or heat significantly reduced the color of the cane juice and the subsequent color intensity in sugar crystals. All transgenic lines, irrespective of the orientation of the PPO gene, showed higher PPO activity and more color units than the nontransformed commercial clones. Although higher levels of PPO activity were correlated to juice with a darker color, the converse was not true in this study. It has been hypothesized that a lowering of PPO activity will lead to a reduction in the color of juice and consequently raw sugar (Vickers *et al.*, 2005a). As pale sugar has a market premium over dark sugar, this has potential economic benefits for the sugar industry.

While most transgenic attempts to improve sugar accumulation in sugarcane have met with limited success, a notable exception was the introduction of a bacterial gene encoding a sucrose isomerase (Wu and Birch, 2007) to produce a high-value sugar isomaltulose.

2.2.4 Alternative products

High-value sugars. Isomaltulose is a sucrose isomer with α -1,6 linkages instead of α -1,2. Although it is only 42% as sweet as sucrose (Li *et al.*, 2004), it is a high-value sugar because it is digested 4–5 times slower than sucrose and so

has major health benefits to consumers (Lina *et al.*, 2002). Not only does isomaltulose reduce the highs and lows in blood sugar (Lina *et al.*, 2002), it also has dental health benefits (Ooshima *et al.*, 1983). Wu and Birch (2007) demonstrated that sugarcane can be engineered to target the production of isomaltulose in the storage vacuoles in sugarcane stalks (see Section 2.1.7). In transgenic lines, when sucrose isomerase was targeted to the vacuole, isomaltulose accumulated without a reduction in sucrose accumulation. Up to a two-fold increase in total sugar accumulation was observed in some lines (Wu and Birch, 2007). The additional sugar was not produced at the expense of structural carbohydrates, as levels of fiber were the same between wild type and transgenic lines. Additional benefits were that the levels of isomaltulose reduced leaf senescence resulting in more leaf biomass on the plants and a reduction in the percent water in stalk tissue from 70% for the nontransformed control mature stalk internode to 60% in the best isomaltulose producing transgenic line (Wu and Birch, 2007). Field trials of these high-sugar lines are currently underway.

Sorbitol. The primary photosynthate of the members of the Rosaceae family including apples (*Malus domestica*), pears (*Pyrus* sp.), peaches and nectarines (*Prunus persica*), plums (*Prunus* subgenus *Prunus*), cherries (*Prunus* subgenus *Cerasus*) is sorbitol. Sorbitol has intrinsic value as a noncaloric sweetener and is also used to manufacture ascorbic acid and personal care products (Kirschner, 2004). Sorbitol synthesis in sugarcane is achieved by a single enzyme conversion step sorbitol-6-phosphate dehydrogenase (S6PDH), which catalyzes the reduction of D-glucose-6-phosphate (G6P) to sorbitol-6-phosphate (S6P). The cytosolic expression of the *M. domestica* sorbitol-6-phosphate dehydrogenase gene (*mds6pdh*) resulted in high accumulation of sorbitol (up 61% of the soluble sugars or 12% of the leaf dry weight) in sugarcane leaves but with 10-fold less sorbitol in the culm (Chong *et al.*, 2007; Brumbley *et al.*, 2004). Sugarcane leaves developed necrosis in a pattern characteristic of early senescence and the severity was related to the relative quantity of sorbitol accumulated. Vacuolar targeting of the same enzyme, while not attempted, may lead to normal plants and higher accumulation of sorbitol in the culm as observed for isomaltulose (Wu and Birch, 2007). However,

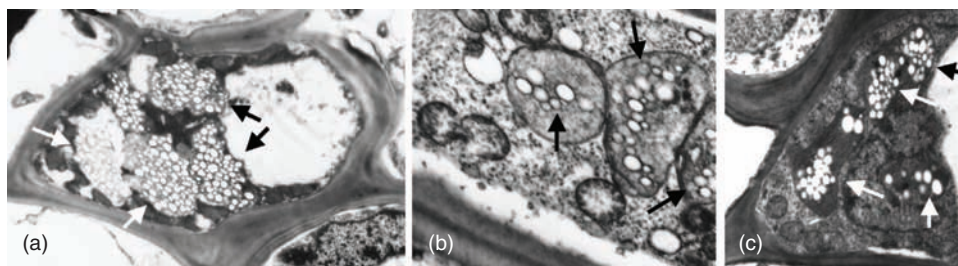


Figure 5 PHB sugarcane line TA4 cells showing plastids containing polyhydroxybutyrate granules. Six-month-old sugarcane plants were sectioned and analyzed for PHB content in (a) leaf tissue taken from the midrib of the lowest green leaf and sampled from older tissue closest to the leaf tip, (b) stem tissue sampled from the basal internode, (c) stem tissue sampled from the basal node. Black and white arrows point to clusters of PHB granules [Reproduced with permission from Todd Werpy and Gene Petersen, the lead authors of “Top Value Added Chemicals from Biomass”. <http://www1.eere.energy.gov/biomass/pdfs/35523.pdf>]

protein stability within the vacuole will continue to be a major challenge for efficient metabolic engineering of the sugar storage compartment in sugarcane. A possible solution would be to modify the enzymes, possibly by using directed evolution (Stemmer, 1994a, b; Chica *et al.*, 2005; Kaur and Sharma, 2006) to function optimally at the pH and to be more resistant to the protease activity in the environment of the storage vacuole.

In addition to the above mentioned sugars, trehalose, a nonreducing disaccharide of glucose, has also been produced in sugarcane (Brumbley *et al.*, 2006b; O'Neill *et al.*, 2006; Zhang *et al.*, 2006). Trehalose may play a role in carbohydrate metabolism impacting on glucose, fructose, and sucrose levels (Rontein *et al.*, 2002).

Biopolymers. To test the ability of sugarcane to be a biofactory, the products from the *Ralstonia eutropha* PHB biosynthetic pathway were targeted to several subcellular compartments of sugarcane (Brumbley *et al.*, 2002, 2004, 2007; Petrasovits *et al.*, 2007; Purnell *et al.*, 2007). PHB is the best-studied member of the polyhydroxyalkanoate (PHA) family, of which approximately 130 naturally occurring members have been identified (see Section 3). PHB is produced from acetyl-coenzyme A (acetyl-CoA) by the successive action of three enzymes [ketothiolase (PHAA), acetoacetyl-reductase (PHAB), and PHB synthase (PHAC)], which are encoded by the genes *phaA*, *phaB*, and *phaC*, respectively (Peoples and Sinskey, 1989a, b). Each gene was on a separate transformation vector and gene expression was controlled by *Ubi-1* promoter and *nos* terminator sequences. The three vectors with the PHB biosynthetic pathway were

biologically transformed into sugarcane callus simultaneously along with a construct containing the selectable marker gene *nptII* (Petrasovits, 2005; Petrasovits *et al.*, 2007).

Previous attempts to produce PHB at high levels in plants resulted in either low levels of PHB accumulation or severe negative phenotypic effects. In sugarcane, the polymer accumulated in the leaves of chloroplast-targeted lines at levels up to 2.5% of dry weight and 0.01% in stems (Figure 5) (Petrasovits *et al.*, 2007). Purnell *et al.* (2007) conducted a replicated glasshouse trial using a random block design with six independent PHB producing transgenic sugarcane lines and found that stalk height and weight and sugar levels were not affected by PHB accumulation.

Sugarcane has also been evaluated as a production platform for *p*-hydroxybenzoic acid (pHBA) using two different bacterial proteins (a chloroplast-targeted version of *E. coli* chorismate pyruvate-lyase and a 4-hydroxycinnamoyl-CoA hydratase/lyase from *Pseudomonas fluorescens*) (McQualter *et al.*, 2005; Brumbley *et al.*, 2004). Both lines provide a one-enzyme pathway from different naturally occurring plant intermediates. The substrates for these enzymes are chorismate (a shikimate-pathway intermediate that is synthesized in plastids) and 4-hydroxycinnamoyl-CoA (a cytosolic phenylpropanoid intermediate). Although both proteins have previously been shown to elevate pHBA levels in plants (Siebert *et al.*, 1996; Mayer *et al.*, 2001), they had never been evaluated concurrently nor had they been used simultaneously in the same plant. The pHBA was quantitatively converted to glucose

conjugates by endogenous uridine diphosphate (UDP)-glucosyltransferases and was stored in the vacuole. The largest amounts detected in leaf and stem tissue were 7.3% and 1.5% of dry weight (DW), respectively, and there were no observable phenotypic abnormalities. However, as a result of diverting carbon away from the phenylpropanoid pathway, there was a reduction in leaf chlorogenic acid, subtle changes in lignin composition, and an apparent compensatory up-regulation of phenylalanine ammonia-lyase (PAL; McQualter *et al.*, 2005).

2.2.5 High-value protein production

GM-CSF, which is used in clinical applications for treatment of neutropenia and aplastic anemia, was used to test the feasibility of using sugarcane as a biofactory for high value protein production (Wang *et al.*, 2005a). Two promoters, *Ubi-1* and sugarcane *ubi9* were tested in transgenic sugarcane lines resulting in production of up to 0.02% total soluble protein as GM-CSF. No significant difference was observed between the *Ubi-1* or *ubi9* promoter line. To achieve higher accumulation of GM-CSF in sugarcane, a C terminal HDEL tag for ER targeting had to be added to the gene construct. The sugarcane-produced GM-CSF showed identical biological activities when compared to commercially available purified protein. In a 14-month old field trial, no abnormal phenotype was observed and the accumulation levels remained relatively stable. This is the first report of GM-CSF production in field-grown plants. No flowering of the trial plants occurred and no pollen or seed was produced during the trial period. Drying, burning, and burial of the test plants effectively blocked possible routes for the transgenic sugarcane to enter the food supply or environment. If GM-CSF accumulation in sugarcane had been as high as Sétamou *et al.* (2002c) reported for the snowdrop lectin (1–1.25% total soluble protein), commercial production would probably have been economically viable because of the high commercial value of this protein.

Sugarcane has also been used for the production of bulk protein, collagen. In vertebrates, the collagen family is made up of approximately 27 proteins (Myllyharju *et al.*, 2000; Kielty and

Grant, 2002; Gelse *et al.*, 2003; Myllyharju and Kivirikko, 2004; Pakkanen *et al.*, 2006). Collagens make up 25% of the total proteins in humans and function to act as connecting structures and to give mechanical stability to the entire body. They form triple-helical structures, giving them commercial importance for the food industry and for a range of medical applications, such as tissue repair and cosmetic surgery (Bulleid *et al.*, 2000). Gelatin is denatured collagen. The yeast *Pichia pastoris* has been engineered successfully for the high level production of triple-helical collagen (Pakkanen *et al.*, 2006). Production has also been attempted in sugarcane and a fragment of human collagen was produced at very low levels (0.025% total soluble protein) (E. Mirkov, personal communication).

2.2.6 Abiotic stress

One strategy for generating abiotic stress-resistant plants has been to express genes for the production of trehalose. *In planta* trehalose production confers stress tolerance in plants (Garg *et al.*, 2002; reviewed in Penna, 2003; Rolland *et al.*, 2006). Abiotic stress normally causes a reduction in photosynthesis. However, trehalose protects the photosynthetic machinery in the plant cells and allows them to remain functional for longer periods of time under a range of abiotic stress conditions (Garg *et al.*, 2002; Rolland *et al.*, 2006). By using two copies of the CaMV 35S promoter in tandem to drive expression of the *Grifola frondosa* trehalose synthase gene in *S. officinarum*, Zhang *et al.* (2006) achieved trehalose accumulation in sugarcane cells at 0.9–1.2% of fresh weight. This low-level production in transgenic plants has been seen in other plant species and has been attributed to native trehalase activities (Garg *et al.*, 2002; Penna, 2003). The transgenic lines showed no negative effects on growth, were drought tolerant, and produced higher yields under drought conditions.

2.2.7 Flowering

In sugarcane cultivars, the time and intensity of flowering are important as they influence the yield and quality of cane (Premachandran, 2006). Sugarcane yield is reduced by cessation of vegetative growth in flowering canes and profusely

flowering clones are undesirable for commercial cultivation, especially when used for late-season crushing.

The identification of genes with major effects on flower induction could lead to new ways to control flowering in sugarcane cultivars (Ulian, 2006). Flower formation depends on the transition of a vegetative meristem to an inflorescence meristem and, subsequently, to a floral meristem. This transition requires the action of a number of genes, including *LEAFY* (*LFY*), a gene that is expressed early and considered to be important in the flowering process (Weigel *et al.*, 1992). *LFY* interacts with another floral control gene, *APETALA1* (*API*), to promote the transition from inflorescence to floral meristem. The ectopic expression of *LFY* induces the ectopic expression of *API* in leaf and axillary flower primordia (Parcy *et al.*, 1998). *LFY* and *API* are pivotal for the switch to the reproductive phase, where, instead of leaves, the shoot apical meristem produces flowers. The *API* promoter is a direct target of *LFY* (Wagner *et al.*, 1999). *LFY* also directly targets the *AGAMOUS* (*AG*) promoter that contains a *LFY*-responsive enhancer necessary for its activity (Busch *et al.*, 1999). *AG* encodes a transcription factor that regulates genes determining stamen and carpel development in wild-type flowers (Yanofsky *et al.*, 1990). *LFY*, therefore, plays pivotal roles in the specification of flowers and in the patterning of floral organs (Hempel *et al.*, 2000).

To study flowering in sugarcane, Ulian (2006) analyzed the SUCEST database and found a DNA EST with significant similarity to the *Arabidopsis* *LFY* sequence with two regions highly conserved in the *LFY* family of transcription factors. The putative *LFY* gene from sugarcane was expressed in the antisense orientation in transgenic sugarcane clone SP87-432 under the control of the constitutive maize *Ubi-1* promoter. Ulian (2006) observed that silencing of the *LFY* gene suppressed flowering in transgenic sugarcane, supporting the notion that the gene plays an important role in sugarcane flower development.

3. FUTURE ROAD MAP

Recent research on sugarcane has started to produce the detailed biochemical and genetic information that will be needed to develop

technologies necessary for sugarcane improvement and to metabolically engineer sugarcane for various traits and compounds.

3.1 Expected Technologies

To successfully manipulate the metabolic processes in sugarcane, a combination of molecular tools is required. In addition, consideration has to be given to both the organizational complexity at the whole plant level and the metabolic compartmentation within cells. Metabolic engineering requires a transformation system, suitable gene(s) and vector constructs, appropriate promoter sequences for cell- and tissue-specific expression, and effective targeting signals to direct the protein to its final destination within the cell. In sugarcane, an efficient transformation system (Bower and Birch, 1992; Bower *et al.*, 1996) and effective targeting signals to direct heterologous proteins to various compartments including the vacuoles, plastids, and mitochondria are available (Gnanasambandam and Birch, 2004; McQualter *et al.*, 2005; Rae *et al.*, 2006; Petrasovits *et al.*, 2007; Gnanasambandam *et al.*, 2007). Future research should focus on improving the efficiency of existing technologies as well as developing new technologies.

3.1.1 Improved nuclear transformation efficiency

Currently, the biolistic method is the most efficient method for the production of transgenic sugarcane plants (Bower and Birch, 1992). However, transformed plants obtained through this method have several shortcomings, including the presence of numerous copies of transgenes with complex patterns of integration (Albert *et al.*, 2003; Wang *et al.*, 2003). This creates some difficulties in obtaining regulatory approval for transgenic crop plants. Multiple transgene integrations may also inhibit transgene expression, cause gene silencing, and/or promote transgene rearrangements. Additionally, biolistic transformation entails incorporation of the entire plasmid into the plant nuclear genome, which is a cause for public concern. Thus, genes essential for plasmid replication (*ori*) in bacteria as well as

antibiotic resistance (e.g., ampicillin) required for selection of the transformed bacterial colony are co-introduced along with the gene(s) of interest. To minimize these problems, two technologies are currently being tested—*Agrobacterium*-mediated transformation (see Section 2) and the use of linearized plasmid DNA (LDNA) containing only the promoter-ORF-terminator cassette.

Agrobacterium-mediated transformation generally produces transgenic plants with fewer copies of the transgenes. *Agrobacterium*-mediated transformation of sugarcane has been reported in a few laboratories (Section 2). Most reports in sugarcane have shown the number of transgene copies to be generally between one and three (see Section 2). This may lead to higher and more stable expression of the transgenes as has been reported in rice (Dai *et al.*, 2001) and maize (Shou *et al.*, 2004) when compared to biolistic transformants. However, the stability of transgene expression using *Agrobacterium*, compared to biolistic transformation, has yet to be verified in sugarcane. Also, the efficiency of transformation is comparatively lower than that for microprojectile bombardment. Future research must aim to increase the efficiency of this transformation method in sugarcane.

In *Agrobacterium*-mediated transformation, transfer of genes of interest (present in the T-region of the *Ti* plasmid of *Agrobacterium*) into the plant nucleus usually occurs between the right and left T-DNA (transfer DNA) borders of the plasmid (Hellens *et al.*, 2000). Generally, this region only contains the regulatory sequences along with the genes of interest. Thus, the transfer is believed to be precise, with none of the plasmid sequences flanking the border regions being transferred. Recently, however, Shou *et al.* (2004) analyzed transgenic maize to determine whether vector backbone sequences of the *Ti* plasmid were also transferred. They showed that in 75% of the R_1 progeny of primary transformants there was some portion of the backbone present. This event may have occurred due to the specific transformation conditions used in the experiment. This needs to be determined when transforming sugarcane with *Agrobacterium*. Field trials of sugarcane plants transformed with *Agrobacterium* are proposed to commence in Australia in 2007 to address many of the concerns raised above (P. Joyce, personal communication).

Concerns raised by activists regarding the release of microbial genes into the environment coincidently with the biolistic transformation procedure has led scientists to use LDNA containing only the promoter-ORF-terminator cassette region for plant transformation. This LDNA method has been tested for gene expression and copy number analysis in rice (Fu *et al.*, 2000; Loc *et al.*, 2002). There was a significant reduction in the number of copies integrated into the rice genome. In addition, a low frequency of transgene rearrangements without any deleterious effect on expression pattern was observed (Fu *et al.*, 2000). Moreover, when two minimal linear genes were co-bombarded, the co-transformation efficiency was similar to that using intact circularized plasmids. Similar experiments in sugarcane, however, showed that it behaved differently to rice (Albert *et al.*, 2004; Joyce *et al.*, 2006b).

3.1.2 Plastid transformation

The current nuclear transformation technology has several limitations, including semi-random and unpredictable incorporation of foreign genes into the genome, and the operation of genetic mechanisms that can switch off foreign genes, resulting in widely varying expression of introduced genes in sugarcane (Ingelbrecht *et al.*, 1999; Gilbert *et al.*, 2005; Vickers *et al.*, 2005a, b). A powerful new strategy, plastid transformation, has been developed in some plants, including *Arabidopsis*, tomato, potato, cotton, carrot, and rice (Bock and Khan, 2004; Koya *et al.*, 2005), with the potential to circumvent these problems by introducing genes into plastid DNA rather than the plant nuclear genome (Maliga, 2002; Daniell *et al.*, 2005b). The plastid transformation strategy utilizes two targeting sequences that flank the foreign genes and that inserts them through homologous recombination at a precise, predetermined location in the chloroplast genome (Maliga, 2004).

Transformation of sugarcane plastid genome (plastome) offers several advantages over nuclear transformation. These include, but are not limited to:

1. High levels of protein production: Because each leaf cell may contain up to 10 000 copies of plastid DNA (each green cell contains

about 50–100 chloroplasts with 60–100 plastid genomes per plastid), plastid-transformed tobacco plants synthesize extraordinary levels of foreign proteins (e.g., 5% of *Bt* toxin that provides resistance against insect pests (McBride *et al.*, 1995)). This concentration is about 10-fold higher than the maximum achieved following transformation of the plant nuclear genome. These high levels of protein production in the plastids can facilitate the development of genetically improved sugarcane plants resistant to insect pests or herbicide damage, and the use of sugarcane plants as efficient bioreactors for production of novel high value biochemicals, pharmaceuticals, and industrial materials (Guda *et al.*, 2000; DeGray *et al.*, 2001; Daniell *et al.*, 2005a).

2. Consistent gene expression: Protein expression from a gene inserted at a specific site in the plastid genome will be more consistent within the leaves of different transformants (Maliga, 2004).
3. Significantly reduced risk of spread of foreign genes (biocontainment): Chloroplasts are predominantly inherited through the maternal line in most important agricultural plants. Hence, the risk of a foreign gene being transmitted in pollen to nontransgenic sugarcane crops or related species is minimal (Lee *et al.*, 2006). This has been experimentally shown by the containment of herbicide resistance in tobacco (Daniell *et al.*, 1998)
4. Absence of gene silencing: Foreign genes can be silenced in sugarcane plants by modification of the inserted DNA in the nucleus, or by specific breakdown of the corresponding mRNA required as a template for protein synthesis in the cytosol. As there are no known similar silencing mechanisms identified in plastids (Dhingra *et al.*, 2004; Lee *et al.*, 2003a), foreign genes should be exempt from silencing in plastid transformed sugarcane plants, in which both transcription and translation occur within the chloroplast, separated from the cytosol.
5. The ability to express polycistronic messages from a single promoter: In nuclear transformation, each encoding sequence must be engineered under the control of a separate

regulatory region, i.e., a monocistron. As a consequence, gene expression levels vary widely among introduced sequences, and generation of a number of transgenic plant lines is required to introduce all of the cistrons into one plant and to get proper coordinated expression in the target biochemical pathway. In contrast, the functioning of the plastid genome (being prokaryotic in nature) permits simultaneous expression of two or more genes from a single plastid promoter region (Quesada-Vargas *et al.*, 2005). Such an expression method makes possible large scale and inexpensive production of some proteins and fine chemicals. In addition, it allows the engineering of metabolic pathways through the introduction of multiples genes in a single operon.

6. Integration via a homologous recombination process that facilitates targeted gene replacement and precise transgene control.
7. Sequestering of foreign proteins in the organelle, which prevents adverse interactions with the cytoplasmic environment. In addition, the ability of chloroplasts to form disulfide bonds and to fold human proteins could allow high-level production of biopharmaceuticals in plants.

Despite its tremendous biotechnological potential, plastid transformation has only been used routinely in tobacco (Maliga, 2004) and attempts to develop plastid transformation in sugarcane have been unsuccessful to date. However, a recent patent issued on plastid transformation in monocots (Daniell, 2006) indicates the potential exists to develop the technology in sugarcane. The complete nucleotide sequence of the chloroplast genome of sugarcane has been determined recently (Asano *et al.*, 2004). The plastome of sugarcane is a circular double-stranded DNA molecule, 141 182 bp in size, and is composed of a large single copy of 83 048 bp, a small single copy of 12 544 bp, and a pair of inverted repeat regions of 22 795 bp each. The sugarcane chloroplast genome is similar to maize, but not to rice or wheat. The availability of sequences for sugarcane chloroplast genome (Asano *et al.*, 2004) could facilitate the development of plastid transformation technology in sugarcane.

3.1.3 Avoiding antibiotic resistance gene in transgenic plants

Elimination of antibiotic resistance selectable marker genes in transgenic plants may increase public acceptance of this technology. The options available include:

1. Use of no selectable marker gene, i.e., only the gene of interest is introduced and transformed tissue is screened for (not a practical solution due to time constraints).
2. Use of a visual marker gene such as *GFP* (which has no harmful biological activities) to select transformed cells (Elliott *et al.*, 1998), which can be cumbersome and prone to contamination.
3. Selecting T₁ progeny that do not contain the antibiotic resistance gene, i.e., they have segregated for the antibiotic resistance gene. This is not an option for sugarcane, because none of the commercial sugarcane cultivars are homozygous. Sugarcane clones are clonally propagated to maintain the elite characters.
4. Use of an antibiotic gene excision system; once again, this will involve retransformation of transgenic cane. However, if more inducible promoters were available, it would be possible to control excision of the antibiotic gene (after selection and regeneration of plants) by switching on the expression of the site-specific recombinase gene (Puchta, 2000).
5. Use of a selection system that is not antibiotic based. The use of alternative marker genes conferring positive selection. For instance, a nontoxic compound to promote the regeneration and growth of transformed cells expressing a transgene that acts upon that compound are being developed. It may even be possible to tailor-make genes that are designed to specific plants.
6. Sugarcane plastid transformation would be one possible way of removing both antibiotic resistance genes and vector sequences from plants using homologous recombination.
7. Bombardment with linear DNA avoids vector-localized genes such as the ampicillin resistance (see Section 3.1.1). Transformation of sugarcane with linearized DNA fragments has been demonstrated (Joyce *et al.*, 2006b).

3.1.4 Suppressing transgene silencing

There are convincing reports indicating that sugarcane displays a high level of PTGS (Liu *et al.*, 2003; Wei *et al.*, 2003). Strategies to suppress this include the use of viral suppressor proteins (Mangwende *et al.*, 2005), addition of putative matrix attachment regions (MARS) (Wei *et al.*, 2003), and presence of introns. The use of viral suppressors, including P0 from ScYLV and HcPro from SrMV, to advance our knowledge of RNA silencing in sugarcane are being explored (Wang *et al.*, 2006, 2007). More research is needed to understand and suppress transgene silencing.

3.1.5 Tissue-specific promoters

Despite several years of research, an effective stem-specific promoter is not yet available in sugarcane. Though a stem-specific promoter has been reported in sugarcane (Hansom *et al.*, 1999), its usefulness remains to be established. Similarly, other tissue-specific promoters that are operative in sugarcane (for example, root-specific promoters) are not available. Attempts to find new promoters for strong expression especially in the mature parenchyma cells of sugarcane stem are ongoing. Recently, Mudge *et al.* (2006) reported the isolation of an EST clone (SMS04) that was selected based on its expression pattern. Further analysis revealed that at least eight variants of this gene existed in sugarcane and that no two had the same sequence. The promoters associated with these genes were isolated and activity tested using the *GUS* reporter gene. Most were functional soon after introduction into the explant, but none maintained activity in mature regenerated plants.

To avoid gene silencing, Potier *et al.* (2006) isolated tissue-specific promoters from the upper stem or roots of maize and sorghum rather than sugarcane. These promoters (with and without the first intron) were used to test the expression of *GUS* gene in sugarcane callus. In general, the presence of an intron showed higher expression in all the promoters studied. Further research should identify effective tissue-specific promoters that can maintain activity in mature transgenic plants. With the available sorghum genome sequences, the search and solution to finding new tissue-specific promoters is more tangible.

3.1.6 Transcriptional regulators

Transcription factors are proteins that interact with the promoter regions of target genes in a sequence-specific manner. They influence the manner in which RNA Polymerase II initiates mRNA synthesis by enhancing or repressing gene expression and, hence, are important regulators of gene expression. Transcription factors tend to control multiple steps in metabolic pathways, meaning that a single protein can affect the expression pattern of a large number of downstream genes (Kinney, 2006). This is an important consideration for plant transformation strategies aimed at significantly affecting the level of end-product accumulation. Although this can be a successful strategy, a lack of knowledge about a particular pathway may mean that additional and hitherto unknown rate-limiting steps may be present that may frustrate the attempt to significantly modify end-product accumulation. Overexpression of transcription factors holds the promise of significantly altering the overall flux through a metabolic pathway by controlling the transcription of multiple genes. The potential of using transcription factors to enhance current and future work into the genetic enhancement of sugarcane is highlighted in Sections 2.2.6, 3.2.6, 3.2.7, and 3.2.9.

3.1.7 Modifying more complex traits

Very few traits that are simply-inherited have been described in sugarcane (Hogarth, 1987; Daugrois *et al.*, 1996; Mudge *et al.*, 1996a, b; Raboin *et al.*, 2006), suggesting that most phenotypic characteristics, such as cane yield and sugar content, are controlled by polygenes. In other organisms, genes that contribute to complex traits (QTL) pose special challenges that make gene discovery and subsequent modification of such traits more difficult. Such problems include locus heterogeneity, epistasis, low penetrance, variable expressivity, and pleiotropy (Glazier *et al.*, 2002). Approaches are even more difficult in sugarcane, where there is the possibility of segregation for three or more alleles at a locus, a lack of chromosome preferential pairing and high levels of heterozygosity (Ming *et al.*, 2001a, b; Aitken *et al.*, 2006), which generally results in numerous

alleles of small effect, some of which cannot be positioned on a genetic map. These factors work together to make claims of linkage discovery notoriously difficult to verify and candidate gene identification challenging. However, prospects for success can be improved through genome sequencing and comparative genomics. Such resources will allow more rapid dissection of complex traits through candidate gene identification and subsequent testing using functional genomics approaches.

3.1.8 Functional and comparative genomics

3.1.8.1 Expressed sequence tags (ESTs)

Sequencing efforts by different organizations in several countries have provided a large set of publicly available ESTs in sugarcane (Carson and Botha, 2000; Casu *et al.*, 2003; Vettore *et al.*, 2003; Ma *et al.*, 2004). The largest of these efforts was undertaken in Brazil by the Organization for Nucleotide Sequencing and Analysis (ONSA). A network of laboratories in all the major research institutions across the state of São Paulo conducted genomics and transcriptomics (Harvey and McMeekin, 2005) on sugarcane ESTs (SUCEST) (<http://sucest.lad.dcc.unicamp.br/en>). The goal of the SUCEST project was to identify 50 000 sugarcane genes or generate a total of 300 000 ESTs. Following sequencing, the data mining groups in SUCEST worked on a cross-section of sugarcane genes including, but not limited to genes involved in amino acid metabolism, carbohydrate metabolism, lipid metabolism, energy metabolism, metabolism of co-factors (vitamins and other substances), nutrient uptake and metabolism, environmental stress, pathogenesis, membrane receptors, phytohormone biosynthesis and regulation, plant morphology, flowering and development of reproductive organs, patterns and levels of gene variation, and comparative genomics.

The outcomes of the SUCEST project were significant. It established Brazil as a biotechnology innovator, developed skills and capabilities throughout research institutions in São Paulo, created international collaborations and networks, and resulted in the formation of new biotech companies (Harvey and McMeekin,

2005). Data from the SUCEST project, and from other sequencing efforts, has been centralized at the Institute for Genomic Research's (TIGR) *Saccharum officinarum* Gene Index (SoGI) (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=s_officinarum; Quackenbush *et al.*, 2001). TIGR aims to integrate research data from international sugarcane EST sequencing and gene research projects. Its ultimate goal is to represent a nonredundant view of all sugarcane genes and data on expression patterns, cellular roles, functions, and evolutionary relationships. The TIGR database has 255 635 ESTs with a total of 78 547 unique sequences. This database was recently moved to the Computational Biology and Functional Genomics group at Harvard University, where it is incorporated into an even bigger gene index project: <http://compbio.dfci.harvard.edu/tgi/plant.html>.

A subset of the available EST sequences have been arrayed on a Sugar Cane GeneChip[®] (Affymetrix) (Casu *et al.*, 2006), which is designed specifically to monitor gene expression. The GeneChip[®] Sugar Cane Genome Array contains 8236 *S. officinarum* probe sets to monitor gene expression for approximately 6024 distinct genes. Expression profiling using this and other cDNA arrays, along with mapping studies, have investigated genes involved in stem development, sugar accumulation, growth at low temperatures, and disease resistance (Casu *et al.*, 2003, 2004, 2005, 2006; Nogueira *et al.*, 2003; Rossi *et al.*, 2003). These, and future studies, should provide targets for functional genomic screens through either overexpression or RNAi-mediated gene knockout strategies. In fact, field testing of a number of lines for increased sugaryield has already begun by the Centro de Tecnologia Canavieira (CTC) and Allelyx, a biotech start up company developed out of the ONSA genomics program (Harvey and McMeekin, 2005).

Importantly, the wealth of sequence information available allows for a robust strategy to incorporate all polymorphic alleles. The EST database is also a starting point for metabolic engineering by helping to understand sugarcane central metabolism. For instance, in a recent approach to produce trehalose in sugarcane, a survey of the EST collection identified a probable trehalose dehydrogenase, the activity of which would have to be inhibited for the strategy to be successful (O'Neill *et al.*, 2006).

3.1.8.2 Genomics

The large database of public ESTs described above is an important resource that has been useful in a variety of ways, providing functional DNA markers, a foundation for development of expression profiling platforms, and a rich resource for evolutionary studies. Available EST sequences may collectively provide tags for half of the sugarcane genes. However, genomic DNA-based systems are also required because (i) many important genes expressed at low levels are unlikely to be found in libraries and (ii) the cDNA approach provides no information about the crucial on/off switches (promoters, enhancers, transcriptional regulators) that control gene expression. Hence, a comprehensive picture of the sugarcane genome including the entire suite of genes, their all important regulatory elements, and their complete arrangement along the chromosomes will eventually require complete sequencing of the genomes of one or more sugarcane genotypes. Ultimately, this could be achieved with whole-genome sequencing.

A major step toward full genome sequencing would be the construction of large sugarcane bacterial artificial chromosome (BAC) libraries and the generation of detailed genomic maps. Brumbley and Brumbley (2005) generated a library of sugarcane cultivar Q200^A containing 98 000 colonies with an approximate 1 × coverage of the genome. Larger libraries of sugarcane cultivar R570 were constructed at Clemson University and at CIRAD in Montpellier, France. Rapid progress in the speed and cost of DNA sequencing will make feasible the sequencing of entire sugarcane genomes within a few years (Paterson, 2006). Once sequenced, the door is open for candidate gene studies using functional genomics. In the interim, much is likely to be learned from comparative genomics studies using the sequences of closely-related sorghum genome both as a source of control elements and/or as a tool to identify likely candidate genes through comparison of QTLs on sugarcane genetic maps with the physical map of sorghum (Guimarães *et al.*, 1997; Ming *et al.*, 1998).

Sorghum is representative of tropical grasses in that it is classified as a C4 photosynthesizer, comprising complex biochemical and morphological specializations that improve carbon assimilation at high temperatures. By contrast, rice is more

representative of C3 temperate grasses. Sorghum's economic and scientific importance, together with progress in characterizing its genome (detailed below) have motivated the sequencing of an elite inbred line of *S. bicolor* L. genotype, BTx623, to 8x genome coverage under the US Department of Energy Joint Genome Institute (JGI) "Community Sequencing Program" (CSP). Sorghum sequencing was completed in early 2007 and the database is available at the same Harvard University site as sugarcane, <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=sorghum>, and annotation is well underway.

The completed sorghum sequence will be an excellent bridge for translating hard-won functional genomics knowledge from *Oryza sativa* L. ssp. *indica* to leading tropical grass crops, with much larger genomes and much more gene duplication. Sorghum and maize diverged from a common ancestor approximately 12 million years ago (Gaut *et al.*, 1997; Swigonova *et al.*, 2004a), versus approximately 42 million years for rice and the maize/sorghum lineage (Paterson *et al.*, 2004). *Saccharum* may have shared ancestry with sorghum as little as 5 million years ago (Sobral *et al.*, 1994) and it retains a similar gene order (Ming *et al.*, 1998), and even produces viable progeny in some intergeneric crosses (Dewet *et al.*, 1976). Maize has undergone a whole-genome duplication since its divergence from sorghum (Swigonova *et al.*, 2004b), and *Saccharum* has undergone at least two (Ming *et al.*, 1998). The low level of gene duplication in sorghum makes it, like rice, an attractive model for functional genomics.

The sorghum sequence will also offer numerous direct opportunities to increase knowledge of sugarcane biology. At the level of genome structure, sorghum and sugarcane chromosomes appear to share very similar gene content and order, and it is expected to be simple to use well-established synteny relationships (Ming *et al.*, 1998), supplemented by a growing body of hybridization marker and end-sequence data to deduce the likely locations in sugarcane of genes identified in the sorghum sequence. Its much closer relationship will make the sorghum sequence of substantially greater value than the rice sequence for such deductions in sugarcane.

A host of classical genetics, supplemented by recent DNA marker studies (Ming *et al.*,

2001a, b, 2002a, b) show heterozygosity resulting from autopolyploidy to be instrumental in the productivity of sugarcane. More generally, genome duplication has long been suggested to play an important role in angiosperm evolution (Stephens, 1951; Ohno, 1970). One of the biggest surprises of the genomics era has been underestimation of the prevalence of ancient whole-genome duplication, finding that even the genomes of diploids such as *Arabidopsis* (Blanc *et al.*, 2000; Initiative, the Arabidopsis Genome, 2000; Paterson *et al.*, 2000; Bowers *et al.*, 2003) and *Oryza* (Paterson *et al.*, 2003, 2004, 2005; Vandepoele *et al.*, 2003; Wang *et al.*, 2005b) have been shaped by genome duplication. Such duplication is associated with a host of rapid responses including loss and restructuring of low-copy DNA sequences (Song *et al.*, 1995; Feldman *et al.*, 1997; Ozkan *et al.*, 2001; Shaked *et al.*, 2001; Kashkush *et al.*, 2002), activation of genes and retrotransposons (O'Neill *et al.*, 1998; Kashkush *et al.*, 2003), gene silencing (Chen and Pikaard, 1997a, b; Comai, 2000; Comai *et al.*, 2000; Lee and Chen, 2001), and organ-specific subfunctionalization of gene expression patterns (Adams *et al.*, 2003, 2004). These mechanisms may be especially important in providing raw material for evolutionary change in sugarcane, in that it appears to have undergone two such genome duplications in about the past 5 million years or so. Sorghum will provide an ideal outgroup for deducing the ancestral states of sugarcane genes that have been affected by genome duplication, in that it is very closely related but diverged prior to the duplication events. Future comparison of the fates of genes in sugarcane to those in *Sorghum halepense*, a polyploid derivative of sorghum, may offer the opportunity to test hypotheses about the degree to which the fates of duplicated genes are determined by a taxon-independent set of molecular principles (Paterson *et al.*, 2006). This could have profound implications for transgenic strategies in the future. For a given trait, are single copies or multiple copies of transgenes more beneficial in sugarcane?

3.2 Expected Products

The future for sugarcane biotechnology is exciting. Because of its narrow genetic base,

there is enormous potential to improve the field performance of this crop. Sugarcane is considered to be an ideal biofactory for the production of a range of sugars, and fine chemicals, including biopolymers, nutraceuticals, industrial enzymes, and pharmaceuticals, for the following reasons: (i) sugarcane is one of the fastest growing and largest biomass-producing agricultural crops in the world; (ii) it accumulates sucrose up to 42% of the dry weight of the stalk. In addition, bagasse (fibrous residue of sugarcane stalks left after extracting the sucrose) is made up of 32–48% cellulose, 19–24% hemicellulose, and 23–32% lignin, all of which all have commercial uses; (iii) it is grown throughout the tropical and subtropical latitudes and is a major crop in many developing nations and can, therefore, be used to help developing economies; (iv) it is usually vegetatively propagated and only produces fertile seed under a specific set of environmental conditions, therefore posing a low risk for transgene flow into the environment; (v) sugarcane crop-cycles run for 4 or more years between plantings. Hence, it does not have to be replanted every year, as it produces new tillers from the stool left in the ground after harvesting; (vi) sugar industries throughout the world already have substantial infrastructure to harvest the large biomass and transport it to mills for processing; and (vii) the tops and leaves of sugarcane, which are traditionally burnt or left behind in the field as a trash blanket, could potentially be used as a production platform for high value biofactory products, as another source of biomass for biorefineries or, more likely, both. In the future, it is highly likely that transgenic sugarcane will be grown not only for the production of sugars for human consumption, but also as a high biomass crop for fuel and energy production, with value adding by metabolically engineering it to produce a range of additional products *in planta*.

3.2.1 Improved sugar

Sugarcane is unusual amongst plants in storing up to 62% dry weight (and 16–25% fresh weight) of sucrose as the primary source of carbon and energy reserve in the storage parenchyma of the mature culm or stalk (Bull and Glasziou, 1963). These are approximate levels obtained in *S. officinarum*, the

major source of commercial hybrid germplasm. In contrast, some of the wild relatives of sugarcane store less than 2% of the fresh weight as sucrose. As the photosynthetic rates on an area basis of *S. spontaneum* have been reported as nearly twice those of *S. officinarum* and 30% greater than that of hybrid cultivars (Irvine, 1975), these striking differences in sink accumulation and storage activity cannot be explained by differences in photosynthetic rates in the leaves (Moore, 2005). Despite many years of research, the process of sugar accumulation is not fully understood.

As more than 70% of the sugar harvested for human consumption is derived from sugarcane (FAO, 2006), improving sugar content in sugarcane will remain as an important focus for future biotechnological innovations. Traditional breeding has improved the yield of sucrose mostly through increased cane yield. Increasing sucrose concentration has not occurred recently through traditional breeding in countries such as Australia, where the levels are already relatively high (Jackson, 2005). Attempts to increase sucrose content in sugarcane cultivars through the modification of plant genes involved in sugar metabolism have been mostly unsuccessful (reviewed by Lakshmanan *et al.*, 2005). For example, a 70% reduction in activity of acid invertase, a key enzyme involved in sucrose breakdown, resulted in no significant change in sucrose yield or purity in the immature internodes of transgenic sugarcane (Botha *et al.*, 2001). A better understanding of sugar-transport pathways will be required for strategies to increase the flux of sugars and, ultimately the amount of sucrose in the storage tissue (Grof and Campbell, 2001; Rae *et al.*, 2005).

The doubled sugar content observed in transgenic sugarcane while attempting to produce isomaltulose (Wu and Birch, 2007) indicates that there is potential to improve sugar content in sugarcane through metabolic engineering of the vacuolar compartment. However, most vacuolar-targeting studies in many crops involving stable transformants have been limited by difficulties in detecting vacuolar-targeted enzyme activities (Ebskamp *et al.*, 1994; van der Meer *et al.*, 1994; Caimi *et al.*, 1996; Ma *et al.*, 2000; Gnanasambandam and Birch, 2004), indicating that the stability of targeted proteins is an important factor for effective metabolic engineering. For isomaltulose

production in sugarcane, a highly efficient sucrose isomerase had to be used (Wu and Birch, 2007).

Reports of experimental efforts to protect foreign gene products from vacuolar degradation are scarce. To understand how to genetically engineer a protein for stability in the vacuole, the “enemies” within the vacuoles—the proteolytic enzymes and their sites of action—need to be characterized (<http://www.proweb.org/other.html>). A method to isolate protoplasts from sugarcane mature stem parenchyma cells has been reported (Gnanasambandam and Birch, 2006), but further optimization is needed for successful vacuole isolation for characterization of proteolytic enzymes. Alternatively, if vacuole isolation proves to be difficult, fluorescent substrates for proteases (from Molecular Probes, Inc.) can be used with isolated protoplasts. Such characterization on proteolytic enzymes may allow for informed modification on foreign proteins to remove sites for attack by sugarcane proteases. Alternatively, directed evolution can be applied to rapidly evolve enzymes for optimal activity under vacuolar conditions (Stemmer, 1994a, b; Chica *et al.*, 2005; Kaur and Sharma, 2006). There may be particular sequences within a protein that are sensitive to proteolytic enzymes, and altering these sensitive sequences may increase protein stability. For example, mutation of protease-sensitive regions of the firefly *luc* yielded larger and more stable signals compared with the wild-type protein in *E. coli* (Thompson *et al.*, 1997).

3.2.2 Alternative or novel sugars

There have already been examples of alternative sugars produced in genetically modified (GM) sugarcane (Section 2). In addition to isomaltulose (Wu and Birch, 2007) and sorbitol (Chong *et al.*, 2007), there is potential to engineer synthesis of a range of other sugar complexes and sugars into sugarcane, for example alternative carbohydrates such as starch and fructans (already demonstrated in sugarbeet).

Tagatose (Kim, 2004) has properties closer to sucrose than any of the other sugar substitutes. D-Tagatose is an isomer of D-galactose and it can function as a low-Joule sweetener. Although

it has relatively the same sweetness as sucrose, it is detected on the tongue sooner.

Xylitol is a sugar alcohol of considerable commercial interest (Granström *et al.*, 2007a, b). Its metabolism in humans is independent of insulin and it is anticariogenic. It also has uses as a pharmaceutical. Xylose is a major component of sugarcane hemicellulose, making up 25% of the w/w dry matter and 91% of the nonfermentable sugars (Pessoa Jr. *et al.*, 1997). Research is currently underway to optimize the conversion xylose to xylitol. *Candida guilliermondii* has been used as a test species on sugarcane bagasse hydrolysate, because it naturally metabolizes xylose (da Silva and Felipe, 2006). However, *Candida* cannot be used in food production, because many *Candida* species are pathogens. An alternative is to use bacteria. When a xylose reductase and a xylose transporter were engineered into the bacterium *Lactococcus lactis*, levels of xylitol production were equivalent to those in the best-producing *Candida* line (Nyyssölö *et al.*, 2005). An alternative would be to engineer a xylose reductase into sugarcane. One strategy might be to have the enzyme incorporated into the sugarcane cell wall structure, so that it is only functional when it is released during sugarcane processing at the mill. Another might be to regulate it with a wound-inducible promoter, so that it is produced in the sugarcane cells after harvesting. In both of these scenarios, the enzyme(s) will have to be engineered to function at the temperatures and pH of a sugar mill and in particular under mill conditions designed to break down hemicellulose (Pessoa Jr. *et al.*, 1997).

Though there has been much research toward the development of low-Joule nonsucrose sweeteners (Modi and Borges, 2005), two such sweeteners (saccharin and cyclamates) were found to be carcinogenic under some circumstances, while others (such as aspartame) exhibit temperature and low pH instability that limit their utility (Knight, 1994). Hence, it may be useful to produce a low-Joule nonsucrose product from sugarcane. Alternatively, there are a range of sweet proteins that may be worth considering. Monellin, Thaumatin, and Brazzein are 100 000, 3000, and 500 times sweeter than sucrose, respectively. These would be a natural replacement for synthetic sugars because they are low-Joule sweeteners and would not trigger insulin production (Kant, 2005).

3.2.3 The biorefinery—biofuels and biochemicals

Sugarcane is expected to become a platform for biorefinery production of fuels and chemicals. Recent demand for renewable energy sources has increased the interest within the research community on transgenic breeding for biomass production. Sugarcane is the crop having the most favorable input/output balance for bioenergy, and with the growing body of research on its genome, may allow the development of a low-input energy crop with key outputs as biofuel (ethanol) and bioenergy (electricity). The production of an alternative energy crop, with high biomass potential, will be a significant step toward the implementation of a biofuels, bioenergy, and, in the not too distant future, bioproducts industries. If biorefineries, producing dozens of products (Figure 6) are integrated into sugar mills, one can envision a sugarcane industry functioning more like petrochemical plants do today. Unlike petrochemical plants, the resource driving this new bioeconomic revolution will be renewable.

In nature, the most prevalent forms of carbon are the biopolymers cellulose and hemicellulose (Houghton *et al.*, 2006). The third most abundant biopolymer is lignin (van Dam *et al.*, 2005). These are considered to be the raw materials for future bio-based industries creating opportunities for sugarcane industries to diversify. Aside from water, sugarcane plants are primarily comprised of cellulose, hemicellulose, and lignin. Traditionally, this resource has been combusted, either in the field to remove the leaf material or at the mill to utilize the waste product bagasse to generate the necessary energy to power mill operations. Since the mid-1980s, it has been proposed that “energy cane” should be introduced as a multiple product alternative to sugarcane grown largely for sucrose (Alexander, 1984, 1985). The time may have come to implement this vision with the inclusion of advances in the fields of biotechnology. Changing the sugarcane industry from primarily a one-product to a multiple-product industry is an enormous challenge, but there are multiple indicators that should make both sugarcane farmers and millers optimistic.

Of all the agricultural crops, sugarcane may be the most ideally suited to benefit from a shift to a bio-based economy because of its large biomass

and, therefore, large quantities of cellulosic and hemicellulosic sugars and lignin (Alexander, 1984, 1985; Brumbley *et al.*, 2004, 2006b, c, 2007). There is good reason for simultaneously developing sugarcane both as a biofactory and as a feedstock for biorefinery processes. As technologies are developed to produce a range of new and diverse products in the sugarcane plant, other technologies are being developed to dismantle the cellulose and hemicellulose to release the large quantities of fermentable sugars from biomass. The breakdown of the tough structural elements that make up sugarcane cell walls will facilitate the extraction of new bioproducts such as the bioplastics described in Section 2.

The biofuel ethanol is becoming increasingly more important as the world attempts to wean itself off its addiction to petroleum-based fuels. The second largest producer of ethanol in the world is Brazil, and sugarcane is their feedstock (Pessoa Jr. *et al.*, 2005; Lorentzen, 2006). In the near future, another bio-based fuel (butanol) may enter the market. DuPont and British Petroleum recently formed a joint venture to produce this biofuel commercially. Butanol resolves many of the problems of ethanol. Not only does it have a higher energy content, it is not as corrosive, uses an air/fuel ratio that is closer to that of gasoline, can be shipped through existing fuel pipelines, is safer to handle, and can replace gasoline at any percentage up to 100%.

Governments and private industry are investing in technologies to convert biomass into biofuels and biochemicals. Cellulose, hemicellulose and lignin interact to form plant cell walls and are responsible for giving plants their structural integrity. Compared to starch, getting to the sugars that make up these polymers is far more difficult and more expensive (Bayer *et al.*, 2004; Houghton *et al.*, 2006). A diverse range of bacteria have been discovered that have multienzyme complexes that bind to and degrade cellulose and hemicellulose. This complex has been termed the cellulosome and the research on these enzyme complexes was reviewed recently (Bayer *et al.*, 2004). Researchers are looking for new organisms with cellulose degrading capabilities. Once discovered, activity and performance of these enzymes and complexes can be improved using directed evolution (Joyce, 2004; Neylon, 2004; Hibbert and Dalby, 2005).



Figure 6 Potential product targets for sugarcane-based biofactories and biorefineries. From just a few building blocks an array of secondary and intermediates chemicals can be produced. The intermediate chemicals are the building blocks of a vast range of end product. [Reproduced with permission from Todd Werpy and Gene Petersen, the lead authors of “Top Value Added Chemicals from Biomass”]

The pool of sugars currently locked up in cellulosic biomass could be used to feed biorefineries both for the production of biofuels and bioproducts. White biotechnology (Paster *et al.*, 2003) is the use of biological processes for the production of chemicals such as plastics, adhesives, and paints. Fermentation technologies could be used to produce building block chemicals that will be the basis for the range of secondary and intermediate chemicals (Figure 6). Currently, the United States and European Union annually produce €1000 billion worth of fine and bulk chemicals from a petroleum feedstock. They plan to shift over 90% of this production from a petroleum to a biorenewable base over the next 50 years. To accomplish this, new enzymes will be required to convert the sugars within the cellulosic biomass to the renewable building blocks required by tomorrow's manufacturing sector.

The vast majority of microbes have never been characterized. This enormous and diverse population is thought to be a rich source of new genes, metabolic pathways, and enzymes. However, most of these organisms are not culturable. Metagenomics is the study of the collective genomes of organisms, independent of growth in culture. The tools for doing metagenomics are advancing rapidly (Tringe and Rubin, 2005; Green and Keller, 2006). For example, Venter *et al.* (2004) used metagenomics to identify over 1.2 million new genes from at least 1800 species, including 148 previously unknown bacterial groups, just in the Sargasso Sea. As our understanding of protein domains advances (Portugaly *et al.*, 2007), it may be possible to design "lab-on-a-chip" devices (Hong *et al.*, 2004) that can be used to rapidly screen different environmental samples for genes encoding specific enzyme domains. Using systems biotechnology, newly discovered enzyme's can be characterized rapidly and their utilities for industrial bioprocessing determined (Lee *et al.*, 2005). Directed evolution tools, such as domain shuffling (Hibbert and Dalby, 2005), can be utilized to combine the new enzyme's functional domains with those of other related enzymes to create an enzyme that will function in the precise environment it is needed, whether that is a fermentation tank inside the cytoplasm, storage vacuole, mitochondria, peroxisome, or plastid of a sugarcane cell.

3.2.4 Biopolymers

A potential major industrial use for sugarcane is the production of biodegradable polymers. These polymers can be produced by fermentation technologies and, as shown in Section 2, can be produced in sugarcane. For example, PHAs are polyesters synthesized naturally by many species of bacteria as carbon sources and energy reserves. PHAs are accumulated to levels as high as 90% of the cell dry weight in bacteria. PHAs have the properties of thermoplastics and elastomers and they are biodegradable (Madison and Huisman, 1999). These classes of biopolymers offer a renewable and environmentally friendly alternative to the 150 t per year of plastic currently produced from petrochemical resources.

In nature, over 130 PHA variations have been identified that produce plastics with properties varying from hard and brittle, too elastomeric, glue-like, and even rubbery (Steinbüchel and Valentin, 1995). PHAs are classified based both on the length of the side chains on the polymer (i.e., whether the side chains are short (C4-6) or medium (C6-16) in length), and on whether the polymer is a homopolymer, or a co-polymer of PHAs with different side chains. In the case of co-polymers, the ratio of medium (mcl) to short side (scl) chains also affects the properties. Co-polymer PHAs of commercial interest range from 2% mcl (hard, brittle) to 20% mcl (soft, elastic). PHB, the most studied of the PHAs, is a short-side-chain homopolymer and displays properties similar to polypropylene (Hocking and Marchessault, 1994).

In Brazil, an important precedent has been established on how to diversify the product base of a sugar industry, first with ethanol, and then with bioplastics (Lorentzen, 2006; Velho and Velho, 2006). In 1992, initial projects were funded to develop a novel platform for the production of PHB from sugarcane through fermentation (Velho and Velho, 2006). The Brazilian company Copersucar (<http://www.copersucar.com.br>) first engineered the bacterium *Ralstonia eutropha* to use sucrose as a carbon source. A pilot plant was then built, integrated into a sugarcane mill (<http://www.biocycle.com.br>), and ultimately produced 50 t per year of high purity PHB. This refinery receives its feedstock (sugar syrup) directly from the sugar mill, which also produces all of the energy necessary to run the fermentation,

extraction, and purification facilities. The solvents for PHB extraction are also sugar mill products sourced from the ethanol production facility (Velho and Velho, 2006). This model is an excellent example of how an existing resource industry can, with government and corporate support, build on its knowledge base to create new business opportunities and a help establish bio-based economy in a developing country (Lorentzen, 2006).

BiopolTM (Choi and Lee, 1999) is the only PHA co-polymer that has been produced commercially. However, Metabolix, Inc. has formed a strategic alliance with Archer Daniels Midland (ADM) to commercialize PHA natural polymers. Metabolix and ADM claim that they can make these natural plastics by fermentation at costs that are competitive, or near competitive, with petrochemical-based plastics (<http://www.metabolix.com>). Metabolix argues that producing these plastics in the cells of living plants will reduce this cost even further. Hence, agricultural crops are regarded as a promising low-cost alternative for the production of PHAs on a large scale (Snell and Peoples, 2002). High-level production (approximately 40% of dry weight) of PHB has been achieved in plants by introducing three *R. eutropha* genes encoding a β -ketothiolase (*phaA*), an acetoacetyl-CoA reductase (*phaB*), and a PHA synthase (*phaC*) (Bohmert *et al.*, 2000). The success in the production of PHB in sugarcane (Petrasovits *et al.*, 2007; Purnell *et al.*, 2007) indicates that other biopolymers can be produced in sugarcane.

3.2.5 Natural products/pharmaceuticals/proteins

There are opportunities to engineer the metabolism of sugarcane to increase production of natural products (Dixon, 2005). For instance, the most functionally and structurally diverse group of plant metabolites is the isoprenoids (also called terpenoids). These diverse compounds have a commercial value as flavors, pigments, polymers, or drugs (reviewed in Rodríguez-Concepción, 2006; Withers and Keasling, 2007). There are other good reasons to learn how to manipulate and/or regulate isoprenoid production in sugarcane, as they play primary roles in respiration, photosynthesis, and regulation of growth and

development. As secondary metabolites, they function in protecting plants against herbivores and pathogens, attract pollinators and seed dispersing animals, and influence competition among plant species (Rodríguez-Concepción, 2006).

As described in Section 2, some work has already been done to engineer sugarcane to produce pharmaceuticals and high-value and bulk proteins. Monoclonal antibodies (mAbs) are one class of pharmaceutical proteins with considerable potential for production in plants. The estimated market size for mAbs is expected to exceed US \$20 billion per year (Reichert *et al.*, 2005). Both IgG- and IgA-type antibodies have been successfully produced in plants (Giritch *et al.*, 2006). Recently a new transient expression technology, “magniffection”, for production of gram quantities (0.5 g kg^{-1} fresh leaf biomass) of mAbs in plant cells was reported (Giritch *et al.*, 2006). The time from gene delivery to production of fully assembled mAb is 14 days and to production of gram quantities is 14–20 days, making it the fastest production platform available (Hiatt and Pauly, 2006). This system uses vectors from two different noncompeting plant viruses, one for producing the heavy chain and one for the light chain, and transfers them to the plant via *Agrobacterium*-mediated transformation.

One area that may hold promise is the production of industrial enzymes. Future biorefineries are going to require a host of enzymes for converting base material into the raw materials required by the manufacturing sector (Figure 6). Some of the enzymes can be produced directly in the sugarcane cells, and potentially at high concentrations once plastid transformation technologies are developed for sugarcane.

3.2.6 Drought tolerance

In most crops, past breeding efforts for drought tolerance have been hindered by the quantitative genetic basis of the trait and the poor understanding of the physiology (reviewed in Tuberosa and Salvi, 2006). For breeders, it is important to select genotypes that are able to optimize water uptake and water use efficiencies and minimize damage incurred by drought stress.

This ultimately will lead to maximized yields. In an effort to select for improved drought tolerance, breeders have identified a myriad of morphophysiological QTLs acting from the cellular to the whole crop level (reviewed in Tuberosa and Salvi, 2006). However, given the genetic complexity of sugarcane, a marker-assisted selection approach for a myriad of improved drought tolerance QTLs would appear a very difficult proposition. An alternative way forward would be to make use of the knowledge gained in simpler systems and identify candidate genes to test in sugarcane. Candidate genes for drought tolerance have been divided into three broad areas including: (i) functional proteins such as enzymes involved in osmotically active compounds, transporters, chaperones, and reactive oxygen species (ROS) scavengers; (ii) transcription factors involved in the plant response to drought stress; and (iii) signaling factors upstream of transcription factors, including protein kinases and proteins involved in phospholipid metabolism, calcium sensing, and protein degradation (reviewed in Umezawa *et al.*, 2006; Valliyodan and Nguyen, 2006). Most of these validation studies have been undertaken with model plants in laboratory or glasshouse conditions. An important next step is to trial these approaches in crop species under field conditions.

In other plants, a variety of transgenes have been used to improve drought tolerance. Examples include, the barley *HVA1* gene (Fu *et al.*, 2006), manganese superoxide dismutase (Wang *et al.*, 2005c), regulator of G-protein signalling protein (RGS) (Chen *et al.*, 2006), and aldehyde dehydrogenase (Rodrigues *et al.*, 2006). Perhaps the most widely and successfully used genes have been the *CBF/DREB* group of transcription factors (Agarwal *et al.*, 2006). *CBF/DREB* is a class of transcription factors that binds to drought responsive *cis*-acting elements and is important in regulating gene expression in response to drought, high salt, and cold stress (Yamaguchi-Shinozaki and Shinozaki, 1994). The ability of *CBF* to enhance tolerance to cold stress (Jaglo-Ottosen *et al.*, 1998) and drought stress (Haake *et al.*, 2002) was first demonstrated in *Arabidopsis* and has subsequently been shown to enhance tolerance to abiotic stress in commercially important crops such as tomato (Hsieh *et al.*, 2002; Lee *et al.*, 2003b) and rice (Ito *et al.*, 2006).

Sugarcane lines have been produced containing a gene encoding drought-induced *Arabidopsis* transcription factor, *AtCBF4* (McQualter and Dookun, 2007). Under glasshouse conditions, plants containing *AtCBF4* driven by the constitutive maize polyubiquitin promoter showed growth retardation. Research is now focused on using drought-inducible promoters to reduce this negative phenotype.

As mentioned in Section 2, trehalose has already been used to engineer drought tolerance in sugarcane (Zhang *et al.*, 2006). There are also a number of other potential osmoprotectants that can be trialed in sugarcane. These include betaines, the amino acids proline and ectoine, and polyols mannitol and sorbitol (Rontein *et al.*, 2002). As mentioned above, transgenic sugarcane plants have been generated that produce sorbitol (Chong *et al.*, 2007) and are being progressed to field trials.

3.2.7 Disease and insect resistance

Estimates of the cost of control and loss due to major sugarcane pests and diseases in Australia are in the order of 10% of the total value of the sugarcane crop (McLeod *et al.*, 1999). Of these diseases and pests, soil-borne pathogens caused an estimated 75% of the losses, followed by canegrubs (larvae of Scarabaeidae) (10%), ratoon stunting disease (caused by *Lxx.*) (6%), and brown rust (caused by *Puccinia melanocephala* H&P Sydow) (3%). Occasionally, disease outbreaks due to the breakdown of disease resistance occur, which can result in much larger losses (40% reduction in cane yield in some sugarcane growing regions in Australia as a result of orange rust (Magarey *et al.*, 2001)). Diseases such as ratoon stunting disease, pineapple disease, and red rot can be largely controlled by farm hygiene processes such as sterilizing cutting and harvesting equipment and by the use of fungicides (Magarey, 2005). However, for most sugarcane diseases, genetic resistance is the most effective method of disease management and sugarcane breeders have done an excellent job in controlling diseases through the use of resistant cultivars.

In the future, it is possible that resistance may break down, and aside from introgressing wild germplasm with novel resistance (if this exists in the gene pool), alternative strategies will be

needed. Such approaches should provide durable resistance, preferably against a range of pathogens. Genetic engineering has the potential to achieve this through inserting carefully selected and possibly multiple genes as transgenes that (i) confer durable broad-spectrum resistance; (ii) are safe for all other organisms; and (iii) cause no yield penalty in the plant (Gurr and Rushton, 2005a). Such approaches have been successful with chewing insect pests, where expression of an insecticidal protein from *B. thuringiensis* has lead to increased yields and reductions in insecticide applications in cotton, soybean and maize (reviewed Gurr and Rushton, 2005b). Recent work in transgenic sugarcane has also indicated that *Bt* proteins are active against stem borers (Weng *et al.*, 2006). Strategies for resistance to sugarcane viruses have also met with some success through RNAi-mediated approaches against the single-stranded RNA SCMV and the double-stranded RNA FDV (Joyce *et al.*, 1997; Ingelbrecht *et al.*, 1999; McQualter *et al.*, 2004). However, similar successes for durable resistance to bacteria and fungi have not been forthcoming (reviewed Gurr and Rushton, 2005a).

Strategies have attempted to boost disease resistance through constitutive overexpression of defense components or single-antimicrobial proteins and have led to a variety of problems including poor quality plants, yield reduction, poor efficacy, or short durability (reviewed by Gurr and Rushton, 2005b). New strategies are being explored to overcome these problems. For example, a two-component system has been suggested, where the manipulation of “master switch” genes (such as kinases and transcription factors), which regulate entire signaling pathways are coupled with pathogen-inducible promoters to precisely regulate spatial and temporal gene expression (Yamamizo *et al.*, 2006). Other strategies have come from the genomics field, for instance the complete genome of *Lxx*, has been fully sequenced (Monteiro-Vitorello *et al.*, 2004) and opportunities now exist to try and control or even eradicate this costly pathogen from sugarcane (Brumbley *et al.*, 2006a). Global transcriptional analysis of plant pathogen interactions has also revealed pivotal steps in defense responses that may be targeted in future strategies (Zabala *et al.*, 2006). Further disease control strategies may involve pyramiding two or more antimicrobial

proteins to improve efficacy and durability of resistance.

Work on transcription factors in other plants may offer some future promise for sugarcane. WRKY proteins are a large family of transcription factors that mainly participate in plant biotic-stress responses (Liu *et al.*, 2007). They share a DNA-binding domain consisting of about 60 amino acids, as well as additional conserved features that distinguish individual subgroups (Eulgem *et al.*, 2000). WRKY transcription factors can bind to domains in the promoters of pathogen-response genes, termed W boxes (TTGACC) (Maeo *et al.*, 2001; Turck *et al.*, 2004). While overexpression of some WRKY transcription factors has resulted in enhanced resistance to various bacterial or fungal pathogens, others can suppress the expression of defense-related genes.

Chen and Chen (2002) transformed *Arabidopsis* plants with AtWRKY18 under control of the CaMV 35S promoter. Transgenic AtWRKY18 plants showed marked increase in the expression of pathogenesis-related genes and resistance to the bacterial pathogen *Pseudomonas syringae*. Thus, AtWRKY18 was shown to positively modulate defense-related gene expression and disease resistance. Conversely, AtWRKY7 transcription factor plays a negative role in defense responses to *P. syringae*. Kim *et al.* (2006) studied the biological function of the *Arabidopsis* WRKY7 gene in both loss-of-function T-DNA insertion and RNAi mutants and gain-of-function transgenic overexpression plants. The T-DNA insertion and RNAi mutant plants displayed enhanced resistance to a virulent strain of the bacterial pathogen *P. syringae*. Transgenic *Arabidopsis* plants that constitutively overexpress WRKY7 showed reduced expression of defense-related genes, including *PR1* and developed more severe disease symptoms than wild-type plants.

A large number of WRKY genes have also been identified in rice. Ryu *et al.* (2006) isolated WRKY transcription factors whose expressions were altered upon attack of the fungal pathogen *Magnaporthe grisea*, the causal agent of rice blast disease. Among 45 tested genes, the expression of 15 was increased in interactions between rice and *M. grisea*. Twelve of this subset of genes were also differentially regulated in rice plants infected with the bacterial pathogen *X. oryzae* pv. *oryzae* (X.o.o) 13751. Ryu *et al.* (2006) suggested that a

large number of *WRKY* DNA-binding proteins are involved in the transcriptional activation of defense-related genes in response to rice pathogens. Several pathogenesis-related genes were induced in *OsWRKY03*-overexpressing transgenic rice plants (Liu *et al.*, 2005), while overexpression of the *OsWRKY71* gene in rice resulted in enhanced resistance to virulent bacterial pathogen X.o.o. 13751 (Liu *et al.*, 2007).

As mentioned above, there are extensive databases at Harvard University covering plants, animals, fungi, and protists. The TIGR has the Comprehensive Microbial Resource (CMR) (<http://cmr.tigr.org/>). Within this free database are the complete genome sequences of 354 bacteria, including a number of plant pathogens. There is currently an effort to reduce the cost of sequencing the genome of a bacterium to under US \$1000, and this is likely to be accomplished in the next decade. When that happens, databases such as the one at TIGR and Harvard University will contain the genomes of the vast majority of plant pathogenic bacterium, fungi, viruses, and mycoplasmas as well as the insect pests that plague agriculture around the world. Studying the plant-microbe or plant-insect interaction in the future will be done at the whole genome, transcriptome, proteome, and metabolome level of the host, and the pests and pathogens.

3.2.8 Modified plant architecture and development

Shoot architecture describes the way in which the aerial portions of plants develop. It is influenced predominantly by shoot branching and plant height. Agronomic interest in shoot architecture has stemmed mainly from potential yield increases associated with some plant ideotypes. For instance, height reduction in cereals has led to yield improvement through decreased lodging, whilst reduction of tillering in some cereals may lead to yield improvements (Duggan *et al.*, 2005).

In sugarcane, complex interrelationships exist among shoot architecture, stalk characteristics, cane yield, and sucrose content (Milligan *et al.*, 1990). For instance, two elements of sugarcane shoot architecture, the number and weight of stalks, determine cane yield. However, stalk number and stalk weight are negatively correlated

and, thus, selecting for either trait alone may not result in increased cane yield (Milligan *et al.*, 1990; Bell *et al.*, 2004; Bell and Garside, 2005). Stalk architecture can also affect cane sucrose content. Taller canes are more likely to lodge under wet or windy conditions, causing a reduction in sucrose content (Singh *et al.*, 2000, 2002), whilst clones with a propensity for producing the low-sugar-content suckers reduce the sucrose content of the harvested crop (Bonnett *et al.*, 2001, 2004a).

Recently, several genes controlling shoot architecture traits, such as stalk height and tillering, have been described (McSteen and Leyser, 2005; Wang and Li, 2006). There is now an opportunity to test how modifying stalk characteristics with these genes will affect cane yield and sucrose content in the same sugarcane cultivar without altering other genes in the plant. Three areas of research have been identified which may lead to increased yields, accelerated biomass production, controlling lodging, and reducing suckering. The genes that are currently being used to address these research areas are the *TB1* gene, which has been shown to affect tiller number and stem width in maize and rice (Doebly *et al.*, 1997; Takeda *et al.*, 2003), the *MAX3* gene that affects axillary meristem outgrowth in rice (Zou *et al.*, 2006) and *Arabidopsis* (Booker *et al.*, 2004), and several gibberellin oxidases that regulate stem elongation (Hedden and Phillips, 2000). Transgenic sugarcane plants have been produced and are currently undergoing extensive glasshouse and field trials to test what effects these genes produce (Pribil *et al.*, 2007).

3.2.9 Other products

3.2.9.1 Products from phenylpropanoid pathway

The phenylpropanoid pathway is an important plant-metabolic pathway involved in the synthesis of defense-related secondary metabolites and in the production of structural-reinforcement polymers, including lignin (Matsuda *et al.*, 2005). The phenylpropanoid pathway produces a large and diverse array of chemicals, both as intermediates and as end products. Enzyme levels in the phenylpropanoid pathway are tightly regulated (Ni *et al.*, 1996) and flux into the phenylpropanoid pathway is controlled, at least

in part, via feedback regulation of PAL, sensed through production of cinnamic acid (Blount *et al.*, 2000). PAL is regulated both transcriptionally and post-transcriptionally (Dixon and Paiva, 1995). This is an important consideration when attempting to over-produce intermediates or end products of the phenylpropanoid pathway by attempting to increase flux through the pathway.

The MYB and bHLH group of transcription factors are involved in regulation of the phenylpropanoid, anthocyanin, and flavonoid pathways. Various branches of phenylpropanoid metabolism are regulated by branch-specific activating and repressing MYB transcription factors, sometimes dependent on bHLH (Vom Endt *et al.*, 2002). MYB transcription factors have been identified, which regulate the first steps in the phenylpropanoid pathway.

Maeda *et al.* (2005) isolated carrot cDNA encoding the R2R3 type of MYB transcription factor (*DcMYB1*) and found that transient expression of *DcMYB1* could up-regulate the PAL gene (*DcPAL1*) promoter activity in carrot protoplasts. Induction of *DcPAL1* expression occurred 1 h after *DcMYB1* expression in carrot protoplasts under various stress treatments, including treatment with a fungal elicitor and by ultraviolet-B (UV-B) irradiation. When *DcMYB1* expression was repressed using RNA interference, up-regulation of *DcPAL1* expression was negated. It was suggested that *DcMYB1* is the main regulatory factor of the *DcPAL1* gene that responds to environmental cues.

Jin *et al.* (2000) produced an *Arabidopsis* line that was a mutant of the R2R3 MYB gene, *AtMYB4*, which showed enhanced levels of sinapate esters in its leaves. The mutant line was more tolerant of UV-B irradiation than the wild type. The increase in sinapate ester accumulation in the mutant was associated with an enhanced expression of the gene encoding cinnamate 4-hydroxylase (C4H), which appears to be the principal target of *AtMYB4* and an effective rate limiting step in the synthesis of phenylpropanoids. *AtMYB4* works as a repressor of target gene expression and includes a repression domain. It belongs to a novel group of plant R2R3 MYB proteins involved in transcriptional silencing (Jin *et al.*, 2000).

The ability to significantly modify flux through the phenylpropanoid pathway would be beneficial

for research targeted at producing novel compounds in sugarcane. McQualter *et al.* (2005) evaluated sugarcane as a production platform for p-hydroxybenzoic acid using 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) from *P. fluorescens* (Gasson *et al.*, 1998) that provides a one-enzyme pathway from a naturally occurring plant intermediate (Mayer *et al.*, 2001). The substrate for HCHL is 4-hydroxycinnamoyl-CoA (a cytosolic phenylpropanoid intermediate). HCHL elevates p-hydroxybenzoic acid levels in plants (Mayer *et al.*, 2001), and its expression in transgenic sugarcane led to the accumulation of p-hydroxybenzoic acid glucosyl conjugates to as high as 7.3% and 1.5% DW in leaf and stem tissue, respectively, with no discernible phenotypic abnormalities (McQualter *et al.*, 2005). However, as a result of diverting carbon away from the phenylpropanoid pathway, there was a severe reduction in leaf chlorogenic acid, subtle changes in lignin composition, as revealed by phloroglucinol staining, and an apparent compensatory up-regulation of PAL. Product accumulation in the leaves at the highest level of gene expression obtained in the study was clearly substrate limited. Increasing flux through the phenylpropanoid pathway in sugarcane by increasing the expression of the *PAL* gene (the first enzyme in the phenylpropanoid pathway) using a sugarcane ortholog of *DcMYB1* could help overcome substrate limitation problems and enable economically viable amounts of p-hydroxybenzoic acid to be produced in transgenic sugarcane. Additionally, silencing the repressor function of a sugarcane ortholog of the *AtMYB4* transcription factor could increase flux through the pathway at the C4H rate-limiting step (the second step in the phenylpropanoid pathway).

3.2.9.2 Flavonoids

Flavonoids form a large group of polyphenolic compounds produced in plants that encompass the wide variety of phytochemicals present in the human diet. Basic research, animal model, and human studies suggest flavonoid intake may reduce the risk of several age-related chronic diseases (Graf *et al.*, 2005). Duarte-Almeida *et al.* (2006) identified phenolic compounds in sugarcane (*S. officinarum*) juice showing the predominance of flavones (apigenin, luteolin,

and tricin derivatives) among flavonoids, and of hydroxycinnamic, caffeic, and sinapic acids, among phenolic acids, representing a total content of around 160 mg l⁻¹. A tricin derivative was present in the highest proportion (>10% of the total). The phenolic extract obtained from sugarcane juice showed a protective effect against *in vivo* MeHgCl intoxication and potent inhibition of *ex vivo* lipoperoxidation of rat brain homogenates, indicating a potential use for beneficial health effects and/or therapeutic applications.

A transgenic approach to enhancing flavonol content in sugarcane has not yet been attempted. However, the maize transcription factor genes *LC* and *CI* have been expressed in transgenic tomatoes, resulting in increased flavonoid levels (Bovy *et al.*, 2002). Tomato fruit normally contain small amounts of flavonoids in the peel, but co-expression of the *LC* and *CI* genes enabled up-regulation of the flavonoid pathway in the flesh of the tomato fruit, a tissue that does not normally produce flavonoids. This resulted in a strong accumulation of flavonols and a modest increase in flavanones.

3.3 Economic Opportunities, Industrial Perspectives, and Political and Economic Consequences

Around the world a paradigm shift is being forced by the concern that we have reached the end of the age of inexpensive crude oil. This is being caused by a shortage of crude oil brought on by (i) the rapid industrial development of China and India (Goodstein, 2004); (ii) the political instability in the major crude oil producing regions of the world; and (iii) the fact that we are either at or very close to the peak for world oil production/reserves (Hubbert, 1956). The process of weaning the world off crude oil has begun, and the transition from a petroleum-based economy to a bio-based economy is underway. Nowhere is this more apparent than in the legislation being enacted in the European Union (EU). Manufacturers in the global automotive, packaging, electronics, and chemical industries will be responsible for the end-of-life processing of their products sold or produced in the EU. This means it will become increasingly more expensive to dispose of

manufactured goods, forcing companies to recycle or reuse (End-of-Life). For instance, 100% of all electronics, most parts of automobiles, and all the packaging sold in the EU will have to be reusable or recyclable.

Clear “Vision Statements” and “Roadmaps” of the EU and the US R&D funding bodies are now in place (Europa, Plants for the future; EPOBIO; [http://www.suschem.org/content.php?_document\[ID\]=2049&pageId=2491](http://www.suschem.org/content.php?_document[ID]=2049&pageId=2491); United States Department of Energy Genomics, GTL roadmap; <http://www.eere.energy.gov/biomass/publications.html#vision>). The corporate world is also investing heavily with the largest focus on biofuels. Biorefineries are being constructed worldwide, primarily to produce ethanol. Additional investments are being made in the production of fine chemicals using fermentation technologies. The production of diverse biopolymers is an area with considerable attention. Examples include: (i) Cargill’s Natureworks, bioplastics made from polylactic acid; (ii) Dupont’s Sorona™ (http://www2.dupont.com/Sorona/en_US), the major component, 1,3-propanediol, is being produced in a joint venture with Tate & Lyle; and (iii) Metabolix’s Natural Plastics based on PHA, which will be produced in a joint venture with Archer Daniels Midland (<http://www.metabolix.com/index.html>). All of these polymers begin with the simple sugars found in corn starch.

What does all this have to do with transgenic sugarcane? The Visions and Roadmaps from the EU and US, the investment in R&D by governments around the world, and by corporations in new bio-based businesses can only mean opportunity (European Union Framework Seven, United States Farm Bill, DuPont, Toyota, Cargill, Abengoa). To make sugarcane attractive for investment, such as is happening in Brazil, the technologies to engineer sugarcane must be advanced. In the future, sugarcane could be genetically engineered to grow more sugar and biomass per hectare, use less water and fertilizer, be resistant to a range of pests and diseases, produce its own bioherbicide to out-compete weeds, be able to grow on marginal land, and be able to grow in drier and cooler climates. The process of harvesting sugarcane could trigger a cascade of enzymes that would start degrading the chains of sugars that make up cellulose and hemicellulose, and modify the lignin, making the sugarcane easier

to mill. This would help produce a larger pool of sugars readily accessible for the biorefineries linked to the sugarcane mills. Sugarcane could also be engineered so that a range of products other than sugars could be produced in its cells while it is growing in the field, adding additional value and further diversifying its product base.

Much of the basic research to accomplish this is already underway. The core lesson from Brazil is that it takes years or even decades to develop the knowledge base, the core skills, and the infrastructure for a bio-based economy (Lorentzen, 2006; Velho and Velho, 2006). The economic prosperity that Brazil is now enjoying because of sugarcane and its byproducts has taken them almost 40 years to develop. This prosperity gives them both the money to invest and a desire to continue this growth cycle. This creates opportunities to commercialize GM sugarcane with improved properties.

The most advanced programs for commercial release of transgenic sugarcane are in Brazil, South Africa, USA, India, China, The Philippines, and Australia. The Centro de Tecnologia Canavieira (<http://www.ctc.com.br>) recently announced it would be field-testing three new transgenic sugarcane varieties with 15% increase in sugar yields (<http://www.inovacao.unicamp.br/english/report/news-sugaralcohol060522.shtml>).

The economic prosperity of the Brazilian sugarcane industry is also being mirrored in the US maize industry. The growing global bioeconomy provides opportunity for any industry that is willing to invest in the infrastructure to produce large quantities of inexpensive sugars. Sugarcane industries are particularly well suited because they already have in place the mechanism to haul very large quantities of biomass from where it is grown to where it is processed. The sugars locked up in this biomass have the interest of governments and industries alike. Many believe that the sugars in the cellulose and hemicellulosic biomass are the replacement for crude oil. The United States and the EU see the tools of the biotechnology revolution as fundamental to this process.

Biology and engineering are merging at the computer interface through the fields of genomics (Sanford *et al.*, 2002; Morgante, 2006), transcriptomics, proteomics (Heazlewood and Millar, 2003), metabolomics (Oksman-Caldentey and Saito, 2005; Rockfort, 2005), and fluxomics

(Sanford *et al.*, 2002), generating enormous data sets that are allowing for the development of systems biology (Lee *et al.*, 2005; Barrett *et al.*, 2006; Joyce and Palsson, 2006). Using these databases, experiments are designed and tested on a computer before being trialed in a laboratory (Lee *et al.*, 2005). One person working in the corner of one laboratory can accomplish more in a few months than was previously accomplished by research teams working throughout their entire careers. These powerful tools could not have come at a better time, as the rising demand for crude oil starts to impact on price, which potentially could play havoc with the world's economies (Goodstein, 2004).

3.3.1 Human health and environment

There is no reason to assume that genetic engineering has a greater potential to harm human health or the environment than any techniques used in classical breeding (Miller, 2007). If there is a lesson we have learned from the enormous experience of commercially growing GM crops on over 1 billion acres (400 million hectares) around the world for over two decades is that if the trait(s) encoded by the transgenes are safe, then there are more than sufficient safeguards in traditional breeding programs to ensure that the GM-derived crop is safe (Bradford *et al.*, 2005).

However, there is still considerable resistance to food derived from GM plants, even though there is no scientific basis for this concern (Miller, 2007). Because of this resistance, the large buyers of raw sugar have been requiring guarantees from the suppliers that the sugar supply is not derived from GM plants. This has created a wait-and-see approach, where sugarcane biotech research and development is ongoing but no one wants to be the first into the market. However, it is likely that sugar from sugarbeets engineered with herbicide and pest resistance traits will enter the market first and that will hopefully open the door for sugarcane. If GM sugarcane lines with doubled sugar production (Wu and Birch, 2007) perform well in field trials, pilot-scale production will most likely be trialed.

Refined white sugar (>99.98% sucrose) is the most chemically pure food produced from agriculture, thus, reducing any foundation for

concern about foreign genes or gene products in this food (Birch and Maretzki, 1993). Taylor *et al.* (1999) analyzed the various products during the crystallization process of sugar from sugarcane juice for the presence of transgene DNA. Their results showed that no PCR amplifiable DNA was present in sugar crystallized from SCMV transgenic plants.

However, when sugarcane is engineered as a biofactory to produce fine chemicals or pharmaceuticals, it is critical that clear safeguards are in place to ensure that there is no conceivable harm to public health or the natural environment. Safety evaluations will have to be done on a case-by-case basis. For instance, sugarcane producing bioplastic such as the pHBA and PHAs discussed in this chapter could still be used for production of sugars for human consumption. Animals already make pHBA and have the capacity to digest it (McQualter *et al.*, 2005), and PHAs have a 40-year history of safety in a variety of medical applications, especially because of biodegradability and biocompatibility. PHAs have been used for surgical sutures and wound dressings. There is ongoing research for the commercialization of PHA-based products for tissue engineering and for controlled release systems, and for bone repair (Zhijiang, 2006). Because these biopolymers in nature are used as a carbon sinks, they are fully biodegradable. So producing them in sugarcane should not be dangerous to human health or the environment.

3.4 Conclusions

Since the first successful report of transgenic sugarcane plants less than 20 years ago, biotechnology has advanced rapidly and been adopted by sugar industries and research organizations worldwide. Research into a range of input traits such as pest and disease resistance, sugar quality and shoot architecture, and output traits such as increased sucrose levels, alternative sugars, biopolymers, pharmaceuticals, and bulk proteins are ongoing. The future looks bright for sugarcane, as market forces drive the development of the bioeconomy, creating opportunities for sugar industry diversification and new partnerships with other global and local industries. A combination of factors, including but not limited to, global warming,

instability in the Middle East, concern over long term oil supplies, and the industrialization of China and India, have caused the world to look for alternatives to petroleum for fuel, energy, and fine chemicals. Governments have made plans, drawn up roadmaps, and set priorities for R&D funding to create ways to shift from a disproportional dependence on the nonrenewable resource petroleum. They are creating new industries and whole new economies based on renewable sources. The sugar derived from lignocellulosic biomass is going to be the renewable feedstock for the bioeconomy. The infrastructure is already being developed. Biorefineries are being constructed all over the United States, Europe and Brazil. Ethanol and a range of biopolymers are being produced in these biorefineries. In the United States, maize is the feedstock; in Brazil it is sugarcane. These nations all have R&D programs underway to switch over to biomass.

Developing new cultivars of sugarcane that will produce more tonnage, use less water and fertilizer, that will not fall down in a storm, can tolerate floods or drought, grow in the heat or the cold, are resistant to pests and diseases, can tolerate salt, can fight off weeds, and supply the majority of the world's needs for sucrose, and other sugars, bioplastics, and a host of other fine chemicals, reduce greenhouse gas, and clean up the environment is going to require considerable genetic manipulation. Systems biotechnology will help in the design strategies to manipulate the metabolic pathways of sugarcane. However, the ability to transform sugarcane with genes and/or metabolic pathways will be instrumental in helping sugarcane industries become drivers of the bioeconomy.

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FURTHER READING

Sugar Beet

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1. INTRODUCTION

1.1 Botanical Description: Distribution, Taxonomy, and Cytology

Beta vulgaris is an herbaceous, allogamous dicotyledon belonging to the Chenopodiaceae family. Annual and biennial genotypes of *Beta* exist; however, cultivated sugar beet is biennial. In its vegetative state, sugar beet produces a close spiral of triangular leaves with conspicuous midribs and petioles in a rosette on a compacted stem (crown) and a large fleshy tap root that is harvested for its sugar (sucrose). Reproduction is initiated by a cold vernalization period followed by long photoperiods (Smith, 1980; Klotz, 2005; Milford, 2006). The reproductive phase begins with the formation of a rosette similar to that described for the vegetative phase followed by the production of an aerial stem with flowering branches. Sugar beet has perfect flowers consisting of a tricarpellate pistil surrounded by five stamens and five narrow sepals. Flowers are sessile and occur singly or in clusters of two to seven. The ovary forms a fruit that contains a single seed. The ovaries of all flowers within a cluster are enclosed in a common receptacle. This produces a, so-called, multigerm seed. When flowers occur singly the fruit is commonly referred to as monogerm seed (Smith, 1987).

Sugar beet can be grown in many environments; however, most commercial production is between 30° and 60° North latitude. It is grown on a wide range of soil types as a summer crop in maritime, prairie, and semicontinental climates, as a winter or summer crop in Mediterranean environments, and under irrigation in semi-arid and arid environments. Sugar beet, a halophyte, exhibits considerable tolerance to soils with high salinity concentrations (Draycott and Christenson, 2003).

It appears that the genus *Beta* originated in middle eastern and eastern Mediterranean countries. Wild forms are found as far east as China and as far west as California (USA). The earliest records of beet domestication indicate that it was cultivated as a leafy vegetable in the Tigris and Euphrates river valleys between 2000 and 1500 BCE (Doney, 1995). The number of species within the genus depends upon the taxonomic treatment, but there is general agreement that *Beta* is comprised of four sections; *Beta*, *Corollinae*, *Procumbentes*, and *Nanae* (Coons, 1954, 1975; Smith, 1987; Jassem, 1991; Doney, 1995).

Section *Beta* is the most widely distributed and includes all cultivated forms of *Beta*. A recent classification scheme divides the section into three species: *B. vulgaris* L., *B. patula* Ait., and *B. macrocarpa* Guss (Letschert *et al.*, 1994). *B. vulgaris* is comprised of three subspecies. Subspecies *vulgaris* includes all cultivated forms

and is further divided into four cultivar groups (cultua): leaf beet, garden beet, fodder beet, and sugar beet. Subspecies *maritima* and *adanensis* are wild forms of *B. vulgaris*. *B. patula* is an isolated species, occurring only on a small island of Madeira. Within this section, *B. macrocarpa* is the most distantly related to sugar beet (Doney, 1995). All species of section Beta produce fertile offspring when intercrossed. Most members of this section are outcrossing due to self-incompatibility.

Section Corollinae species are found in areas of the Balkan Peninsula, Turkey, Transcaucasia, and Iran, generally at higher elevations than species of section Beta. This section is comprised of five species and includes diploid, autotetraploid, allotetraploid, and allohexaploid forms of which some are apomitic (Ford-Lloyd, 2005). All species are characterized by a corollalike perianth. There is little homology between chromosomes of sugar beet and species of Corollinae, and the interspecific hybrids are almost always sterile (Smith, 1987). Polyploid sugar beet parents and bridging hosts can sometimes be useful in introgressing traits from species in this section into sugar beet (Coons, 1975; Hadley and Openshaw, 1980).

Three species comprise the Procumbentes section. Some evidence suggests that two of these, *B. procumbens* Sm. and *B. webbiana* Moq., are not distinct species and the third species, *B. patellaris* Moq., is an autotetraploid derived from the *B. procumbens* morphological form (Ford-Lloyd, 2005; Skaracis, 2005a). The Procumbentes section is characterized by its annual growth habit and monogerm seed. Its distribution is limited to the Canary Islands. Species of this section usually produce sterile offspring in interspecific hybrids with sugar beet, but bridging hosts and polyploid sugar beet lines may facilitate the transfer of desirable traits (Coons, 1975; Hadley and Openshaw, 1980). Applied breeders are especially interested in this section because of the beet cyst nematode (*Heterodera schachtii* Schm.) resistance observed in some populations of Procumbentes (Coons, 1975; Halldén *et al.*, 1997; Skaracis, 2005b; Panella and Lewellen, 2006).

There is only one species, *B. nana* Boiss. et Heldr., in the Nanae section. *B. nana* is a diploid, characterized by small plants with rosettes of leaves less than 10 cm across (Smith, 1980). It is endemic to a few high-elevation environments in

Greece (Ford-Lloyd, 2005). No crosses between *B. nana* and sugar beet have been reported.

Sugar beet is a true diploid species with a haploid chromosome number of nine. The small size of the chromosomes, the absence of differentiating characteristics, and the unavailability of genetic stocks with chromosome aberrations made identification of individual chromosomes difficult without recent technology (Skaracis, 2005a). A complete set of primary trisomics (Romagosa *et al.*, 1986, 1987; Schondelmaier and Jung, 1997) is available for assigning genes to chromosomes and establishing linkage groups. Furthermore, a full set of *B. procumbens* and *B. webbiana* monosomic addition lines and eight *B. corolliflora* addition lines have been identified (Skaracis, 2005a). Colchicine has been used for at least 50 years to produce tetraploid sugar beet (Savitsky, 1966). When tetraploid pollinators are crossed with a diploid parental line, approximately 10% aneuploids will be present in the resulting commercial triploid hybrid seed. The advent of molecular markers has greatly expanded knowledge of linkage relationships in sugar beet (Pillen *et al.*, 1993; Barzen *et al.*, 1995; Halldén *et al.*, 1996; Schondelmaier and Jung, 1997; Rae *et al.*, 2000).

1.2 Economic Importance

Sugar beet is a significant industrial crop of the temperate zone, the worldwide production of which exceeded 240 million tons in 2000 (FAO, 2000). Worldwide, sugar from sugar beet provides about a third of all sugar consumed. Used as a sweetener in foods, beverages, and pharmaceuticals, sugar makes up about 11% of all food consumed in the world and its consumption has been growing at 2% per year, the same rate as the increase in world population (Wozniak, 1999). World acreage of sugar beet harvested is currently greater than 7.7 million hectares and is expected to increase to meet world demands. Market value of this crop is in the billions of US dollars. About half of all the sugar consumed in the United States comes from sugar beet roots. Over 26 million tons of sugar beet valued at over \$1.2 billion is produced each year in the United States on more than 0.6 million hectares. Due to its high productivity, sugar beet is increasingly being scrutinized not only as a source of sugar, but also

as a possible “green bioreactor”, i.e., for synthesis and accumulation of new metabolites in roots (Monger *et al.*, 1995; Sévenier *et al.*, 1998; Menzel *et al.*, 2003). The increase in world population taken together with the increased demand for alternative energy sources suggests a growing need for increased production of sugar beet not only as a source of food but also as a source of biofuels. This anticipated demand provides the motivation for development of innovative approaches, such as genetic modification through engineering to augment and complement classical breeding techniques.

1.3 Traditional Breeding

The transformation of fodder beet into sugar beet through selection was the basis for the early survival of the beet sugar industry. Since that time traditional plant breeding efforts have made notable contributions to reducing labor costs through the incorporation of the monogerm trait into commercial varieties, reducing the impact of diseases and pests on the crop, and increased productivity through the improvement of elite parental lines and the development of hybrid varieties.

Utilization of commercial sugar beet hybrids has allowed breeders to capitalize on the general occurrence of nonadditive genetic effects for root yield. Pollen sterility in the lines used as females depends upon a genetic-cytoplasmic interaction (cytoplasmic male sterility; CMS). Male-sterile plants have a sterile cytoplasm (S) and homozygous recessive alleles (xxzz) at two loci that control pollen development (S: xxzz). CMS lines require an equivalent pollen-fertile maintainer line (referred to as an O-type line in sugar beet) that has normal (N) cytoplasm and is recessive at the same two loci (N: xxzz). The development of CMS parental lines and their corresponding maintainer lines is facilitated by the introduction of a dominant gene that results in almost obligate self-fertility. Genetic male sterility, controlled by a single nuclear gene, is often introduced into O-type breeding populations to allow crossing among otherwise self-fertile plants (Smith, 1987; Biancardi *et al.*, 2005; Bosemark, 2006). Because monogerm seed is required in commercial production, all female parental

lines must be homozygous for the recessive gene that produces the monogerm phenotype. Parental lines to be used as pollinators in the production of commercial hybrids are almost always multigerm, because they produce more pollen than comparable monogerm plants. The genotype of pollinator parental lines with regard to the monogerm–multigerm trait or pollen-fertility restoration genes is not important.

A significant portion of the increase in sugar beet productivity over years can be attributed to improved varieties, and sustained profitability of the industry requires a continued emphasis on yield enhancement. Increasing sucrose concentration enhances processing efficiency but root yield (weight per area) is often the major component influencing sucrose yield per hectare. An inverse relationship between root yield and sucrose concentration has made simultaneous improvement of both difficult (Campbell, 2002). Furthermore, the optimum balance between root yield and sucrose concentration may depend upon payment calculations that often include premiums for roots with relatively high sucrose concentration. Genetic variance for sucrose concentration is predominately additive. In contrast, nonadditive variance and specific combining ability are significant in determining root yield. In addition to sucrose concentration, sugar beet quality also takes into account naturally occurring compounds that impede sucrose crystallization during normal factory operations (Dutton and Huijbregts, 2006). Selecting for increased sucrose concentration usually results in a concurrent reduction in these undesirable compounds.

Host plant resistance to diseases and pests has contributed to sugar beet productivity and in some cases allowed the crop to thrive in areas where the diseases and pests could not be controlled economically by other means (Smith, 1987; Biancardi *et al.*, 2005; Bosemark, 2006). For many diseases, resistance is not simply inherited and is not complete (immunity). As a result, resistance is usually combined with other control strategies to minimize damage. Identifying sources of resistance and selecting resistant plants from segregating populations frequently entails the establishment of specialized nurseries and/or the development of dependable inoculation techniques. Wild relatives of sugar beet are a potential source of pest resistance genes (Lewellen, 1992; Panella and

Lewellen, 2006); however, their use is complicated by the extended time and effort required to transfer the desired resistance to otherwise adapted genotypes (Stander, 1993). In some cases the triploid hybrid produced by crossing a resistant tetraploid pollinator with a susceptible CMS line may be more resistant than the comparable diploid hybrid (Hecker and Ruppel, 1976).

Mass selection based upon phenotype has been effective in enhancing resistance to some diseases, increasing sucrose content, and selecting for desirable root shape. Half-sib or full-sib (pair crosses) selection is often used by breeders, especially when self-incompatibility precludes selfing. Including a CMS tester in a small tent with the two plants in a pair cross will provide seed for initial combining-ability tests. Reciprocal recurrent selection, a procedure used in other cross-pollinated crops, is not readily applied to sugar beet as it requires plants be selfed as well as crossed. Doney and Theurer (1978) outlined a method that overcomes these obstacles by incorporating genes for self-fertility and genetic male sterility into source populations. Gametophytic (pollen) screening has been suggested as a selection method (Smith and Moser, 1985). In some situations, vegetative propagation or cloning may be used to perpetuate genotypes that are difficult to maintain with seed production (Saunders, 1982; Mezei *et al.*, 1990; Tsai and Saunders, 1995).

1.4 Limitations of Conventional Breeding

Although sugar beet breeders often can rely on the experiences of hybrid breeders in other allogamous crops for guidance, sugar beet has a unique combination of complicating characteristics that must be considered in the implementation of an applied breeding program. Developing a productive commercial sugar beet hybrid requires more time and resources than most other field crops.

The biennial growth habit of sugar beet may lengthen the time required for a selection cycle, compared to annual crops, and possibly limits the size of a breeding program. Vernalization requires refrigeration facilities or nurseries that frequently must be located in environments at some distance from production areas. *BvFL1* (*Beta vulgaris* flowering locus 1), the homolog of *Arabidopsis*

FLC, a repressor of flowering, was recently characterized in sugar beet. During vernalization, the gene is down-regulated permitting flowering to occur in beet. Efforts are underway to create a transient system for knocking out *BvFL1* to stimulate flowering in the absence of vernalization; a tool that could speed up the breeding process in the future (Reeves *et al.*, 2007). Sugar beet is normally self-sterile. This can be a problem when selfing would be beneficial, especially in the identification of O-type individuals in populations from which candidates for CMS parental lines are to be selected (Campbell, 2000; Biancardi *et al.*, 2005; Bosemark, 2006). Self-sterility can be overcome by the introduction of a self-fertility gene; however, if intermating is desired within or between populations homozygous for the self-fertility gene it is customary to introduce genetic male sterility. In addition, to be commercially viable, CMS lines must be homozygous for the gene controlling the monogerm trait. These requirements for female parental lines complicate the introduction of traits related to adaptation and productivity.

The origin and subsequent development of sugar beet varieties suggest that the genetic base of the crop is less diverse than many open-pollinated crops. The wild relatives of sugar beet likely possess many characteristics, particularly resistance to diseases, nematodes, insects, and abiotic stresses that would be beneficial in sugar beet (Lewellen, 1992; Doney, 1995; Panella and Lewellen, 2006). However, populations derived from crosses between sugar beet and its wild relatives have many undesirable traits that must be eliminated by selection and backcrossing to sugar beet (Stander, 1993). Marker-assisted selection (Skaracis, 2005b) can be helpful in selecting for one or a few genes that control a particular trait of interest (Halldén *et al.*, 1997) but are of limited value in eliminating most of the undesirable characteristics in an interspecific hybrid population. Selection for resistance to specific pests becomes more complicated as the number of loci conditioning the resistance increases (Bosemark, 2006). Most of the economically important traits and resistance to numerous pests and diseases are either quantitative or conditioned by multiple genes (Biancardi *et al.*, 2005).

Multienvironment trials with candidate sugar beet hybrids are similar to those used in other crops but require more resources than many other

crops (Fauchère *et al.*, 2002). The volume of seed required for widespread trials generally entails production at a site with winter temperatures sufficient for vernalization but not cold enough to significantly reduce winter survival (Kockelmann and Meyer, 2006). Often sugar beet harvest equipment for field plots is not commercially available and must be fabricated or modified locally.

2. DEVELOPMENT OF TRANSGENIC SUGAR BEETS

2.1 Rationale for Transgenic Sugar Beet

Transgenic sugar beet has the potential to overcome some of the constraints and difficulties associated with conventional breeding and accelerate the development of adapted productive commercial hybrids. In addition to allowing the transfer of genes from other *Beta* species without the introduction of undesirable traits (Cai *et al.*, 1997), genes from unrelated organisms could be utilized in sugar beet improvement. The successes in introducing genes from microorganisms that resulted in resistance to broad-spectrum herbicides have demonstrated the potential of transgenic sugar beet (Pidgeon *et al.*, 2005a, b). When sugar beet is grown in areas outside the natural range of the species, it is vulnerable to pathogens and pests that it had not encountered previously. When the attacking organism and the progenitors of sugar beet have not coevolved, resistance genes in *Beta* may be rare or nonexistent. In these cases, other organisms may contribute particularly valuable resistance genes. The possibilities of transgenic sugar beet are not limited to the introduction of resistance genes but include the modification of physiological process related to adaptation and yield, the production of new products of economic value, and the advancement of knowledge of basic sugar beet physiology (Wozniak, 1999; Lathouwers *et al.*, 2005; Skaracis, 2005b).

2.2 Tissue Culture and Regeneration

Genetic improvement via biotechnological approaches has progressed slowly in sugar beet because this crop is quite recalcitrant to regeneration

following genetic transformation (Snyder *et al.*, 1999; Ivic *et al.*, 2001; Ivic and Smigocki, 2003; Zhang *et al.*, 2004; Ivic-Haymes and Smigocki, 2005a; Norouzi *et al.*, 2005). To successfully apply gene transfer techniques for crop improvement, an efficient and reliable tissue culture regeneration system is generally a prerequisite. Attempts to enhance the regeneration potential of sugar beet via improved tissue culture conditions have been the subject of numerous studies reporting a range of outcomes. Various sugar beet cell and tissue explants used for regeneration of whole plants include axillary buds, cotyledons, epicotyls, hypocotyls, leaves, petioles, thin-layer epidermal strips, zygotic embryos, unfertilized ovaries, and leaf main veins of plantlets derived from cultured apical meristems as well as suspension cells derived from some of these various tissues (Hooker and Nabors, 1977; Rogozinska and Goska, 1978; Saunders, 1982; Bhat *et al.*, 1986; Steen *et al.*, 1986; Detrez *et al.*, 1988; Krens and Jamar, 1989; Ritchie *et al.*, 1989; Jacq *et al.*, 1993; Gurel and Gurel, 1998; Zhang *et al.*, 2001, 2004; Norouzi *et al.*, 2005). The potential for direct uptake of foreign DNA through electroporation or chemical (e.g., polyethylene glycol) treatment has been demonstrated in sugar beet with leaf and petiole-derived protoplasts (Bhat *et al.*, 1985; Lindsey and Jones, 1987, 1989; Krens *et al.*, 1990; Hall *et al.*, 1993, 1995, 1996b, 1997; Lenzner *et al.*, 1995). Hall *et al.* (1995, 1996a) demonstrated that stomatal guard cells were totipotent and useful source of cells for transformation and regeneration of transformed sugar beet plants from protoplasts (Hall *et al.*, 1996b). The manipulation of these wall-less cells, however, is troublesome as osmotic potential and physical handling become a primary concern prior to wall regeneration. Similarly, new culture conditions need to be established for protoplasts as they are distinct from those used with tissue or organ culture.

The regenerative capability of cultured sugar beet and other plant cells has been accomplished by alteration of growth regulators, e.g., type and concentration (Tetu *et al.*, 1987; Toldi *et al.*, 1996; Zhang *et al.*, 2001); physical factors, e.g., light, temperature, relative humidity, tissue support matrix, (Saunders and Shin, 1986; Owens and Wozniak, 1991; Elliot *et al.*, 1992; Hall *et al.*, 1993); chemical components, e.g., inorganic and organic nutrients (DeGreef and Jacobs, 1979; Freytag

et al., 1988); and physiological status of the explants (Saunders and Shin, 1986; Jacq *et al.*, 1992; Toldi *et al.*, 1996). A regeneration medium that was considered superior to other formulations in both callus induction and adventitious shoot formation was reported by Freytag *et al.* (1988). The medium included inorganic salts, 10 vitamins, 6 amino acids, sucrose, benzyl adenine, and indole-3-butyric acid. It was the combination of amino acids and vitamins that was determined to be critical for the observed enhancement. However, despite the success of this medium, highly variable rates of adventitious shoot formation were still observed and could not be overcome via tissue culture manipulation. Findings that emerged from all of these studies point to a more serious obstacle for establishing reproducible regeneration protocols, i.e., the influence of a particular genotype.

A major consideration when establishing a reliable and efficient transformation protocol is the recognition that transformation methods developed with model plants often fail when applied to elite lines; therefore, it is advantageous to initiate molecular breeding programs with lines of interest (Christou, 1995; Ivic and Smigocki, 2001). Early attempts to develop a model plant for sugar beet genetic manipulation studies yielded a tissue culture clone, REL-1, which was selected for its high frequency of shoot regeneration from leaf-derived callus (Saunders, 1998). However, since REL-1 does lack many agronomically important traits, numerous cycles of backcrossing are required to introduce transgenes into favorable genetic backgrounds (Saunders, 1998; Snyder *et al.*, 1999). Therefore, the identification of desirable germplasm with high regeneration potential is considered an important element for development of an efficient plant transformation method, particularly for recalcitrant crops, such as sugar beet. Since regeneration in sugar beet is strongly genotype dependent, selection of highly regenerative individuals from favorable genetic backgrounds is considered a prerequisite (Miedema, 1982a, b; Freytag *et al.*, 1988; Jacq *et al.*, 1992, 1993; Ivic-Haymes and Smigocki, 2005b). In addition, regeneration frequencies are significantly reduced, more so in the recalcitrant crops, by the transformation process. Inhibition of regeneration in sugar beet was shown early on for a highly regenerative sugar beet line, AN5, following co-cultivation with *Agrobacterium*

tumefaciens that was used to transform it (Krens *et al.*, 1988).

Variations in regeneration from leaf cultures of up to four individual plants from five breeding lines were reported by Saunders and Doley (1986). Similar results were also obtained when petioles from individual plants of 12 breeding lines were cultured (Saunders and Shin, 1986). A study focused on a larger number of individual plants (up to 34) of seven elite lines characterized by relatively high sugar content and disease resistance traits (Smith and Ruppel, 1980; Panella, 1999; Lewellen, 2000) identified a wide range of regeneration frequencies (Ivic-Haymes and Smigocki, 2005b). Individual plants within each line were phenotypically identical, but they still exhibited a wide range of shoot regeneration responses (Table 1). The widest range was observed within FC607 (19–100%), in contrast to REL-1 (63–100%). In addition, large deviations were observed in the mean percentage of adventitious shoots that regenerated from leaf disks derived from plants that were preselected for a positive regeneration response (Table 1). Shoot regeneration frequencies ranged from 8% to 56% as compared to greater than 80% for REL-1. FC607 had the highest mean number of shoots, 8, which regenerated per excised explant (Table 1).

Determination of the regenerative potential can be made at an early stage of plant development (Ivic-Haymes and Smigocki, 2005b). Highly regenerative plants were identified among 3-week-old seedlings using explants from fully expanded

Table 1 Adventitious shoot formation on leaf explants from seven sugar beet breeding lines and a highly regenerative, tissue culture-selected line REL-1^(a)

Breeding line	Plants with shoots (%)	Explants with shoots (%)		Shoots/explant
		Mean	Range	
FC607	61	56	19–100	8
C78	10	38	25–50	1
Z731	50	26	8–69	3
C76-89-5	50	17	4–50	1
C69	35	14	3–75	1
7911-4-10	58	8	6–13	0
FC709-2	0	–	–	–
REL-1	100	83	63–100	4

^(a) Source: Ivic-Haymes and Smigocki (2005b). © Springer

first pairs of true leaves. This regeneration potential was stably maintained for at least a few years in vegetatively propagated plants. Once regenerated shoots were rooted and transferred to the greenhouse, vegetative propagation through tissue culture was employed to expand the source of highly regenerative donor material needed for transformation experiments (Ivic-Haymes and Smigocki, 2005b). Even though other studies have shown that the stability of the adventitious regenerants from leaf explants may exhibit somaclonal variation (Saunders and Doley, 1986; Saunders *et al.*, 1990), *in vitro* leaf explant responses were found to be comparable to the respective source genotypes with no detectable phenotypic changes (Ivic-Haymes and Smigocki, 2005b).

Variations in morphogenic capacity among individuals within a cultivar have been reported in spinach, cotton, alfalfa, and red clover (Keyes *et al.*, 1980; Matheson *et al.*, 1990; George and Tripepi, 1994; Kumar *et al.*, 1998; Ishizaki *et al.*, 2001; Mishra *et al.*, 2003; Moltrasio *et al.*, 2004). The variable response observed within the sugar beet breeding lines reflects the fact that sugar beet is an open-pollinated species and most lines are a heterogeneous mixture of genotypes (Bosemark, 2006). However, even though genetically identical plants like REL-1 were obtained by axillary shoot propagation *in vitro*, they still exhibited differences in their regeneration potential that likely were the result of differences in the physiological state of these plants. The interactions between genetic background, physiology, medium composition, and growth conditions on the regeneration potential are well documented. It is imperative, therefore, that individual plants within a line of interest be tested in order to identify the high regenerators that could be used for molecular breeding and improvement programs. In addition, the selection of lines with enhanced regeneration capacity should advance our understanding of *in vitro* capabilities, particularly as they relate to regeneration of plants from callus tissue or suspension culture.

2.3 Transformation Methods

A reproducible and efficient method for regeneration of transgenic sugar beet plants has

not been developed, although years of efforts have been expended to improve its recalcitrance to transformation (Table 2; see for review Wozniak, 1999; Lathouwers *et al.*, 2005). Most recently, reported methods include *Agrobacterium*-mediated transformation of shoot-base tissues (Lindsey and Gallois, 1990; Hisano *et al.*, 2004), cotyledonary node explants (Krens *et al.*, 1996; Joersbo *et al.*, 1998; Lennefors *et al.*, 2006), shoot explants (Zhang *et al.*, 2001) or embryogenic callus (D'Halluin *et al.*, 1992; Zhang *et al.*, 2001) as well as particle bombardment of embryogenic callus (Snyder *et al.*, 1999; Ivic and Smigocki, 2003), and polyethylene glycol-mediated transformation of guard cell protoplasts (Hall *et al.*, 1996a; Lauber *et al.*, 2001). Frequency rates for obtaining transformants ranged from 0.0001% to 0.0007% for guard cell protoplasts (Hall *et al.*, 1996b) and were higher than 30% for bombarded embryogenic hypocotyl callus (Snyder *et al.*, 1999). In general, sugar beet transformation methods are labor intensive and require a relatively long time for explant preparation and subsequent regeneration of transgenic plants. For example, the method of D'Halluin *et al.* (1992) requires 2–3 months for generation of seedling-derived callus for transformation, followed by sequential transfer of tissues to five different media throughout the procedure. The method of Snyder *et al.* (1999) similarly entails several lengthy (2–3 months) and labor-intensive steps for plant material preparation, including a seed germination step that is often plagued by high levels of contamination. As with the method reported by D'Halluin *et al.* (1992), several transfers of tissues were required and plants regenerated only after about 6 months in culture. Most methods have not been found readily applicable in laboratories other than those in which they were developed (Krens *et al.*, 1996; Snyder *et al.*, 1999; Smigocki, unpublished results). The method of Snyder *et al.* (1999) proved ineffective for transformation of several commercial sugar beet breeding lines (Ivic and Smigocki, 2001). Although proprietary methods that include *Agrobacterium*-mediated transformation of cotyledons have been reported to generate transgenic plants, in general, these methods are not well described and require high input due to their low efficiency (Fry *et al.*, 1991; Krens *et al.*, 1996). Although low in efficiency

Table 2 Transgenic sugar beets derived using various transformation methods, explants, and selection conditions

Method variety/cultivar	Explant	Selection/marker gene and promoter ^(a)	Beneficial gene ^(a)	Phenotype	Transformation frequency ^(b)	Reference
<i>Agrobacterium tumefaciens</i>						
—	Cotyledon	NOS _p <i>npII</i> , 35S _p <i>gusA</i>	35S _p <i>epsps</i>	Herbicide ^R	—	Fry <i>et al.</i> , 1991
HIAB1, HIAB2/HIAB3, HIAB4, HIAB5	Cotyledon	NOS _p <i>npII</i> , 35S _p <i>gusA</i>	e35S _p <i>epsps</i> , FMV _p <i>gox</i>	Herbicide ^R	0.005–1.5%	Mannerlöf <i>et al.</i> , 1997
O-type 272	Cotyledon	35S _p <i>pmi</i> , <i>npII</i> , <i>gusA</i>	—	—	—	Joersbo <i>et al.</i> , 1998
REL-1	Cotyledon	NOS _p <i>npII</i> , <i>Osm_pgusA</i>	Pat _p <i>ipt</i>	Insect ^R , carbon partitioning Cercospora ^R	1.8–3.5%	Snyder <i>et al.</i> , 1999
REL-1	Cotyledon	NOS _p <i>npII</i>	35S _p <i>cfp</i>	—	0.3%	Kuykendall <i>et al.</i> , 2003
31-188	Cotyledonary node	NOS _p <i>npII</i> , 35S _p <i>gusA</i>	—	—	0.9%	Krens <i>et al.</i> , 1996
G018	Cotyledonary node	35S _p <i>pmi</i>	Ubq3 _p dsRNA-replicase	BNYVV ^R	—	Lennefors <i>et al.</i> , 2006
Kwerta, Sharps Poly 4, AMP1	Shoot base	NOS _p <i>npII</i> , 35S _p <i>gusA</i> , 35S _p <i>cat</i>	—	—	3.7–19%	Lindsey and Gallois, 1990
Bella, Dippie Ero, SVP	Shoot base	NOS _p <i>npII</i> , 35S _p <i>gusA</i>	—	—	2.5–10%	Konwar, 1994
31-188	Shoot base	NOS _p <i>npII</i> , <i>cabII_pgusA</i>	—	—	—	Stahl <i>et al.</i> , 2004
VRB	Shoot base	NOS _p <i>bar</i> , <i>HIS1-r_pluc</i> , <i>Mll_pluc</i> , <i>Tlpp_pluc</i>	—	—	—	Oltmanns <i>et al.</i> , 2006
8T0015, KWS SAAT AG	Shoot base	NOS _p <i>npII</i> , 35S _p <i>gusA</i> , NOS _p <i>hpt</i>	35S _p <i>cryIA (b)</i> , 35S _p <i>cryIC</i> , 35S _p chitinase	Insect ^R	6.8-(161%)	Hisano <i>et al.</i> , 2004
NK150, TK 80 (<i>B. vulgaris</i>)	Leaf midvein shoot base	—	—	—	—	—
Acc. France (<i>B. maritima</i>) 028	Petiole	NOS _p <i>npII</i>	35S _p <i>sod</i>	Cercospora ^R herbicide ^R / oxidative stress	—	Tertivanidis <i>et al.</i> , 2004

KS3, KS7	Seedling	NOS _p bar, 35S _p gusA	–	–	Kishchenko <i>et al.</i> , 2005
Several breeding lines	Embryogenic seedling callus	NOS _p npII, 35S _p bar, TR2 _p bar, 35S _p alds	35S _p bar, 35S _p alds	Herbicide ^R	D'Halluin <i>et al.</i> , 1992
2 breeding lines	Embryogenic seedling callus	TR _p bar, TR _p npII	35S _p cp	BNYVV ^R	Mannerlöf <i>et al.</i> , 1996
SDM 3 CMS 22 003	Shoot, embryogenic callus	35S _p gfp	–	2–5%	Zhang <i>et al.</i> , 2001
Hutian207, Gantang1, Xintian6, kws9306, kws9204 (sugar beet)	Bud tip	35S _p hpt	35S _p Atnhx1	Salt tolerance	Yang <i>et al.</i> , 2005
49–975, 52-028, 49–863 (fodder beet)					
Polyethylene glycol					
Bv-NF, SES 1	Guard cell protoplasts	35S _p pat, 35S _p gusA	–	0.0001–0.0007%	Hall <i>et al.</i> , 1996b
Bv-NF	Guard cell protoplasts	35S _p pat	35S _p 1-sst	Fructans	Sévenier <i>et al.</i> , 1998
Bv-NF, 4D6834	Guard cell protoplasts	35S _p pat	Ubq3 _p 15	BNYVV ^R	Lauber <i>et al.</i> , 2001
Bv-NF, 4D6834	Guard cell protoplasts	35S _p pat	Ubq3 _p 1-sst, Ubq3 _p 6g-fft	Fructans	Weyens <i>et al.</i> , 2004
Particle bombardment					
Rel-1	Embryogenic hypocotyl callus	NOS _p npII Osm _p osm, PinII _p thi, PR-S _p thi	Osm _p cec, PinII _p cec	Pathogen ^R , defense genes	Snyder <i>et al.</i> , 1999
FC607	Leaf	Osm _p gusA, PinII _p gusA	–	0.9–3.7%	Ivic-Haymes and Smigocki, 2005a

^(a) *alds*, acetolactate synthase; *AtNHX1*, Na⁺/H⁺ antiport gene from *Arabidopsis*; *cec*, cecropin MB39; *cfp*, cercosporin export gene; *cp*, BNYVV coat protein gene; *crpIA (h)*, cryIC, crystal protein genes from *Bacillus thuringiensis*; *epsps*, 5-enolpyruvylshikimate-3-phosphate synthase; *dsRNA*-replicase, modified BNYVV replicase; *gdx*, glyphosate oxidase reductase; *ipt*, isopentenyl transferase; *osm*, osmotin; *P15*, BNYVV movement protein; *SOD*, superoxide dismutase; *thi*, DB4 thionin; 1-SST, 1-sucrose:sucrose fructosyl transferase; 6G-FFT, fructan:fructan 6G-fructosyl transferase

^(b) Calculated as number of transformed shoots per number of explants or protoplasts

relative to some other dicotyledonous plants, the method of D'Halluin *et al.* (1992) was the first to provide a sufficient number of transgenic sugar beets suitable for further evaluation in an agronomic field test.

A recently developed method uses highly regenerative leaf callus suspension cultures derived from commercial breeding lines that were first identified as being highly regenerative (Table 1; Ivic and Smigocki, 2003). When these suspension cells were subjected to particle bombardment, transformed embryos and callus lines regenerated starting at 5 months of culture but no transgenic plants were recovered. Such long tissue culture periods have been associated with the inhibition of the organogenic potential in transformed tissues (Cassells and Curry, 2001) as well as higher frequencies of abnormal phenotypes and reduced fertility in transgenic plants (Altpeter *et al.*, 1996; Cassells and Curry, 2001; O'Kennedy *et al.*, 2001). To circumvent these problems and reduce the time in culture, leaf disks from the highly regenerative plants were used as target tissue for particle bombardment immediately after excision from the leaves (Ivic-Haymes and Smigocki, 2005a). This relatively simple and time-saving procedure yielded transformed plants as early as 3 months after bombardment at a frequency of 0.9–3.7%. In addition, since frequent transfer of leaf disks to fresh medium was shown to have a negative impact on regeneration (Ivic-Haymes and Smigocki, 2005b), this method also proved to be less labor-intensive and costly since bombarded explants were only transferred to fresh media every 3–4 months.

Unlike the method of Ivic-Haymes and Smigocki (2005a), Hisano *et al.* (2004) recently developed yet another method that is entirely dependent on tissue culture. This *Agrobacterium*-mediated gene transfer method utilizes shoot-base cultures as target tissues for transformation but is deemed simpler than the previously published shoot-base method (Lindsey and Gallois, 1990; Konwar, 1994). Leaf blades excised from young plants are cultured on shoot formation medium until shoots regenerate on the veins. Shoot bases are then stripped of the shoots and used as explants for transformation. The regeneration frequencies were reported to be consistent and as high as 30%, thus contributing to the production of uniform transgenic plants from several lines

of *B. vulgaris* as well as *B. maritima* accessions. Two different *Bacillus thuringiensis* (*Bt*) genes and a chitinase gene were introduced along with an *nptII* (neomycin phosphotransferase II) gene as the selectable marker for kanamycin resistance. In addition, the *hpt* (hygromycin phosphotransferase) gene was used as a selectable marker to regenerate the first hygromycin resistant transgenic sugar beet. An important advantage of this method is the reported lack of an intervening callus phase that often is linked to the occurrence of somaclonal variation. Other reports of direct shoot regeneration include *Agrobacterium*-mediated transformation of cotyledons with a soybean cercosporin toxin export gene, *cfp*, which is being evaluated for its potential role in *Cercospora* leaf spot resistance (Kuykendall *et al.*, 2003).

A method reported by Yang *et al.* (2005) utilized tissue-cultured bud tips derived from immature inflorescence to introduce salt tolerance into sugar beet. Vigorously growing bud clumps were induced on the cultured inflorescence. Buds were excised from the clumps and after 7 days of culture, bud meristems were exposed by removal of the leaves from the buds. *Agrobacterium*-mediated gene transfer method was used to transform the exposed meristems. Attempts to optimize the transformation frequency included the use of various concentrations of *Agrobacterium* for infection, varying the duration of the co-cultivation period, infecting the buds under variable negative pressure and adding varying concentrations of a surfactant (Silwet L-77) during co-cultivation. These permutations enhanced the overall frequency of regeneration of transgenic plants that were able to grow at higher salt concentrations.

Transgenic sugar beet resistant to a glufosinate herbicide have also been produced using an *Agrobacterium* vacuum infiltration transformation method (Kishchenko *et al.*, 2005). Target tissues included etiolated seedlings or suspension cultures that were prepared from friable hypocotyl and cotyledon callus of two breeding lines. After the seedlings were vacuum infiltrated, a similar procedure was used to generate friable callus from the hypocotyls and cotyledons. Transgenic plants regenerated from the transformed callus after 4–10 weeks on phosphinothricin-containing selective medium.

2.3.1 Hairy root cultures

The regeneration of transformed sugar beet hairy root cultures has provided an *in vitro* approach for detailed studies of a variety of agricultural problems associated with sugar beet production. Hairy root cultures are derived via *Agrobacterium rhizogenes*-mediated transformation of seedling hypocotyls or petioles (leaf stalks) (Tepfer, 1984; van der Salm *et al.*, 1996; Kifle *et al.*, 1999; Cai *et al.*, 2003; Smigocki *et al.*, 2006). The advantage of hairy root cultures is that they provide a rapid means (generally less than 3 weeks) for obtaining transformed sugar beet tissues that express foreign genes of interest. This approach provides for a rapid functional analysis of gene function *in planta*. Generation of transgenic plants that express the most interesting genes will still require the traditional labor-intensive and time-consuming transformation approaches since sugar beet hairy roots are recalcitrant to regeneration (Smigocki, unpublished results). The beet necrotic yellow vein virus (BNYVV) coat protein (CP) gene was initially evaluated in sugar beet hairy roots as a precedent for introducing BNYVV resistance into transgenic sugar beet (Ehlers *et al.*, 1991; Mannerlöf *et al.*, 1996). Similarly, Cai *et al.* (1997) generated transformed hairy roots with a complementary DNA clone *HsI^{pro-1}* from *B. procumbens*, a beet cyst nematode (*H. schachtii*)-resistant species, and observed incompatible reactions (no cyst development) in several independent transformants derived from susceptible lines.

Sugar beet hairy root cultures have been effectively utilized for studying plant–pathogen and plant–pest interactions *in vitro*. Mugnier (1987) examined the infection of transformed roots of sugar beet and table beet by *Polymyxa betae* and of brassicas by *Plasmodiophora brassicae*. He was able to identify two distinct fungal phases in the infected hairy root cultures. The culture of these obligate parasites on transformed roots allowed for detailed life cycle descriptions not previously obtainable under axenic conditions. Preliminary experiments with *H. schachtii* indicated that the larvae were capable of forming new cysts without loss of pathogenicity when cultured on sugar beet hairy root cultures (Paul *et al.*, 1987). An *in vitro* approach was also used to demonstrate resistance to *H. schachtii* in a modified nematode-

resistant sugar beet line, AN5 (Paul *et al.*, 1990). Detailed observations of sugar beet root maggot (SBRM, *Tetanops myopaeformis*) larval-feeding behavior and evaluation of the influence of bacterial additions on larval development were similarly evaluated using axenic hairy root cultures (Wozniak, 1993). In the same way, hairy root bioassays were used for *in vitro* testing of toxic *Nicotiana tabacum* extracts on SBRM survival (Smigocki *et al.*, 2003).

As the methods presented above and those under development are refined, it is clear that steady increases in transformation efficiency will occur. Improved transformation efficiencies will lead to a more rapid development of transgenic sugar beet with agriculturally desirable traits.

2.4 Selection of Transformed Tissue

2.4.1 Selectable marker genes

2.4.1.1 Antibiotics

As summarized above, the efficiency of sugar beet transformation is influenced by a multitude of factors, among them plant genotype and explant source (Table 2) being of paramount importance. Methods employed for selection of transformed cells appear to be equally important in the success of all transformation techniques. Antibiotic selection, which allows for specific enrichment for transformed cells, is generally considered an integral part of a transformation protocol. A widely used selection scheme in transgenomics incorporates an *nptII* selectable marker gene into the targeted cells making them resistant to kanamycin and other related antibiotics such as the aminoglycoside G418 (Geneticin). A high rate of nontransgenic escapes is often reported when antibiotics or herbicides are used as selective agents due to high levels of inherent tolerance to the selective agent (Lindsey and Gallois, 1990; D'Halluin *et al.*, 1992; Ivic-Haymes and Smigocki, 2005a). Another major drawback to the use of antibiotics is that they have been reported to severely repress the subsequent regeneration of transgenic plants (Everett *et al.*, 1987; Mullins, 1990). Use of kanamycin for selection of transformed sugar beet tissues has also proven to be difficult. A general delay in

regeneration of transformed calli on kanamycin-containing media was observed when sugar beet suspension cultures were subjected to particle bombardment transformation (Ivic-Haymes and Smigocki, 2005a). Culture of explants for about 4 months on 100 mg l^{-1} kanamycin yielded no calli or shoots. Contrary to these findings, Hisano *et al.* (2004) attributed their high transformation rate in part to the relatively high (150 mg l^{-1}) concentration of kanamycin in the selection medium. They report that supplementing the medium with phytohormones (6-benzylaminopurine (BA) and indole-3-butyric acid (IBA)) and using half-strength Murashige and Skoog (MS) salts contributed to the improved selection efficiency without disturbing the morphogenic ability of the sugar beet cells on kanamycin-containing media. Krens *et al.* (1996) selected transformed cells on even higher concentrations of kanamycin (250 mg l^{-1}) and maintained regenerated shoots on 200 mg kanamycin per liter. The variable responses of sugar beet tissues to kanamycin selection underscore the important roles that genotype and explant source play in the regeneration potential of transformed sugar beet.

To date, most reports on sugar beet transformation utilize vectors that carry the *nptII* selectable marker gene (Table 2). These transformation vectors represent the first generation of vectors that are widely distributed and, therefore, generally easily obtainable because their use is often not restricted by intellectual property rights. A relatively new class of transformation vectors that carry the *hpt* gene for selection of transformed cells on hygromycin-containing media is being used in sugar beet transformation (Hisano *et al.*, 2004; Yang *et al.*, 2005; Smigocki, 2008). Hisano *et al.* (2004) reported that hygromycin selection was effectively utilized for regenerating transgenic *B. maritima* plants from one of the two accessions that they tested. They were not successful at regenerating transgenic plants from three *B. vulgaris* lines. Yang *et al.* (2005), however, did regenerate hygromycin-resistant plants from several *B. vulgaris* accessions transformed with the *hpt* gene. Similarly, hygromycin-resistant hairy roots were regenerated from two *B. vulgaris* breeding lines (Smigocki, 2008). The observed differences in the generation of transgenic sugar beet most likely reflect genotypic differences, explant source and transformation method employed.

2.4.1.2 Herbicide

As a desirable field trait and a useful selectable marker, the advent of herbicide resistance genes is considered a significant enhancement in the selection of transformed cells (Perez *et al.*, 1989; Catlin, 1990; Joersbo and Okkels, 1996). Herbicide resistance has been introduced into sugar beet using three different genes that are involved in mediating the resistance: mutant acetolactate synthase (*als*) or 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) genes and the phosphinothricin acetyl transferase (*pat*; *bar*, bialaphos resistance) gene (Table 2; D'Halluin *et al.*, 1992; Mannerlöf *et al.*, 1997; Kishchenko *et al.*, 2005). D'Halluin *et al.* (1992) transformed embryogenic calli derived from cotyledons, hypocotyls, petioles, and leaves of sugar beet seedlings with *A. tumefaciens* carrying the *bar* or mutant *als* genes. They used kanamycin (200 mg l^{-1}) for initial selection of *bar* or mutant *als* transformants followed by selection for resistance to broad-spectrum herbicides glufosinate-ammonium (phosphinothricin, 20 mg l^{-1}) or to sulfonylurea (chlorosulfuron, 2 mg l^{-1}) compounds, respectively. The PAT enzyme acetylates and inactivates the herbicide (De Block *et al.*, 1987) while the mutant ALS makes the transgenic plant insensitive to the herbicidal compounds. Similarly, Mannerlöf *et al.* (1997) utilized the mutant *epsps* and a glyphosate oxidase reductase (*gox*) gene for selection of transformed cells resistant to the herbicide glyphosate (N-phosphonomethyl-glycine). The introduction of a mutant EPSPS enzyme into plants impedes the action of the herbicide that normally targets and inhibits the endogenous EPSPS in plants (Steinrücken and Amrhein, 1980). Gox provides an additional offensive line of protection by degrading glyphosate and reducing the total amount of the herbicide that can reach the target EPSPS (Dill, 2005). Herbicide selection proved effective for recovery of transgenic sugar beet that also possesses functional utility in realistic field and greenhouse situations.

2.4.1.3 Cytokinin

Selection schemes that rely on antibiotic or herbicide resistance genes for production of field-released transgenic crops have raised concerns

regarding their impact on the environment and their safety for human consumption. An alternate selection system introduces a β -glucuronidase gene as a selectable marker that releases the active growth hormone cytokinin from inactive cytokinin glucuronides in transgenic cells (Joersbo and Okkels, 1996; Okkels *et al.*, 1997). Release of the cytokinin stimulates growth and regeneration of transformed shoots. Transformation was reported to be two to three times more efficient than with kanamycin selection. Although sugar beet contains an endogenous β -glucuronidase (Wozniak and Owens, 1994), this should not preclude application of this system to sugar beet. Conjugated glucuronides may afford an alternative selection technique that avoids some of the potential problems associated with cosuppression and *trans*-inactivation. On the other hand, this approach is sensitive to variations in endogenous cytokinin levels as well as those used in *in vitro* culture of sugar beet, and fine-tuning it for generating transgenic plants may prove to be more complicated and time consuming. Another cytokinin approach based on the expression of a bacterial isopentenyl transferase (*ipt*) gene that catalyzes the first committed step in cytokinin biosynthesis has been developed for generating marker-free transgenic plants (Ebinuma *et al.*, 1997). Increased synthesis of cytokinin mediated by the *ipt* gene has been reported to enhance regeneration of transformed cells (Smigocki and Owens, 1988).

2.4.1.4 Mannose

A novel selection technique, based on the concept of favoring the growth of transgenic cells while starving, rather than killing, the nontransgenic cells, is the mannose selection system. The approach exploits the inability of most plant cells to utilize mannose as a carbon source and the expression of a bacterial gene coding for a phosphomannose isomerase (*pmi*) as a selectable marker gene (Okkels and Whenham, 1993; Okkels *et al.*, 1994). Mannose selection was demonstrated to be particularly efficient for regeneration of transgenic sugar beet (Joersbo *et al.*, 1998, 1999; Lennefors *et al.*, 2006). Transformed sugar beet cells that were capable of using mannose as a carbon source were positively selected for

enhanced growth while nontransformed cells were nutrient deficient, but not killed initially. This last point, wherein nontransformed cells do not die and release their potentially toxic cellular contents during selection, is believed to be of major significance for providing a more compatible environment for growth of transformed cells.

2.4.2 Reporter genes

Several markers have been developed for use as a visually screenable phenotype in transgenic plants. Marker genes that have been expressed in sugar beet for detection of transformed cells are encoded by the *gusA* (β -glucuronidase; *uidA*, GUS), *luc* (luciferase, LUC), and *gfp* (green fluorescence protein). The *gusA* gene is being used extensively for optimizing transformation conditions and for the confirmation of stable integration of newly introduced transgenes into plant genomes. By far, *gusA* has been the most frequently expressed reporter gene in transformed sugar beet (Table 2). In addition to monitoring for transformation, the *gusA* reporter gene serves as a powerful tool for determining temporal and tissue-specific expression patterns of gene promoters. Analysis of GUS to LUC activity in bombarded sugar beet cell suspension cultures revealed that the expression driven by the osmotin (*Osm*) gene promoter was about 2.5 times higher than that driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Ingersoll *et al.*, 1996). In addition, when embryogenic suspension cultures were bombarded with the *Osm-gusA* gene construct, chimeric expression was detected in transformed calli suggesting that the calli arose from both transformed and normal cells or that the *gusA* gene became silenced in certain parts of the transformed calli (Ivic and Smigocki, 2003). Similarly, expression of the *Osm-gusA* gene in sugar beet plantlets, greenhouse-grown plants, and hairy roots revealed various patterns and levels of GUS activity useful for construction of transgenes in order to achieve spatial and/or temporal regulation of expression *in planta* (Figures 1a–e; Ivic-Haymes and Smigocki, 2005a).

Recently, *luc* gene expression was documented in transgenic sugar beet taproots using root-specific gene promoters (Oltmanns *et al.*, 2006). The *luc* gene was chosen over the *gusA* reporter

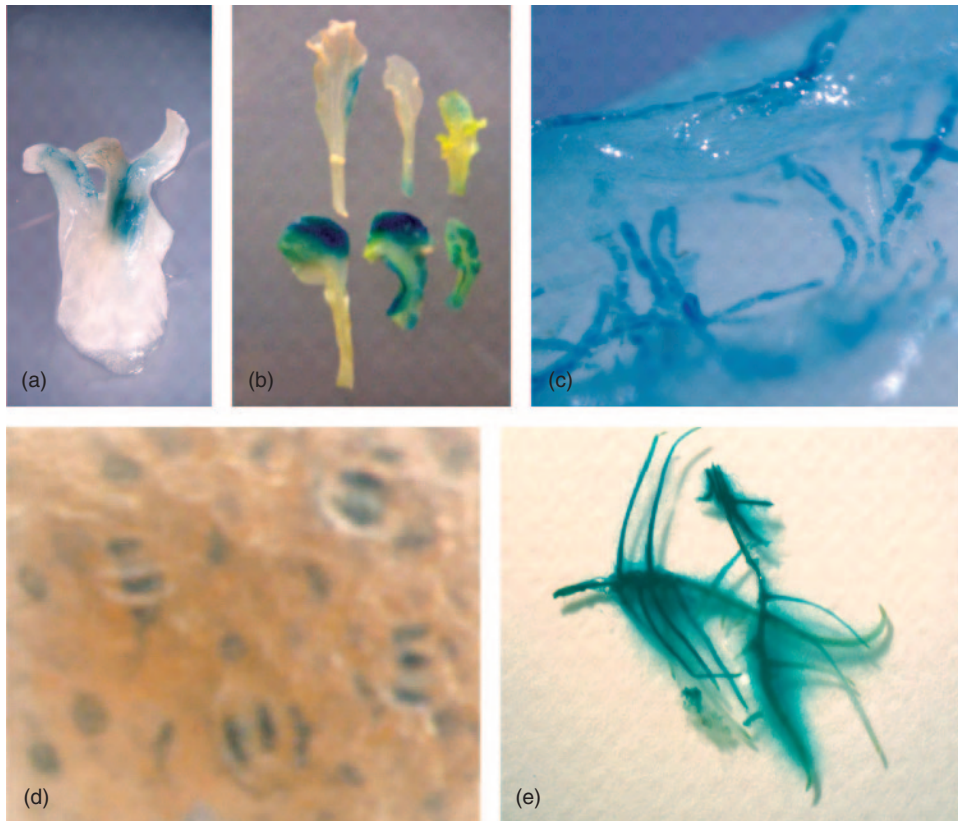


Figure 1 (a) GUS activity in Osm-*gusA* shoot, (b) leaves and (c) trichomes of tissue culture-propagated Osm-*gusA* sugar beet plantlets; (d) epidermal and stomatal cells of a greenhouse-grown plant; and (e) hairy roots transformed with a *Medicago truncatula* root-specific promoter fused to *gusA* [Source: Ivic-Haymes and Smigocki (2005a). © Springer; Smigocki, unpublished results]

gene because sugar beet roots contain high levels of phenolic compounds that have been reported to inhibit the β -glucuronidase enzyme encoded by the *gusA* gene (Vainstein *et al.*, 1993). The GUS and LUC activity assays, however, are invasive and cannot be used to monitor gene expression *in vivo* in the same material over time. The *Aequorea victoria gfp* gene can function in a range of heterogenous environments and it is not a destructive assay. Analysis for GFP activity is monitored with blue or UV light for emission of fluorescence thus no tissues are destroyed in the process. The first and only successful detection of *gfp* gene expression in sugar beet was reported by Zhang *et al.* (2001). They employed the *sgfp-S65T* construct that had been optimized to give improved signals in plant cells (Chiu *et al.*, 1996). This approach for identifying transgenic sugar beet is considered advantageous

for enhancing regeneration and plant regeneration in the absence of selectable agents such as antibiotics and herbicides that are known to reduce regeneration frequencies and negatively impact public acceptance of genetically modified (GM) plants.

2.4.3 Promoters

Use of appropriate promoters for regulating transgene expression in plants is critical to the success of the transformation. Constitutive as well as tissue-specific and/or inducible promoters have been used to direct transgene expression in sugar beet (Table 2). The constitutive promoters 35S from the CaMV as well as the ubiquitin3 (Ubq3) from the ubiquitin gene have been used extensively in sugar beet to drive expression of

selectable marker, reporter, and beneficial genes. However, comparison of the 35S constitutive promoter to several inducible promoters for potential impact on gene expression revealed other strong promoters that may be more useful for driving the expression of beneficial genes in sugar beet cells (Ingersoll *et al.*, 1996). When *gusA* gene expression from the 35S, PR-S, osmotin, and a proteinase inhibitor (PinII) promoter was followed over a 48-h period, steady state levels of expression were reached with all promoters 12 h after DNA transfer (Ingersoll *et al.*, 1996). The 35S and PinII promoters reached similar levels of expression; the PR-S promoter, from a systemic acquired resistance gene, expressed at approximately 50% higher level than the 35S and PinII promoters; and the osmotin promoter proved superior in that the expression levels at 12–48 h after bombardment were approximately 2.5 times those of 35S.

Several sugar beet promoters have been cloned and characterized for tissue-specific expression. A chlorophyll a/b binding protein promoter expressed the *gusA* gene at high levels in leaves of transgenic sugar beet (Stahl *et al.*, 2004). Three other promoters had a root-specific expression pattern (Oltmanns *et al.*, 2006). A linker histone variant and a thaumatinlike protein gene promoter had expression restricted to the vascular tissue of the root, while the major latexlike gene promoter was expressed throughout the entire root. This technology holds promise for expressing genes in a tissue-specific manner without imparting deleterious effects on nontarget tissue.

2.5 Transgenics Developed

2.5.1 Herbicide resistance

Weed control is a costly and necessary part of sugar beet production, relying heavily on several postemergence herbicide treatments with or without a pre-emergence herbicide applied at planting. Herbicide costs ranged from 171 to 319 US dollars per hectare (Kniss *et al.*, 2004) with additional costs for cultivation and hand labor required to remove weeds not controlled with herbicides. Use of transgenic herbicide-resistant sugar beet could provide producers with broad-spectrum weed control at a fraction of the current cost for efficacious weed control in

this crop (Kniss *et al.*, 2004). The glyphosate-resistant sugar beet (Roundup Ready[®]) developed using a combination of an *Agrobacterium* sp. *cp4 epsps* and a *gox* gene (Mannerlöf *et al.*, 1997) was approved for production in the United States in 1998 (Duke, 2005). Transgenic sugar beet resistant to a glufosinate herbicide (Liberty Link[®]) has also been produced (Kishchenko *et al.*, 2005). Glufosinates inhibit glutamine synthase, thereby disrupting nitrogen metabolism. Introduction of the *bar* gene creates resistance to the glufosinate herbicide. The *bar* gene encodes a phosphinothricin acetyl transferase, which acetylates and inactivates the herbicide (De Block *et al.*, 1987). Although not extensively examined in sugar beet, *bar* expression in other crops did not affect nutritional composition (Oberdoerfer *et al.*, 2005).

2.5.2 Disease resistance

Cercospora beticola is a devastating, photoactive toxin-producing foliar fungal pathogen of sugar beet (Daub and Ehrenshaft, 2000). Tolerance to the fungus is a quantitative trait with low heritability (Nilsson *et al.*, 1999; Schäfer-Pregl *et al.*, 1999). Additionally, *C. beticola* has developed resistance against several major fungicides used for control (Bugbee, 1995; Windels *et al.*, 1998), therefore, novel disease control strategies are highly desirable. *A. tumefaciens* was used to deliver a chloroplastic and cytosolic superoxide dismutase from tomato into sugar beet (Tertivanidis *et al.*, 2004). Overexpression of the antioxidant superoxide dismutase created greater tolerance to *Cercospora* leaf spot and the fungal toxin cercosporin. Transgenic plants exhibited 70% less damage following exposure to cercosporin, and disease severity caused by *C. beticola* was reduced by 50%. In addition, tolerance to oxidative stress was enhanced in transgenic plants exposed to the herbicide methyl viologen. Another recently reported approach for imparting resistance to *Cercospora* leaf spot in sugar beet is based on the expression of the cercosporin toxin export (*cfp*) protein gene from *C. kikuchii*, the causative agent of purple seed stain in soybean (Kuykendall *et al.*, 2003). *Agrobacterium*-mediated gene transfer was employed for the introduction of the *cfp* gene into sugar beet, but the

resistance of the transgenic plants to *Cercospora* leaf spot was not reported.

Plants harboring defense-related genes encoding cecropin MB39, osmotin, DB4 thionin, and PR-S were regenerated from bombarded embryogenic hypocotyl calli of the tissue culture line REL-1 (Snyder *et al.*, 1999). These transformants were generated in an effort to provide resistance to a variety of bacterial and fungal pathogens, particularly *Erwinia carotovora* ssp. *betavascularum* that causes root rot, *C. beticola*, the causative agent of leaf spot, and *Rhizoctonia solani* AG2-2 that causes seedling root rot. However, preliminary analysis of transgenics for *Cercospora* leaf spot resistance showed that these plants were more susceptible to this pathogen than the controls. In addition, since the defense-related genes were introduced into the REL-1 tissue culture line, transgenic plants that show promising levels of resistance will need to be crossed with elite breeding lines. Otherwise, the genes will need to be transferred directly into advanced breeding lines for further evaluation.

BNYVV causes rhizomania, a devastating disease of sugar beet, which is transmitted by a soilborne fungus, *Polymyxa betae*. Moderate levels of BNYVV resistance have been introgressed to commercially important breeding lines (Lewellen and Whitney, 1993). Most recently, genetic engineering approaches are being applied to enhance tolerance to BNYVV (Mannerlöf *et al.*, 1996; Maleki *et al.*, 2003; Lathouwers *et al.*, 2005; Lennefors *et al.*, 2006). Expression of modified and, therefore, dysfunctional BNYVV movement protein (P15 TGB) genes driven by a constitutive promoter were introduced into sugar beet (Lauber *et al.*, 2001). When challenged with the virus, a number of the transformants were resistant. CP gene-mediated resistance was also reported by Maleki *et al.* (2003) and Mannerlöf *et al.* (1996), where the titer of BNYVV was shown to be reduced in the transgenic plants. A double-stranded RNA (dsRNA)-based resistance has recently been reported to be superior to the CP-mediated resistance (Lennefors *et al.*, 2006). Transgenic plants expressing an inverted repeat of a short fragment derived from the BNYVV replicase gene displayed higher levels of resistance than the CP-transformed plants. Even though the replicase gene fragment was obtained from a B-type isolate of BNYVV, transgenic

plants showed high levels of resistance against the A- and P-types BNYVV, thus illustrating the broad spectrum of the obtained resistance. This cross-reactivity between pathotypes may be a result of the high degree of conservation of the targeting sequence between viral isolates (Koenig and Lennefors, 2000). RNA silencing induced resistance does not rely on the accumulation of viral proteins, therefore, extensive toxicity or allergenicity studies on the transgenic plants may not be required thus facilitating their more rapid regulatory approval for large-scale commercial production and acceptance by the general public.

2.5.3 Alternative sweeteners

Low-calorie sweeteners are in high demand. Laboratory-based production of artificial sweeteners can be time consuming and expensive. To produce fructans in sugar beet, Sévenier *et al.* (1998) and Weyens *et al.* (2004) introduced the gene encoding 1-sucrose:sucrose fructosyl transferase (1-SST) from Jerusalem artichoke or onion, respectively, into sugar beet. This enzyme catalyzes the degradation of sucrose into low molecular weight fructans, a sugar substitute that is not metabolized by humans and promotes growth of beneficial digestive microorganisms. Introduction of 1-SST had no deleterious effect on plant growth and the tap root dry weight was unaffected in the transgenic line. Sévenier *et al.* (1998) reported that on average the transgenic plants produced 110 μ M (micromole) fructan per gram fresh weight, which is equivalent to 40% of the root total dry weight. An overall decrease in storage carbohydrates was observed in the Sévenier *et al.* (1998) study, and Weyens *et al.* (2004) speculated that this may have been due to the different expression level of the gene driven by two different constitutive promoters, 35S versus Ubq3. These observations suggest the fructan producing sugar beet may be a viable alternative to laboratory production of alternative sweeteners.

2.5.4 Salt tolerance

Sugar beet normally grows in soils with <103 mM salinity. In order to grow sugar beet in high-salt soils in China, Yang *et al.* (2005) overexpressed

the *Arabidopsis thaliana* Na^+/H^+ antiport gene (*Atnhx1*) in beet plants. The *Atnhx1* gene transports superfluous Na^+ from the cytosol to the vacuole thus making the plants more salt tolerant. Using three cultivars of beet that already were more salt tolerant when grown in 171 mM NaCl, Yang *et al.* (2005) generated transgenic plants expressing the *Atnhx1* gene that were able to tolerate up to two and a half times higher salt concentrations (427 mM NaCl). Using this approach, new high-salt-tolerant cultivars are likely to be created for exploiting saline soils for cultivation of sugar beet.

2.5.5 Frost tolerance

Chinese researchers from the Inner Mongolia Sugar Beet Manufacturing Research Institute and Department of Biology of Inner Mongolia University have transferred a frost tolerance gene from fish to sugar beet, which has made the plant more tolerant to cold temperatures (Yuancong, 1997). Using this transgenic technology will allow for a longer growing season and later harvest of beets in the northern growing regions. The transgenic lines extended the growing season 30 days beyond that of the control varieties and were able to withstand temperatures reaching -6°C to -6.5°C .

2.5.6 Regeneration potential

In hopes of improving the regeneration capacity of *B. vulgaris* by increasing endogenous cytokinin levels, Krens and colleagues (Krens *et al.*, 1988) were first to utilize “shooter” mutants of *A. tumefaciens*, wherein the auxin locus of the transfer-DNA region was inactivated but the cytokinin biosynthesis (*ipt*) gene was left intact. Although nine sugar beet lines were evaluated, no shoot regeneration from inoculated leaf discs was observed. When seedling tissues were used as explants, shoot regeneration was observed at low frequencies, but transformation was not confirmed. In an analogous attempt to enhance regenerative ability of transformed REL-1 cells (leaf discs, suspension cells) and “FC607” (petioles, leaf discs), explants were inoculated with a supervirulent *A. tumefaciens* strain EHA101

carrying the *ipt* gene on a binary vector (Smigocki and Owens, 1988; Wozniak and Owens, 1989). Although transformed cells were obtained, the overall effect on shoot regeneration of the two lines was negative. On the other hand, Snyder *et al.* (1999) observed higher transformation efficiency when the *ipt* versus the reporter *gusA* gene was introduced via *Agrobacterium*-mediated gene transfer. They speculated that this may reflect an enhancing effect of cytokinin on regeneration due to expression of the introduced *ipt* gene. Taken together, these results suggest that fine-tuning *ipt* transgene expression in sugar beet cells may be a tool for enhancing regeneration of transgenic plants.

2.5.7 Sucrose accumulation

There is a strong negative correlation between sugar concentration and dry root mass in sugar beet (Hosford *et al.*, 1984), a barrier for increasing sugar beet productivity by conventional breeding methods. High-sugar yield and concentration could potentially be achieved in roots containing more cambial rings with shorter diffusion paths between phloem and accumulating cells (Milford, 1973; Wyse, 1979). Cytokinin and auxin plant growth regulator concentrations are correlated with the formation and expansion of secondary cambia during storage root growth (Hosford *et al.*, 1984). High-cytokinin levels were similarly correlated with cambial initiation and rapid cell division in developing tap roots and just prior to cytokinesis in synchronized tap root cell suspension cultures (Elliott *et al.*, 1984, 1986). Additionally, cytokinins have long been known to modulate assimilate movement in plants and that photosynthate transport increased to the site of cytokinin application. Taken together, these results suggest that higher cytokinin levels may increase cell division, the number of vascular rings and sucrose accumulation in taproots (Monger *et al.*, 1995).

As part of an effort to alter carbohydrate partitioning and activate otherwise dormant supernumerary cambia, which typically contribute little to root growth in *B. vulgaris* (Elliot *et al.*, 1996), sugar beet cells were transformed with the *ipt* gene. Expression of the *ipt* gene fused to a potato tuber-specific promoter from a class

I patatin gene (*Pat*) in transgenic sugar beet increased zeatin riboside concentrations up to 18-fold in the leaves while a corresponding 2-fold increase was observed in the tap roots (Ivic *et al.*, 2001). Leaf concentrations of major nonstructural carbohydrates, sucrose, glucose, and starch were not significantly altered. Higher sucrose levels were found in young tap roots, however, in mature plants, tap roots were greatly reduced in size and had lower carbohydrate concentrations. It is possible that both high cytokinin and sucrose content in younger plants subsequently had a negative effect on normal tap root development leading to a decrease in sucrose accumulation during later stages of growth. Adjusting the expression of the *ipt* gene with precisely regulated promoters may promote expansion of the cambial rings for increased sucrose accumulation.

2.6 Testing of the Activity and Stability of Inheritance of the Gene and Adverse Effects on Growth, Yield, and Quality

Laboratory and field evaluations of GM herbicide tolerant (GMHT) beets have indicated variability among transformants in terms of transgene expression (Steen *et al.*, 1986; Steen and Pedersen, 1995a; BBA, 2002), but given the insertion of a gene cassette into complex genome, this is not surprising. Early field trials of GMHT beets noted considerable variability in field evaluations of herbicide tolerance to glyphosate as well as in testing of resistance to BYNVV using a CP gene (Steen and Pedersen, 1993, 1995a; Tenning *et al.*, 1995). Some of the variance was ascribed to position effects based on the area of the genome where transgenes had inserted, however, evidence of breaks or crossovers were not uncommon (Pedersen and Steen, 1995; Steen and Pedersen, 1995b); this phenomenon is in no way unique to sugar beet.

Variation in insertion number and environmental influences were noted with respect to glyphosate tolerance, however, ploidy effects and the number of insertions did not appear to correlate with or influence observed tolerance to the herbicide. Gene silencing due to methylation of transgenes and post-translational mechanisms also likely played a role in observed variation of transformants evaluated in the greenhouse

and field. In some instances, adequate transgene expression in the greenhouse-screening protocols was lost or reduced in field evaluations. Alterations in morphological and physiological characters not associated with the transgenes were noted, but were generally rare among GMHT beet plants tested in the field for herbicide tolerance. Field trials in Denmark, France, England, and the United States in the early 1990s did not indicate any association between the presence of a transgene for glyphosate tolerance and alterations in root yield, sugar yield, polarization, or juice purity (Steen and Pedersen, 1995a). That is, while there were evidences of a yield enhancement from increased weed control, the transgene itself did not result in a significant metabolic cost or yield drag on the lines evaluated.

One of the early Roundup Ready beet lines submitted for approval of commercial use, Monsanto and Syngenta's GTSB 77, contained a stable insertion of a single copy of the *cp4 epsps* and *gusA* genes, however, the *gox* gene was found to be truncated during insertion and not capable of acting as a template for protein expression (FDA, 1998a). The line was eventually deregulated by the United States Department's Animal and Plant Health Inspection Service Agriculture (USDA-APHIS) for commercial use, but withdrawn from further consideration for approval by the European Union (EU), presumably due to questions with the remaining 69% of the *gox* gene present, but nonexpressed, in this line. It was never released commercially in the United States either despite the deregulation status obtained in 1999 (FR, 1999).

The more recently deregulated GMHT beet line approved for commercial use in the United States and Canada, H7-1, contains a single insertion of the *epsps* gene and does not contain the *gox* or *gusA* genes. Inheritance and gene expression with this line have proven to be stable and predictable (CFIA, 2005; FR, 2005). This sugar beet line (or any other GM beet) has not been approved for commercial cultivation in the EU as of this writing, but is awaiting food and feed approval. Compositional analyses of the H7-1 GMHT beet plants indicated that when comparing H7-1 to nontransgenic control plants, no differences were observed for ash, crude fiber, crude protein, dry matter, soluble carbohydrates, saponins, crude fat, acid detergent fiber, and neutral detergent fiber.

Processed sugar beet (brei) was also analyzed for sucrose, sodium, potassium, amino nitrogen, and invert sugar levels with none of the values exceeding the literature ranges for sugar beet (FDA, 1998a).

As sugar beets are selected in the course of a breeding scheme for agronomic characteristics and specifically for appropriate expression of the transgene(s), many individual transformants with varying copy number of insertions and possibly deleterious effects from tissue culture, have been rouged out of the population. Hence, the lines promulgated for experimental field trials are reasonably well characterized, predictable, and agronomically suitable. As regulatory authorities examine inheritance and stability of gene expression at this stage, most of the unwanted variability has been selected out and transgenes are typically single copy inserts which follow predicted Mendelian inheritance patterns (FR, 1998, 2005; CFIA, 2005).

3. FUTURE ROAD MAP

3.1 Expected Products

3.1.1 Disease resistance

Wider acceptance of transgenic technology within the sugar beet community will pave the way for the introduction of transgenic beets with improved defenses. The genetic base of the commercial sugar beet crop is quite narrow; the early beet sugar industry was based upon a few open-pollinated lines that had been selected for high sugar concentration. Additionally, heritability of some resistances is low and breeding efforts often come at the expense of other agronomic traits. Transgenic technology will allow for the introduction of foreign resistance genes to create new, more durable sources of resistance. In many instances, plants recognize invading pathogens through a gene-for-gene interaction in which a plant R gene (receptor) recognizes a pathogen avirulence (*Avr*) gene (effector) that activates downstream defense responses (DeFeyter *et al.*, 1993; Romeis *et al.*, 1999). Transfer of receptors using transgenic technology has been an effective means of generating new sources of resistance in crops. However, there are conflicting reports of

how well plant R genes function when transferred between plant families. The flax genes *L2*, *L6*, and *L10*, which confer resistance to *Melampsora lini* were transferred to tobacco. Only one allele, when overexpressed, had efficacy against tobacco pathogens. However, the resistance was broad spectrum and effective against *Cercospora nicotianae* and *Phytophthora parasitica* (Frost *et al.*, 2004). This is similar to an observation that a resistance gene from common bean conferring resistance to bean dwarf mosaic virus, when transferred to tobacco provided resistance to CaMV (Seo *et al.*, 2006). Both instances suggest some receptors have broad-range efficacy and function in diverse plant families. In contrast, *Bs2* from pepper, which confers resistance to *Xanthomonas campestris* retains function in solanaceous plants, but is nonfunctional in other families suggesting that some R gene functions are taxonomically restricted (Tai *et al.*, 1999). Within the same family, overexpression of an identified defense-related gene, *Pto*, in tomato increased resistance against the bacterial pathogen *Pseudomonas syringae* by constitutive activation of plant defense responses in the transgenic lines (Tang *et al.*, 1999).

Transfer of broad-spectrum resistance from other crops could provide a needed alternative for achieving durable levels of resistance in sugar beet. Some sugar beet resistance against the fungal pathogen *Fusarium oxysporum* appears to lack durability and their efficacy varies by geographic location (Linda Hanson, personal communication). Type I resistance against *Fusaria*-causing head blight in cereals, some of which infect sugar beet (i.e., *F. graminearum*, Hanson, 2006) is coded for by a major gene (Steed *et al.*, 2005). This gene would be an ideal candidate for use in transformation, because, currently, there is no resistance against *F. graminearum* in sugar beet. However, since the efficacy of R genes has been shown to be variable in other systems, candidates from other crop systems should also be evaluated including those from corn (Isebaert *et al.*, 2005) and soybean (Iqbal *et al.*, 2005).

Transgenic technology can similarly provide approaches for the transfer of resistance genes between sugar beet varieties to generate lines with resistance to multiple pathogens. Transfer of resistance from wild relatives to cultivated crops has been a successful approach for cereals

(Rubiales *et al.*, 2001). Several wild relatives of sugar beet contain resistance to major sugar beet pathogens. Introgression of these genes through traditional breeding can be very difficult, especially when trying to transfer genes between an annual relative and the biennial cultivated beet. In addition, when cyst nematode resistance from a wild relative (*B. procumbens*) was introgressed into sugar beet, it had a significant negative impact on yield (Schulte *et al.*, 2006). Transfer of the resistance gene through genetic modification may provide a cleaner transfer that does not impart the same deleterious effects. Rhizomania, caused by BNYVV, is a major threat to sugar beet production worldwide. Resistance sources within sugar beet are limited (Lewellen *et al.*, 1987) and appear to be losing efficacy in certain areas. The wild beet *B. vulgaris* subsp. *maritima* contains additional sources of resistance (Gidner *et al.*, 2005) and genetic modification may provide the best avenue for integrating these genes into sugar beet, including possible pyramiding of the R genes. There are great efforts being made to identify novel sources of resistance to a wide array of pathogens in wild beet relatives for future use in sugar beet (Francis and Luterbacher, 2003; Panella and Lewellen, 2006), which may be introduced with transgenic technology.

Lastly, to create novel disease control strategies, transgenic technology can be used to overexpress resistance-associated proteins to increase host resistance. Expression of an allele of tobacco chitinase in strawberry created a new line of defense against gray mold, for which strawberry has no natural lines of defense (Vellicce *et al.*, 2006). Even though chitinase reduced disease severity by approximately 50%, overexpression of two other defense-related proteins, a glucanase and thaumatinlike protein, did not enhance resistance, suggesting that not all proteins induced during the resistance response may be directly responsible for disease control. Furthermore, even though chitinase is associated with antifungal responses, overexpression of the tobacco gene did not confer increased resistance against *Colletotrichum acutatum*, suggesting some pathogen specificity of the chitinase alleles. Transfer of a defense-related protein from pepper to *Arabidopsis* created transgenic lines with accelerated growth, and increased tolerance to several biotic and abiotic stresses (Lee and Hwang, 2006). Overexpression of

defense-related proteins never provides complete immunity against invading pathogens, therefore, transformation of lines with existing resistance capabilities may result in greater efficacy of the overall defense response.

Several researchers are investigating key components induced in sugar beet in defense against an array of different pests and pathogens, including: sugar beet root maggot (Puthoff and Smigocki, 2007), *C. beticola* (Nielsen *et al.*, 1997; Lee Panella, personal communication), *R. solani* (Nagendran and McGrath, 2006), BNYVV, and *F. oxysporum* (Larson *et al.*, 2007). Cross-comparisons between resistance responses of sugar beet to several pests and pathogens will allow for selection of a wide array of resistance-associated genes that can be manipulated in hopes of developing broad-spectrum resistance in transgenic sugar beet.

3.1.2 Insect resistance

Cytokinins are known to influence numerous physiological and biochemical plant processes as well as plant defense responses. Cytokinin applications have been correlated with the subsequent accumulation of secondary metabolites, many of which are known to be insecticidal (Hallahan *et al.*, 1992; Decendit *et al.*, 1992; Chilton, 1997). In field trials, an overall increase in yields was attributed in part to reduced insect populations which were correlated with the concomitant accumulation of four secondary metabolites with known insecticidal properties (Hedin *et al.*, 1988; Hedin and McCarty, 1994). To specifically focus on the effects of elevated cytokinin levels on insect resistance, the *ipt* gene was fused to a wound-inducible promoter for expression in transgenic plants (Smigocki *et al.*, 1993; Smigocki, 1995). The 70-fold higher cytokinin levels in late-flowering *Nicotiana* were correlated with resistance to a number of insects (Smigocki *et al.*, 1993). Insecticidal activity was localized primarily to leaf surfaces that upon chemical analysis revealed compounds suggestive of oxygen-containing aliphatic molecules in the molecular weight range of diterpenes (Smigocki *et al.*, 1997, 2000). These *Nicotiana* compounds killed almost all SBRM larvae, a major sugar beet pest in the central and western United States and Canada. These results suggest that cytokinin-mediated

insect resistance could potentially provide effective SBRM control.

Assimilation of dietary nutrients and proteins from ingested plant tissues is critical to insect survival. Inhibition of digestive proteases, therefore, is yet another potential target for development of effective strategies for insect control. Digestive proteinase inhibitors (PI) are enzymes that occur naturally in many plants (Jongsma and Bolter, 1997; Christeller *et al.*, 1998; Haq *et al.*, 2004). PI genes have been cloned, reconstructed and expressed in transgenic plants, and shown to enhance insect or nematode tolerance (Maqbool *et al.*, 2001; Cowgill *et al.*, 2002a, b; Samac and Smigocki, 2003). Several coleopteran pests that commonly use cysteine proteases for protein digestion, as well as nematodes, were inhibited by cysteine PI genes of the oryzacystatin I (*OCl*) and II (*OClI*) PI gene family (Kondo *et al.*, 1990; Michaud *et al.*, 1995; Samac and Smigocki, 2003). Clones of trypsin/serine PIs were shown to specifically enhance resistance to pests that use serine digestive proteases (Heath *et al.*, 1997). A *Nicotiana glauca* gene, post-translationally cleaved into five individual trypsin (serine) PIs, was overexpressed in transgenic plants and effectively targeted insects representing four different insect orders (Charity *et al.*, 2005). Simultaneous overexpression of two classes of PI genes provided resistance to more than one insect in tomato (Abdeen *et al.*, 2005). Similarly, additive effects of a cowpea trypsin inhibitor paired with a pea lectin or a *Bt* gene and of a serine PI gene with a *Bt* gene enhanced resistance to several insects (Boulter *et al.*, 1990; MacIntosh *et al.*, 1990; Zhao, 1996). These studies suggest that pyramiding the expression of PIs with defense genes with different mechanisms of action should provide an advanced strategy in pest management as it should be more effective in overcoming a general adaptive response in the insect. Gene pyramiding is also proving to be useful for addressing the limitations that are being encountered with cultivation of genetically engineered plants, mainly buildup of resistance in the targeted pest or pathogen (Mehlo *et al.*, 2005).

SBRM is the most devastating insect pest of sugar beet in North America. Developing larvae feed on tap and feeder roots throughout the growing season inflicting significant crop damage either by completely severing the roots of seedlings or badly scarring the larger roots (Dregseth *et al.*,

2003). Further damage is inflicted on damaged tap roots by opportunistic pathogens such as *E. betavascularum*, *Aphanomyces cochlioides*, and *R. solani*. *Erwinia* infections alone can reduce sugar yields by as much as 10–15%, not including losses during postharvest storage of the roots. Effective SBRM control measures are lacking (Yun, 1986; Campbell *et al.*, 2000). Two major classes of digestive enzymes were identified as targets for control of the SBRM (Wilhite *et al.*, 2000). They were shown to be effectively blocked by several plant PIs that included a soybean trypsin-chymotrypsin and a squash aspartyl inhibitor. Pyramiding the expression of the corresponding PI genes in sugar beet roots is an approach that may prove effective for enhancing resistance to SBRM. Various PI gene constructs have been introduced into sugar beet hairy roots and are in the process of analysis for insect and disease resistance (Smigocki, 2008).

3.1.3 Defense response genes

Research efforts focused on discovering and characterizing sugar beet root defense responses will lead to the discovery of the underlying genetic and molecular mechanisms controlling resistance. An in-depth understanding of molecular basis of interactions between arthropods and plants is crucial for development of novel control strategies for invasive and re-emerging pests that are not dependent on environmentally persistent and damaging pesticides (see reviews Stotz *et al.*, 1999; Baldwin *et al.*, 2001; Kaloshian, 2004; Kaloshian and Walling, 2005). Studies on root responses to insect attack are less represented primarily due to the limitations imposed by the complexity of the rhizosphere environment (Wu *et al.*, 1999; Bird and Kaloshian, 2003; Puthoff *et al.*, 2003; Williamson and Gleason, 2003; Bird, 2004). Sugar beet and SBRM are being utilized as a model system for studying the molecular aspects of root–insect interactions and to gain knowledge of root resistance mechanisms (Puthoff and Smigocki, 2005, 2007). Profiling of genes whose expression is modulated by insect feeding has been primarily studied utilizing *Arabidopsis* gene chips, and only a few studies examined the transcriptional profiles induced in the insect's natural host (Hui *et al.*, 2003; Puthoff *et al.*,

2005; Wang *et al.*, 2005). In each of these studies, specifically up-regulated genes were identified in a pool of responsive genes.

Expressed sequence tag (EST) libraries enriched for genes involved in the initial responses of sugar beet roots to insect herbivory were generated from a breeding line showing moderate resistance to SBRM and from a susceptible line (Puthoff and Smigocki, 2007). Many of the identified genes were found to be regulated by other pathogens including insect pests. These included a polyphenol oxidase gene (Voelckel and Baldwin, 2004), a β -glucosidase gene (van de Ven *et al.*, 2000), a glutathione S transferase gene (Reymond *et al.*, 2000), a subtilisinlike serine protease gene (Tornero *et al.*, 1996), and osmotinlike protein gene (Zhu *et al.*, 1995). In addition, regulation of pathogenesis-related (PR) protein genes was found and included a chitinase gene, oxalate oxidaselike genes, peroxidase genes, osmotinlike genes, and a PR10-like gene. While PR protein genes are typically induced after microbe infections they have also been found to be regulated by insect feeding (Hui *et al.*, 2003; Voelckel and Baldwin, 2004). Only a single gene coding for a PI with similarity to serine PIs was cloned from the moderately resistant line. Interestingly, since serine protease activity has been shown to be one of the major activities in SBRM larval guts (Wilhite *et al.*, 2000), these results strongly suggests that it may play a role in resistance and warrants further investigation (Smigocki, 2008). The identification of genes following SBRM larval feeding on moderately resistant varieties allows for the formulation of testable hypotheses to gain knowledge on root defense responses and the mechanism of resistance. This valuable information will lead to development of alternate control measures based on biotechnological approaches thus providing new tools to breeders that will ultimately broaden pest and disease resistance of sugar beet and be more generally applicable to other crops and diseases.

3.1.4 Sucrose accumulation

In order to enhance storage and utilization of sucrose in the tap root, an understanding of basic mechanisms controlling the metabolism of carbon in sugar beet is needed. A plant SNF-1

(sucrose nonfermenting-1, SnRK1) protein kinase has been shown to play a central role in the signal transduction pathway linking the perception of cellular glucose levels with the derepression of a range of glucose-repressible genes (Monger *et al.*, 1997; Purcell *et al.*, 1998; Schwachtje *et al.*, 2006). It has been demonstrated in transgenic potato that expression of an antisense *SnRK1*-related sequence decreased sucrose synthase gene expression and eliminated sucrose inducibility of the sucrose synthase enzyme. SnRK1 protein kinases have also been implicated in regulation of other important metabolic enzymes, among them nitrate reductase that affects assimilation of nitrogen into amino acids and sucrose phosphate synthase, the most important enzyme for sucrose synthesis in plants (Halford and Hardie, 1998). Taken together, these findings strongly implicate a role for the SnRK1 enzyme as a significant regulator of carbohydrate metabolism that may be exploited to increase sucrose storage and accumulation in sugar beet (Purcell *et al.*, 1998). Interestingly, Schwachtje *et al.* (2006) recently demonstrated that antisense suppression of SnRK1 in *N. attenuata* transgenic plants altered resource allocation to the roots. Transgenic plants were more tolerant to insect attack, suggesting that this tolerance mechanism complements the likely defensive value of diverting resources to a less vulnerable location within the plant.

3.1.5 Synthesis of novel starches

Preliminary research regarding starch synthesis in sugar beet is being addressed (Monger *et al.*, 1995). Even though starch is transiently made in sugar beet leaves, no starch accumulates in the roots of any *Beta* species. Understanding starch synthesis in sugar beet may enable novel types of starches suitable for industrial purposes to be made. This would provide yet another alternative role for producing value added products in sugar beet.

3.1.6 Biofuels

Due to its high productivity, sugar beet is becoming increasingly interesting not only as a source of sugar, but also as a possible “green

bioreactor”, i.e., for synthesis and accumulation of new metabolites in roots (Sévenier *et al.*, 1998; Menzel *et al.*, 2003). Reports at the Third Annual World Congress on Industrial Biotechnology and Bioprocessing indicated that industrial technology is expected to account for about 10% (\$125 billion in value) of sales within the chemical industry (Agricultural and Environmental Biotechnology Conference, Melbourne, Australia, 2006). Much of the projected growth is expected to come from industrial biotechnology that includes products made from bio-based feedstocks or through fermentation or enzymatic conversion. Biofuels such as ethanol and biodiesel will significantly contribute to the growth. Currently, the production of biofuels is not cost effective because more energy appears to be required to produce alcohols (ethanol, butanol, methanol) from sugar beet than can be extracted from it. However, with the application of biotechnology, higher conversion technology at a lower cost is anticipated to allow for broader commercialization with meaningful production rates.

3.2 Addressing Risks and Concerns

Field trials of GMHT sugar beet have been conducted since 1990 in Europe and shortly thereafter in the United States; however, concerns over consumer acceptance of sugar in human foods and pulp products and molasses as feed has led most sugar processors to forego the technology and agronomic enhancements offered (Bennet *et al.*, 2004). While the sucrose produced from processing GMHT sugar beet cannot be differentiated with sensitive diagnostic tests from that produced by organic or conventionally cropped beets, the EU and Japan both mandate that sugar from sugar beets be labeled as GM (EP, 2003; MAFF, 2006), however, the processed molasses and sucrose will not require labeling for Japan (BSDF, 2006; FAS, 2006). Japan, the EU, and other countries have previously raised concerns over importation of sugar beet pulp derived from GM beet, but progress in resolving these issues continues to evolve. Still, despite the lack of detectable DNA or protein associated with this genetic modification in processed sucrose and pulp or molasses products (Klein *et al.*, 1998; FSANZ, 2006), EU directives will require labeling

of all foods that may contain products derived from GM beets. In accord with the Japanese ruling, the Australian and New Zealand Food Standards agency has required that only materials derived from GM beet, which indicate the detectable presence of GM protein or DNA, need to be labeled. Hence, molasses, sugar, and pulp wherein the DNA or protein cannot be detected do not require special labeling procedures (FSANZ, 2004).

With respect to risk assessment and management of GMHT sugar beet, there is a startling contrast in approach between the EU and US regulatory systems with respect to weed management and environmental considerations. The Farm Scale Evaluations (FSE) conducted in the United Kingdom, which considered GMHT beet, potato, and oilseed rape as comparators to their nonengineered counterparts, assessed the impacts of weed management (i.e., decline in weed type and number) on wildlife associated with the agroecosystem (Heard *et al.*, 2003; Squire *et al.*, 2003). While the FSE compared GMHT beet to nontransgenic controls over a substantial growing area, the real comparator was weed control and not the transgene directly. That is, a decrease in weeds and the ensuing effects (e.g., availability of seeds for granivorous birds and insects, biodiversity within agricultural fields) may well be attributed to any mechanism (e.g., cultivation, traditional chemical herbicides, planting dates, etc.), which controls weed populations regardless of the mode of action. A resultant measure stemming from the FSE is the introduction of cropping schemes wherein a small percentage (e.g., 5%) of the GMHT beet field is intended not to receive glyphosate or glufosinate applications as a means of maintaining a food source for birds and other wildlife. It is not clear if effects of traditional herbicide regimes were previously evaluated for their impacts on wildlife prior to the advent of GM technology or if new chemical herbicides will undergo the same scrutiny in the EU system as GMHT crops.

In contrast to this situation in the EU, in the United States, herbicides are registered under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) by the US Environmental Protection Agency (US EPA) with the expectation that efficacy in weed control may result in an absence of weeds throughout an agricultural field. FIFRA is a cost:benefit statute, which relies on a standard

of no significant adverse environmental effect in decision making relative to pesticide registration. As such, use of broad-spectrum herbicides like glufosinate and glyphosate are expected, even intended, to result in a relatively clear field with respect to weed population numbers. This difference may be in part a result of differing perspectives on what constitutes “nature” or wild lands between the EU and the United States (Madsen *et al.*, 2005).

Another dichotomy noted between the EU and United States regards the issue of coexistence of crop types that are engineered with those intended to be GM free (i.e., organically cultivated). The USDA’s National Organic Program (NOP) is process based, meaning that if all aspects of planting, cultivation, and harvest were performed according to a NOP certified organic program, the resulting produce derived may be labeled as organic despite the possible presence of GM traits from wind or insect vectored pollen. There is currently no organic sugar production in the United States to speak of, but this is not the case in the EU and other countries. The EU has established separation distances for GM beet of 1.5 m from conventional beet and 3.0 m from certified GM-free beet (i.e., organic) or seed production fields. This distance is established with the expectation that the GM beets will not contain bolters (i.e., flowering plants) as a source of pollen and the GM-free beets will similarly not contain flowering beets.

Gene flow from commercial beet plantings to weed beets (e.g., *B. vulgaris* ssp. *maritima*; *B. macrocarpa*) can be affected by wind-borne pollen over distances of at least a few kilometers (Boudry *et al.*, 1993; Bartsch and Pohl-Orf, 1996; Bartsch *et al.*, 2002). Weed beets are present in coastal areas in Europe and in portions of sugar beet growing areas of California in the United States. Although most commercial fields and experimental trials with GMHT or rhizomania resistant beet are not likely to contain bolters, nevertheless, there are occasions when pollen may be exchanged in either direction between *B. vulgaris* ssp. *vulgaris* and weed beets with the expression of transgenes in the hybrid progeny (BBA, 2002).

While the presence of a transgene expressing an herbicide tolerance mechanism in a weed beet appears to offer little or no selective advantage to the recipients of this trait in the absence of

herbicidal treatment, the establishment of feral or ruderal beets with herbicide tolerance can lead to weediness issues in subsequent crops including beets (Bartsch *et al.*, 2002). Additionally, flowering weed beets or chard present in seed production areas can contribute to genetic stock used in cultivar development. Sequential cropping with Roundup Ready and Liberty Link crops may further select for resistant weed type beets. Those with an annual habit may exacerbate this issue as they continue to flower in field wherein commercial beets typically would not. Due in part to this situation, applicants to the EU have agreed to follow a stewardship plan in which deployment of GMHT beet and other HT crops are managed and monitored so that selection of weeds for herbicide tolerance are precluded or minimized (Bartsch *et al.*, 2002; EFSA, 2006).

While these types of stewardship and monitoring plans may not be a formal part of the requirements stemming from deregulation of GMHT beets or the labeling amendments for the herbicides applied to HT crops in the United States, applicants for deregulation will be monitoring for development of herbicide-tolerant weeds within the agroecosystem. As Bartsch *et al.* (2002) point out, the EU comprehension of nature conservation includes agricultural areas. This again may explain in part the differences in regulation and assessment present in the EU and the US relative to GM crops.

3.3 Expected Technologies

3.3.1 Tissue-specific promoters

Transformation technology is evolving at a rapid pace. Not only is the transformation efficiency increasing, but the timing and tissue specificity of expression can be controlled by using specialized promoters thus minimizing the possible negative effects that may be associated with constitutive transgene expression in engineered plants. In sugar beet, several tissue specific promoters have been characterized that include the chlorophyll a/b binding protein promoter for high level of leaf-specific expression (Stahl *et al.*, 2004) and several root-specific promoters showing either restricted expression in the vascular tissue or throughout the root (Oltmanns *et al.*, 2006). Additional source

of root-specific gene promoters was identified when clones derived from a root-specific EST library were analyzed for expression patterns in various root tissues (Smigocki, unpublished results). Heterologous promoters are also useful for expressing beneficial genes in sugar beet. A *Medicago truncatula* root-specific promoter from gene *MtHP*, which shares sequence similarity to PR10 multigene family (Xiao *et al.*, 2005) was shown to be constitutively expressed at high levels in sugar beet hairy roots and may prove to be a useful alternative to the 35S promoter for high levels of constitutive expression in roots of transformed plants (Figure 1e; Smigocki, unpublished results). Tissue-specific promoters are highly desirable for maximizing expression at site of interest and will be effective for targeting beneficial transgene expression to tissues most prone to insect and pathogen attack and for manipulating sucrose production in sugar beet roots.

3.3.2 Inducible promoters

As mentioned above, constitutive overexpression of some genes can have detrimental effects on plant growth and development. Some genes need only be induced at certain stages in growth. Tighter control of gene expression will be achieved by using inducible promoters. Using a promoter from a heat-shock protein allows for heat-induced gene expression in *Arabidopsis* (Masclaux *et al.*, 2004). This system requires incubation at high temperatures with high humidity and has worked better with *in vitro* cultures than mature plants, which would be more suited for laboratory studies rather than large-scale field production. Several chemical inducible promoters are also available including those activated by application of tetracycline, dexamethason, copper, tebufenozide, oestrogen, and ethanol (Moore *et al.*, 2006). Accumulation of hexoses at the expense of sucrose in beet taproots during storage is caused by invertase activity (Rosenkranz *et al.*, 2001). Additionally, the concentration in sugar beet tap roots is negatively affected by sucrose synthase activity (Klotz and Campbell, 2004). The potential use of inducible promoters to produce inhibitors of these enzymes during storage could be of substantial benefit to sugar beet processors since upwards of 250 g of sucrose is lost per ton

per day while the roots are held in storage, in some cases for longer than 200 days.

3.3.3 Excision of antibiotic resistance

Antibiotic resistance as a means of selection has been a staple tool in the development of transgenic organisms. However, antibiotic resistance is not desired in the final product. It raises concerns about gene flow to soil and gastrointestinal microorganisms and weeds within the neighboring fields. Elimination of the selectable marker may increase the willingness of the public to accept transgenics. Several methods widely employed in the past were time consuming and transformation process dependent, therefore, the procedures were not widely applicable across plant species. Recently a chemical-inducible DNA excision system was developed that allows for site-specific excision of marker sequences (Zuo *et al.*, 2001). With this system, treatment with β -estradiol activates transcription of a Cre recombinase. This enzyme excises the unwanted sequence through recombination using the *loxP* sites that flank the ends of the selectable marker coding region (Dale and Ow, 1991) and now the selectable marker and *cre* coding sequence as well (Zuo *et al.*, 2001). This approach has been successful in *Arabidopsis* (Zuo *et al.*, 2001), rice (Sreekala *et al.*, 2005), and tomato (Zhang *et al.*, 2006).

3.3.4 Virus-based gene vectors

Plants provide cost-effective platforms for production of therapeutics or other value added products. A limiting factor for this approach has been the inability to produce large quantities of the product in plants. Most recently, Giritch *et al.* (2006) demonstrated that the production of heavy and light chains of the human IgG antibody was expressed in 85% versus 5% of the *N. benthamiana* cells if each gene (i.e., one for heavy and one for light chain) was expressed on two different viral plant vectors (tobacco mosaic virus and potato virus X). The overall yield of the protein product was increased 10-fold using this dual-vector system. Similar approach can be explored in sugar beet for production of value added products using, for example, the beet mosaic virus

(BtMV). BtMV is a member of the economically important potyvirus group of plant viruses that infects sugar beet and its close relatives. It is distributed worldwide in all major beet-growing areas and, in mixed infections with certain other viruses, causes severe stunting and yield losses on susceptible sugar beet varieties. The complete nucleotide sequence of BtMV genomic RNA has been determined and will facilitate accurate diagnosis and differentiation of the virus as well as development of virus resistant plants (Nemchinov *et al.*, 2004). In addition, the BtMV virus can be engineered to transiently express useful proteins of interest in sugar beet. Production in sugar beet of effective biomedical products for control of human and animal diseases, as well as other useful products, should provide an environmentally friendly and relatively inexpensive approach for production of new and raw materials.

3.4 Specific Regulatory Measures

Within the United States' Coordinated Framework for Regulating Biotechnology (<http://usbiotechreg.nbii.gov>), three agencies have principal authority and oversight for experimentation, culture, and food or feed production involving transgenic sugar beets. USDA-APHIS performs environmental assessments for genetically engineered crops like sugar beet under authority of the National Environmental Policy Act and the Plant Protection Act. USDA-APHIS has performed an environmental assessment on both Roundup Ready and Liberty Link sugar beets, reaching a finding of no significant impact in both instances. Both Roundup Ready sugar beet (FR, 1999, 2005) and Liberty Link sugar beet (FR, 1998) have been deregulated by the agency, meaning that they are cleared for commercial purposes to be cultivated without further regulation by the USDA. The Monsanto beet line GTBS77 was deregulated in 1999, but has not been commercially cultivated (Russell P. Schneider, Monsanto Company, personal communication). Line H7-1 has recently been approved by the agency for commercial use (FR, 2005).

Approval has been granted by the US EPA under FIFRA to amend the pesticide labels for glyphosate and glufosinate so that they may be applied to GMHT sugar beets. As part of

this process, the US EPA has also performed an analysis to determine that a food and feed tolerance has been granted for herbicide residues that may exist on plant materials derived from these GMHT beets under the Federal Food, Drug and Cosmetic Act (FFDCA).

Voluntary consultations with the Food and Drug Administration (FDA) have also been conducted for the use of the herbicide tolerance mechanisms (e.g., EPSPS, PAT proteins) resulting from the genetic modifications of Roundup Ready beets (FDA, 1998a, 2004) and Liberty Link (FDA, 1998b) sugar beets. The FDA consultation reviews the similarity of the GMHT beet to conventional beet cultivars to determine if the genetically engineered line has significantly altered nutritional or biochemical constitution. Given the use of these herbicide resistance mechanisms in other crops previously, it is not surprising that FDA did not find any cause for concern with respect to food or feed safety from using GMHT beet.

In 2006, Roundup Ready beets were grown on approximately 250 acres in the United States and will be processed through a standard processing plant in the spring of 2007 (BSDF, 2006). A similarly sized acreage of Roundup Ready beets will be cultivated in the United States in 2007 and again processed through a standard sugar beet processing facility with segregation of products such that study and distribution may proceed. Larger acreages of Roundup Ready beets are planned for 2008 in the United States. The situation and progress in the EU are less clear.

In a similar finding, the Canadian Food Inspection Agency has approved Roundup Ready sugar beet for unconfined environmental release and use as livestock feed (CFIA, 2005). Health Canada has separately reached a conclusion of safety for food uses of Roundup Ready sugar beet (HC, 2005).

Several field trials with GM beet expressing resistance to BNYVV have been conducted in the United States, Sweden, Denmark, France, the United Kingdom, Spain, Greece, Iran, and other countries under various regulatory authorities and guidelines. Regulatory variances among countries and even within a country or collective regulatory body (e.g., EU/EC) have had an impact on the development and deployment of GM technology in sugar beet. Following legal changes stemming from political influences, sugar beet variety trials

with GM beet in the EU were abandoned in 2000 (Märländer, 2005). Subsequently, the glufosinate-tolerant beet was removed from official application for consideration of market access.

The US EPA is currently revising its policies regarding registration of plants expressing viral CPs for resistance to viral infection. It is not clear at this juncture if the BNYVV-resistant sugar beet lines field tested for suitability will need to undergo a full registration process prior to approval for commercial release or meet established exemption criteria. Previous viral-resistant plants were assessed through the USDA-APHIS and were considered as exempt by US EPA from the requirement of a food tolerance action under FFDCA without the need for FIFRA Section 3 registration, so US EPA action on these products was minimal. That may well change as new measures are instituted in 2007. This action could seriously impact the timing of deployment of BNYVV and other virus resistant sugar beets in the United States.

Differences inherent in the statutory mandates and in guideline tests required for approval of field releases and food/feed use require significantly more time to satisfy on a country-by-country basis with the lack of regulatory harmonization. Although the US EPA, USDA-APHIS, Canada's CFIA and Health agency, Organization of Economic Cooperation and Development (OECD), and others strive to unify the requirements for commercial application of this technology, on a global scale, relatively little progress has been made. In addition to adding cost to the process, these differences lend themselves to fostering a lack of confidence in regulatory officials and managers making decisions about environmental and health issues. This, in part, may explain much of the dichotomy between the EU and the United States with respect to GM approvals of beet and other crops (FR, 2004; DeVuyst and Wachenheim, 2005; Madsen *et al.*, 2005).

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Stevia

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1. INTRODUCTION

The worldwide demand for high-potency sweeteners is expected to increase. With the new practice of blending different sweeteners, the demand for alternatives is expected to increase. The sweet herb of Paraguay, *Stevia rebaudiana* Bertoni, produces in its leaves just such an alternative with the added advantage that stevia sweeteners are natural plant products. In addition, the sweet steviol glycosides have functional and sensory properties superior to those of many other high-potency sweeteners. Stevia is likely to become a major source of high-potency sweetener for the growing natural food market in the future. The task at hand is to convert stevia from a wild plant to a commercial crop well suited to efficient mechanized production. The necessary steps are the development of seed, seedling, and crop production system, including information on optimized crop inputs, weed and disease control, harvest and handling methods, and a breeding program aimed at optimizing glycoside content and sensory characteristics. Understanding the biology of the stevia plants and the chemistry of the sweet glycosides together with application of biotechnology techniques are prerequisites for conversion of stevia to a commercial crop.

1.1 History, Origin, and Distribution

Stevia is a small bush native to the valley of the Rio Monday in highlands of Paraguay, between 25°

and 26° South latitude, where it grows in sandy soils near streams (Katayama *et al.*, 1976). The leaves have been traditionally used for hundreds of years in Paraguay and Brazil to sweeten local teas, medicines, and as a “sweet treat”. Dr M.S. Bertoni botanically described and named the plant in 1905 in honor of Paraguayan chemist Dr Ovidio Rebaudi while “stevia” was adopted from the name of Dr Peter James Esteve (a Spanish botanist) who died in 1566. Stevia is one of the 950 genera of the Compositae (Asteraceae) family. There are now more than 154 *Stevia* species but *S. rebaudiana* is the only one species with significant sweetening properties due to sweet steviol glycosides (Robinson, 1930; Soejarto *et al.*, 1982, 1983); other species do contain other biochemicals of interest. The first reports of commercial cultivation in Paraguay were in 1964 (Katayama *et al.*, 1976; Lewis, 1992). A large effort aimed at establishing stevia as a crop in Japan was begun in 1968. Since then, stevia has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, the United States, Indonesia, Tanzania, and since 1990 Canada (Shock, 1982; Goenadi, 1983; Brandle and Rosa, 1992; Fors, 1995). The species of stevia are found in the wild in semiarid habitats ranging from grassland to mountain terrain.

For nearly 20 years, millions of consumers in Japan and Brazil, where stevia is approved as a food additive, have been using stevia extracts as safe, natural, and noncaloric sweeteners. Japan is the largest consumer of stevia leaves and extracts

(steviosides) in the world, and there it is used to sweeten everything from soy sauce to pickles, confections, and soft drinks. Even multinational giants like Coca-Cola and Beatrice Foods use stevia extracts to sweeten foods (as a replacement for NutraSweet and saccharin) for sale in Japan, Brazil, and other countries where it is approved as a food additive. Other countries use lesser quantities of steviosides. Steviosides have zero calorie and can be used wherever sugar is used, including in baking, etc. They are ideal for diabetic and low-calorie diets; in Japan “Diet Coke” uses steviosides.

Currently, stevia production is centered in China and the major market is in Japan (Kinghorn and Soejarto, 1985). No large-scale mechanized production has been established and stevia sweeteners are not yet found in mainstream food products in most countries of the world. Progress toward large-scale commercialization has been slow, largely due to difficulties in producing the crop, the poor quality of stevia extracts, and the absence of regulatory approvals essential for stevia sweeteners in the North American and European markets.

Both the sweetener and the stevia plant *S. rebaudiana* Bertoni (also known as *Eupatorium rebaudianum* Bertoni) are known and pronounced as “stevia” in English-speaking countries as well as in France, Germany, Italy, Portugal, and Sweden; although some other countries use different names. Similar pronunciations occur in Japan (“sutebia” in Katakana), and in Thailand (“satiwia”). In some countries, India, for example, the name translates literally as “sweet leaf”. Below are some names for the stevia plant in various regions of the world:

- China: *Tian ju*—sweet flower and *Tian ju ye*—stevia leaf.
- English-speaking countries: candy leaf, sugar leaf, sweetleaf (the United States), sweet honey leaf (Australia), and sweet herb of Paraguay.
- German-speaking countries (also Switzerland): *Süßkraut*, *Süßblatt*, and *Honigkraut*.
- India: *Madhu Parani* (Marathi), *Madhu Patra* (Sanskrit), *Seeni Tulsi* (Tamil), and *Madhu Patri* (Telugu).
- Italy: *Piccolo arbusto con foglia dolce*.
- Japan: *Amaha sutebia*.

- Portuguese-speaking countries: *Capim doce*, *Erva doce*, *Estêvia* (Brazil), and *Folhas da stêvia*.
- Spanish-speaking countries: *Caá-ché*, *Hierba dulce*, *Ka’a he’ê* (Guaraníes—natives of Paraguay), *Stevia del norte de Paraguay*, and *Yerba dulce*.
- Thailand: *Satiwia* (*Ya wan*—Bangkok).

1.2 Botanical Description

Stevia is a member of the Compositae (Asteraceae) family (Gentry, 1996). It is a small shrubby perennial growing up to 65 cm in height, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrated above the middle. Trichome structures on the leaf surface are of two distinct sizes, one large (4–5 μm) and one small (2.5 μm) (Shaffert and Chetobar, 1994). The flowers are small (7–15 mm), white, and arranged in an irregular cyme. The seed is an achene with a feathery pappus (Robinson, 1930).

Stevia is an obligate short-day plant with a critical day length of about 13 h. Extensive variability within populations for day-length sensitivity has been reported (Valio and Rocha, 1966; Zaidan *et al.*, 1980). Plants can initiate flowering after a minimum of four true leaves have been produced (Carneiro, 1990). The reproductive anatomy of the male and female gametophytes is typical for angiosperms (Shaffert and Chetobar, 1992, 1994). Stevia is diploid and has 11 chromosome pairs ($2n = x = 22$), with regular karyotype. This is characteristic of most of the South American members of the genus (Frederico *et al.*, 1996), and reported widely. However, numbers of $2n = 24, 33, 34, 44, 48, 66$, and 70 have also been observed (Darlington and Wylie, 1955; Bolkhoviskikh *et al.*, 1969; Moore, 1973, 1974, 1977; Golblatt, 1981, 1984, 1985, 1988; Golblatt and Johnson, 1990, 1991, 1996, 1998). Recent studies from Oliveira *et al.* (2004) have also confirmed the chromosome number of $2n = 22$ previously reported by Frederico *et al.* (1996) and Monteiro (1980, 1982).

1.3 Economic Importance

The natural habitat of stevia is semihumid subtropics on the Tropic of Capricorn (22–23° South latitude), 200–400 m above the sea level,

with 1500–1800 mm of rainfall and temperature extremes of -6°C to 43°C . It naturally grows in low-lying areas on poor sandy, acidic soils adjacent to swamps, and so is adapted to and requires constantly wet feet or shallow water tables (Shock, 1982; Colombus, 1997; Oddone, 1999). Under cultivation and on more fertile soils, the mature plant can be larger, up to 1.8 m with up to 20 branches per plant (Jia, 1984). Vegetative growth is reduced when temperatures are below 20°C and when day length is less than 12 h. Increasing day length to 16 h and increasing light intensity can increase vegetative growth and stevioside levels (Metivier and Viana, 1979; Yermakov and Kochetov, 1996). Flowering is photoperiod dependent and is enhanced by reducing day length and short-day length. Responses of flowering, and also stevioside content, to day length appear to be variable with some eight cultivars/selections being obligate short-day plants (Brandle *et al.*, 1998); however, some lines appear to be photoperiod insensitive. Stevia has been successfully taken to a wide range of climatic locations around the world and apparently grown successfully, although often by using vegetative propagation methods and seedling establishment in a greenhouse before planting in the field. The typical agronomic research locations have been listed from Russia, Ukraine, North China, Canada, Georgia, California, Korea-Suweon, central China, Japan, Mexico, Taiwan, Thailand, India, Brazil, and Paraguay (Shock, 1982; Goenadi, 1987; Shu, 1989; Gvasaliya *et al.*, 1990; Matejka, 1992; Shyu *et al.*, 1994; Fors, 1995; Chalapathi *et al.*, 1997; Colombus, 1997; Dzyuba, 1998; Oddone, 1999).

Shading has been shown to reduce growth (Slamet and Tahardi, 1988), though factors other than latitude and solar radiation usually limit production. Humidity is reported to be important at some locations as the plant has poor tolerance to water stress; it often visibly wilts under hot, dry conditions even with good soil moisture levels. Rainfall in many locations is supplemented by irrigation to avoid any moisture stress. Only in the more humid and wet areas, rainfall may alone be sufficient for adequate growth for commercial production.

Climatic requirements, of day length and temperature, are different for maximum vegetative production and for maximum flowering and seed production. Soils of the natural habitat are

generally low-fertility, acidic sands (pH 4–5) with shallow water tables and little organic matter, where plants grow to 0.6–0.7 m in height. Under cultivation on better, more fertile soils, growth can increase up to 1.0 m and even up to 1.8 m (Jia, 1984). Soils should be well drained but with reasonable water-holding capacity and preferably with pH 5–7; alkaline soils should be avoided (Oddone, 1999).

1.3.1 Propagation

In the wild, stevia regenerates from seed, from the rooting of plant stems touching the ground (and trampled into the ground), and from regeneration at the base of the plant (crown division). Seed germination is notably very poor, commonly due to sterile seed. Some plant varieties/selections produce virtually no viable seed due to self-incompatibility.

Under cultivation, stevia can be propagated by seed, tissue culture, and vegetative cuttings (and plant separation). A comparison of these three methods (Tamura *et al.*, 1984) showed neither significant difference in growth nor glycoside content; cuttings showed the least and seed the greatest variation among individual plants in chemical composition.

1.3.1.1 Seed propagation

Establishing stevia crop by using seed is more successful in tropical climates where there is no climatic restriction on the length of the growing season. In northern climates, the shorter growing season necessitates seedling establishment in a glasshouse/greenhouse prior to the growing season. Germination and establishment from seed are often poor and sometimes unsuccessful (Shaffert and Chebotar, 1994). Stevia flowers need to be fertilized by pollen from another plant to produce viable seed. A high density of bees (3–4 hives per hectare) is recommended for good seed production (Oddone, 1999). Harvesting of immature seed may also contribute to poor germination (Colombus, 1997).

Fertile seeds are usually dark colored, whereas sterile seeds are usually pale (Felippe, 1978; Oddone, 1999). Seeds are very small (1000 seeds

weigh 0.3–1.0 g) and as a result seedlings are slow to develop, reaching a size suitable for transplanting to the field at 45–60 days (Colombus, 1997; Brandle *et al.*, 1998; Oddone, 1999). Seeds are usually germinated in a greenhouse where a range of bedding materials have been trialed (Carneiro *et al.*, 1997).

Germination rates vary greatly. It can take 4–6 days to reach two-thirds of the final germination of 62–90% at 25 °C (Carneiro and Guedes, 1992). Germination requires at least 20 °C and often more than 25 °C; light generally increases germination.

1.3.1.2 Vegetative propagation

Significant research efforts have been made toward vegetative propagation including *in vitro* multiplication. Cuttings of new stems and shoots can be propagated successfully (Shock, 1982; Gvasaliya *et al.*, 1990; Nishiyama *et al.*, 1991) and rooting of cuttings can be stimulated, but not always, by use of growth regulators (Zubenko *et al.*, 1991; Carvalho and Zaidan, 1995; Kornilova and Kalashnikova, 1996); some growth regulators can sometimes influence (increase) the concentration of steviolosides in the leaves (Acuna *et al.*, 1997). The size (number of leaves) of cuttings and day length during cutting can influence rooting and growth. Cuttings taken in late winter rooted better than those taken at other times (Carvalho and Zaidan, 1995).

For tissue cultured propagation many different parts of the plant can be used successfully—leaves, auxiliary shoots, root-neck sprouts, shoot primordia, internodal explants, etc. *In vitro* multiplication has been used frequently to multiply individually selected or bred clones and successful procedures have been documented (Handro *et al.*, 1977; Ferreira and Handro, 1988a, b; Carneiro *et al.*, 1992; Kornilova and Kalashnikova, 1996; Uddin *et al.*, 2006).

1.3.2 Economic attributes and industrial uses

Stevia products are known to be used as artificial low-calorie (nonsucrose) sweeteners and for most other purposes where sugar can be used. The primary use is as a sweetener to enhance the

palatability of foods and drinks. Unlike aspartame, stevia sweeteners are heat stable to 200 °C, are acid stable, and do not ferment, which makes them suitable for use in a wide range of products including baked/cooked foods. Stevia products have also beneficial uses as herbal and medicinal products and for some more unusual uses, for example, in tobacco products. A fermented extract of stevia showed bactericidal activity against food-borne bacteria including *Escherichia coli* (Tomita *et al.*, 1997). Over 100 phytochemicals have been discovered in stevia. It is rich in terpenes and flavonoids. The constituents responsible for stevia's sweetness were documented in 1931, when eight novel plant chemicals called glycosides were discovered and named. Of these eight glycosides, the one called stevioside is considered the sweetest and has been tested to be approximately 300 times sweeter than sugar (Ishima and Katayama, 1976; Tanaka, 1982). Stevioside, comprising 6–18% of the stevia leaf, is also the most prevalent glycoside in the leaf. Other sweet constituents include steviolbioside, rebaudiosides A-E, and dulcoside A.

In a research, stevia has demonstrated antimicrobial, antibacterial, antiviral, and antiyeast activity. A water extract was shown to help prevent dental cavities by inhibiting the bacteria *Streptococcus mutans* that stimulates plaque formation. Additionally, a US patent was filed in 1993 on an extract from stevia that claimed it to have vasodilatory activity and deemed it effective for various skin diseases (acne, heat rash, and pruritus) and diseases caused by blood circulation insufficiency. Some of the accepted uses, worldwide ethnomedical uses, and nutritional composition are listed in Tables 1–3.

1.4 Traditional Breeding

There is a great variation in phenotype and in leaf analysis among the wild populations of *S. rebaudiana*. The collections made as part of the various breeding and selection research programs have invariably included a range of genotypes and selections of plants with differing levels of steviolosides (Table 4) in their leaves, for example. Shock (1982) planted out 200 lines for survival testing and screened 17 lines for productivity. The stevioside content of leaves can vary substantially (4–16%) between individual plants. This natural

Table 1 Examples of some uses for stevia products and extracts (Midmore and Rank, 2002)

Food and culinary uses	
Soft drinks, cordials, fruit juices	Table top sweetener—for tea, coffee, etc.
Cakes, biscuits	Ice creams, yogurts, sherbets
Jams, sauces, pickles	Pastries, pies, baking
Chewing gum	Jellies, desserts
Seafoods, vegetables	Candies, confectioneries
Diabetic diets	Weight-watcher diets
A source of antioxidants	Flavor, color, and odor enhancers
Alcoholic beverage enhancer (aging agent and catalyst)	
Medicinal uses	
Skin care—eczema and acne control, rapid healing agent	Toothpaste, mouthwashes—plaque retardant/caries preventor
Hypertension treatment and blood pressure control	Diabetic foods and weight loss programs
Bactericidal agent	Pill and capsule additive to improve taste
Calcium antagonist	
Other uses	
Production of plant growth regulators (potential use)	Tobacco additive and flavorant

Table 2 Worldwide ethnomedical uses

Brazil	For cavities/tooth decay, depression, diabetes, fatigue, heart support, hypertension, hyperglycemia, infections, obesity, sweet cravings, tonic, urinary insufficiency, wounds, and as a sweetener
Paraguay	For diabetes, and as a sweetener
South America	For diabetes, hypertension, infections, obesity, and as a sweetener
United States	For candida/thrush (yeast infections), diabetes, hypertension, hyperglycemia, infections, and as a vasodilator and sweetener

variability could be partially due to the largely self-incompatible nature of the flowers. The heritability for yield, leaf/stem ratio, and stevioside content has been studied by Brandle and Rosa

Table 3 Stevia nutrient composition per 100 g (dry weight basis)

Nutrient composition	Per 100 g
<i>Proximate</i>	
Moisture (g)	7
Energy (Kcal)	270
Protein (g)	10
Fat (g)	3
Total carbohydrate (g)	52
Ash (g)	11
Crude fiber (g)	18
<i>Minerals</i>	
Calcium (mg)	464.4
Phosphorus (mg)	11.4
Iron (mg)	55.3
Sodium (mg)	190.0
Potassium (mg)	1800.0
<i>Antinutritional factors</i>	
Oxalic acid (mg)	2295.0
Tannins (mg)	0.010

(1992) and Brandle (1999). Studies correlating the plant leaf yield, branch number, and plant height have been reported (Buana and Goenadi, 1985; Shu and Wang, 1988; Buana, 1989). Other studies included correlation between stevioside content and leaf/stem ratio (Tateo *et al.*, 1998), leaf thickness with rebaudioside A (R-A)/stevioside (St) ratio (Shyu *et al.*, 1994). Most breeding programs are based on crossbreeding and selection. Few of the varieties/cultivar selections and releases have been listed in Table 4. Some of these selections, although very high yielding, are self-incompatible and can only be reproduced vegetatively. This limits their commercial use, although they may be useful for breeding new hybrids.

Even with clonal reproduction there can be some variability. An Indonesian study (Nurhaimi and Mathius, 1995) showed variations in DNA fingerprints between six groups of plantlets from an *in vitro* culture indicating some clonal variation. In China, in a sample of plants, one clone varied from the other by 10.26–19.57% in total stevioside content (Huang *et al.*, 1995).

A variety of plant breeding procedures have been used to improve leaf yield and rebaudioside

Table 4 Variety/cultivar selections and releases (Midmore and Rank, 2002)

Year	Location	New variety	Features	References
1979	Korea	Suweon 2	Yield + 22%, steviosides + 12%	Lee <i>et al.</i> , 1978
1982	Korea	Suweon 11	Thick leaves, high rebaudioside A (R-A)%	Le <i>et al.</i> , 1982
1989	China	Yunri, Yunbing		Shu, 1989,
1995	China	Zongping 1	Highest R-A% and St%	Shu, 1995
1994	Taiwan	K1, K2, K3	High yield, better R-A/St ratio	Shyu <i>et al.</i> , 1994
1994	Indonesia	BPP 72		Suhendi, 1989
1996	China	SM4	Yield increases 1.5%, R-A/St ratio up	Weng <i>et al.</i> , 1996
1996	Russia	Ramonskaya Slastena		Kornienko and Parfenov, 1996

A content in the leaves. Based on cultivar descriptions from Japan, China, and Korea, it appears that sufficient genetic variability exists to make significant genetic gains in leaf yield, rebaudioside A content, and the ratio of rebaudioside A to stevioside (Morita, 1987; Brandle and Rosa, 1992; Shyu *et al.*, 1994; Shizhen, 1995).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Stevia is generally self-incompatible. Therefore, it is difficult to increase the commercial production (Monteiro, 1980; Toffler and Orio, 1981) by means of the conventional sexual seed production. In general, propagation of stevia is largely employed by the means of seeds but it is not an adequate procedure because it results in populations with a wide random variation (Tamura *et al.*, 1984; Nakamura and Tamura, 1985) in several important characters, such as glycoside content. Variation in glycoside content has been reported to vary between 5% and 20%. Germination and establishment from seed are often poor and sometimes unsuccessful. Stevia is a cross-pollinated species requiring the presence of pollen from other plant to produce a viable seed, which often is an obstacle in the absence of appropriate means of pollen transfers. On the other hand, limited vegetative propagation (Sakaguchi and Kan, 1982) is also a major constraint in stevia production.

Stevia is likely to become a major source of high-potency sweeteners for the growing natural food market in the future, but the crop yields are compromised by such inherent failures that pose a problem where conventional breeding is desirable to design alternative methods for the

rapid production of plants with high and uniform yields of sweeteners. On this account, cloning of selected plants seems to be the desirable approach for obtaining homogeneous populations. Available data on tissue culture of this species indicate that *in vitro* propagation may be a useful alternative for this purpose. Moreover, exploration of *in vitro* generated variability and *in vitro* selection methods may also be advantageous as a source of new stevia genotypes. Clonal propagation is practical for small-scale production, but is probably not economically viable for large-scale stevia production where labor costs are high.

To overcome these constraints, it is important to develop alternative methods for rapid production of transgenic homogenous population with a high and uniform yield of sweetener plants.

2. DEVELOPMENT OF TRANSGENIC STEVIA

At present, several innovative methods are available to insert genes into plant genome and generate transgenic plants. The types of approaches chiefly used are mechanical, electrical, and chemical (Table 5). These approaches involve the specific techniques such as microinjection, particle bombardment or biolistic gun (Christou, 1996), electroporation (Potrykus, 1995), and *Agrobacterium*-mediated transformation (Hooymaas, 1995). These techniques are widely used and are versatile and safe, but are substantially less efficient than viruses. The major challenge of DNA delivery is to develop a system that is both highly efficient in delivery/expression and applicable to basic research as well as clinical settings. Albeit the availability of all these techniques, least work has

Table 5 Summary of DNA transfection methods

Approach	Method
Mechanical	Microinjection
	Pressure
	Particle bombardment
Electrical	Electroporation (low voltage)
	Electroporation (high voltage)
Chemical	DEAE (diethylaminoethyl)-dextran
	Calcium phosphate
	Artificial lipids
	Proteins
	Dendrimers
	Other polymers (including controlled-release polymers)

been done on stevia. Dhir *et al.* (2005) have taken an initiative in the transgenic research of stevia.

2.1 Electroporation-Mediated Gene Transfer in Stevia Protoplasts

Electroporation is a technique widely used for gene transfer in animal, plant, or microbial systems. It has the advantages of being simple, effective, and applicable to a wide range of plant species, and can be used to deliver DNA into large numbers of cells within a very short time (Wu and Feng, 1999). Until recently, success with electroporation has been limited to protoplasts (Potrykus, 1995); however, it is now feasible to deliver DNA via electroporation directly into certain intact plant cells.

Electroporation is the use of short high-voltage pulses to overcome the barrier of the cell membrane. By applying an external electric field, which just surpasses the capacitance of the cell membrane, transient and reversible breakdown of the membrane can be induced. This transient, permeabilized state can be used to transfer a variety of different molecules into the cells, either through simple diffusion in the case of small molecules or through electroporetically driven processes allowing passage through the destabilized membrane as is the case for DNA transfer. Electroporation was initially started for gene transfer but now is in use for delivery of a large variety of molecules starting from ions to drugs, dyes, tracers, antibodies, and oligonucleotides to RNA and DNA. Electroporation-mediated gene transfer has been achieved in stevia protoplasts (Figure 1). The typical procedure for the gene transfer can be subdivided as follows.

2.1.1 Protoplast isolation and purification

In vitro raised plants were used for protoplast isolation. Different explants (leaves, petioles, roots, stems, and hypocotyl) from 3-week-old plants were collected and thinly sliced with a surgical knife. Approximately 1 g (fresh weight) of each explant was incubated in 10 ml of filter-sterilized (Millipore, 0.22 μ m pore size) enzyme solution consisting of different concentration of cellulase “Onozuka” R-10, macerozyme, driselase, and pectolyase Y-23, and 9% mannitol in cell protoplast wash (CPW) salt mixture (MgCl_2 , CaCl_2 , and KNO_3). The pH of the enzyme solution was adjusted to 5.8. Cell wall digestion of explants was carried out in a disposable multivessel dish overnight in dark at $26 \pm 2^\circ\text{C}$ with gentle swirling (50–60 rpm) on a gyratory shaker.

After cell wall digestion, the protoplasts were carefully observed with an inverted microscope. The crude protoplast suspension was filtered through mesh and centrifuged. The pelleted protoplasts were resuspended and washed once with Kao and Michayluk (KP8; 1975) culture medium to remove the enzyme solution and centrifuged for another 10 min.

2.1.2 Electroporation and protoplast culture

Viable protoplasts obtained from the previous step will then be transferred into the electroporation cuvettes, previously filled with 1 ml of electroporation buffer containing 0–25 μ g of DNA per milliliter. To optimize the electric pulse parameters, different voltages (50–400 V) were tested by the 960 μ F capacitor (Bio-Rad Gene Pulse, Hercules, CA, USA). After the electric pulse was delivered, cuvettes containing the protoplasts were incubated for 10 min on ice, and were subsequently cultured for 48 h at $24 \pm 2^\circ\text{C}$ before being harvested for gene expression assay. Electroporated and nonelectroporated protoplasts were diluted and cultured with KP8 media to a concentration of 1 million protoplasts per milliliter (5 ml per dish). The petridishes were wrapped with parafilm to maintain humidity and prevent contamination. The sealed dishes were kept in dark at $26 \pm 2^\circ\text{C}$ in a culture box. Control protoplasts were either untreated, incubated in

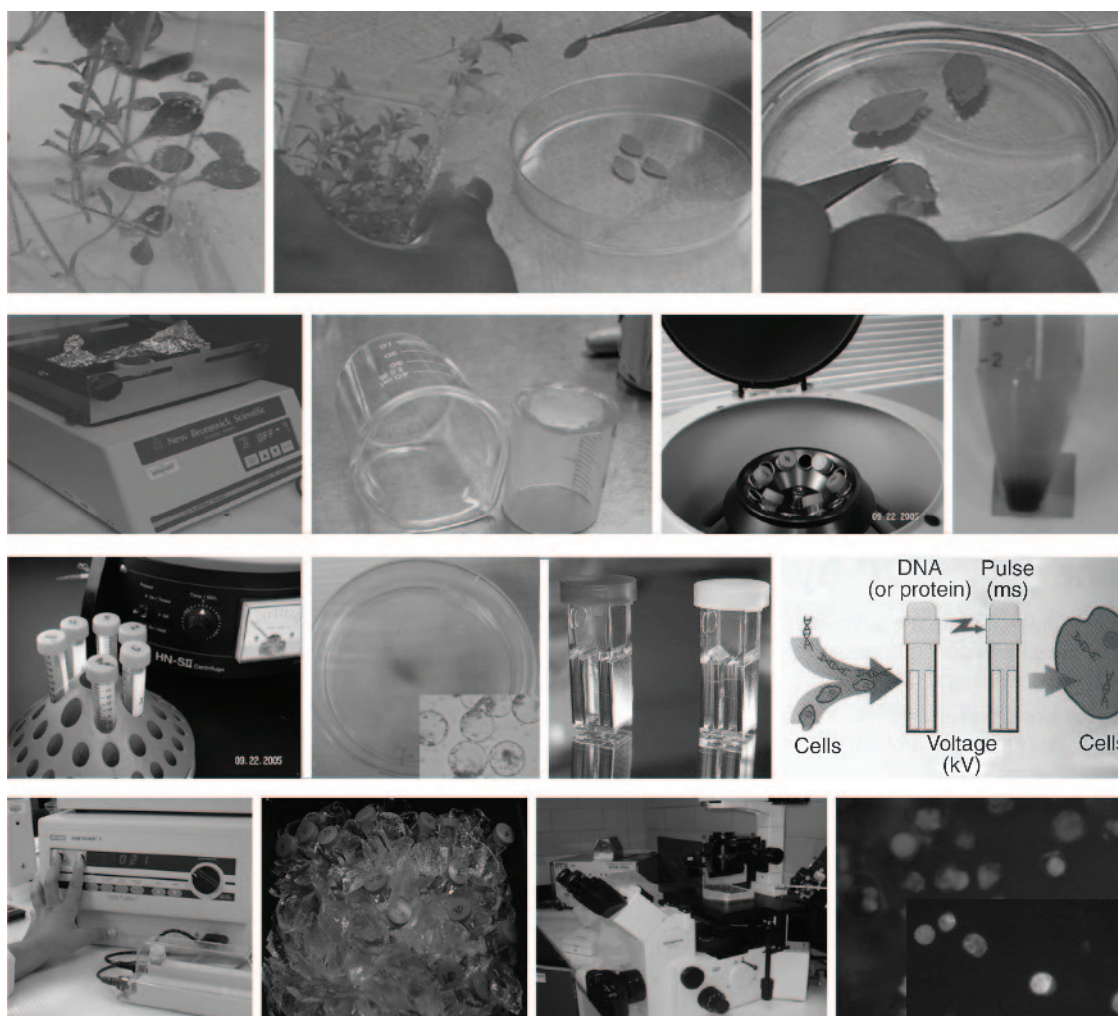


Figure 1 Stevia protoplast isolation, purification, and culture for genetic transformation through tissue electroporation

buffer and plasmid DNA but not electroporated, or electroporated with an equal volume of buffer to replace the DNA solution.

Five different buffers were used to study their effect on gene expression: (1) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered mannitol (HBM)—0.55 M mannitol, 1 mM HEPES pH 7.0 (Nagata, 1989); (2) HEPES-buffered saline (HBS)—5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4 , 600 mM glucose, 21 mM HEPES pH 7.2 (Dhir *et al.*, 1991); (3) EPR (electroporation)—9.0% glucose, CaCl_2 , 4 mM N-2 hydroxyethyl-piperazine-N2-ethanesulfonic acid (HEPES) and 0.2 spermidine; (4) KP8—medium,

9 M CPW (9% mannitol, MgCl_2 148 mg/l and KNO_3 101 mg/l; (5) 13 M mannitol 150 mM NaCl, and 5 mM CaCl_2 . Samples were preincubated in each buffer for 1 h and later prepared for electroporation as described above.

2.1.3 Factors affecting electroporation

2.1.3.1 Effect of different explants on protoplast viability

Direct observations under the inverted microscope have revealed that maximum digestion of cell walls

Table 6 Effect of enzyme combinations on protoplast yield and viability

Cellulase Onozaka-10 (%)	Macerozyme Onozaka-R (%)	Driselase (%)	Pectolyase Y-23 (%)	Yield of protoplast (gram fresh weight)	Viability of protoplasts (%)
1.5	1.0	0.2	0.1	7×10^6	75–85
1.5	1.0	0.5	0.1	5×10^6	85–90
2.0	1.5	0.2	0.1	5×10^5	85–90
2.0	1.5	0.5	0.1	5×10^5	95

occurred in the leaf tissue (approximately 90% converted protoplasts were viable). In comparison to the leaf explants, the conversion of cells into protoplasts for the petiole was 80%, hypocotyls 50%, stem 70%, and root 30%. The protoplasts, which were measured using the micrometer, varied in size from 20 to 40 μm . A range of enzyme combinations have been tested to digest the tissue and isolate the protoplast (Table 6); leaf explants were found to yield higher amount of protoplasts. The released protoplasts settled to the bottom of the multivessel dish and can be used for downstream processes (Figure 1).

2.1.3.2 Plasmid DNA

The transient expression assay vector pCambia 1304 (CAMBIA GPO Box 3200, Canberra ACT 2601, Australia) carries a selective marker gene (*hptII* (hygromycin phosphotransferase)) conferring hygromycin resistance and a fusion

between the reporter genes coding for β -glucuronidase (GUS) (*uidA*) and a green fluorescent protein (GFP) (*mgfp5*), both driven by a 35S promoter from cauliflower mosaic virus (CaMV) (Figure 2). Plasmid DNA was independently transformed into *E. coli* JM109 (Dower *et al.*, 1988) or DH5a (Hanahan, 1983) cells and purified by the cesium chloride method (Sambrook *et al.*, 1989). Supercoiled plasmid DNA was directly employed in transformation experiments or linearized by digestion with *EcoRI* (GIBCO BRL). Concentrations of plasmid DNA were spectrophotometrically determined at 260 nm and confirmed by tris-borate-ethylene diamine tetraacetic acid gel electrophoresis in 0.8% agarose gels (Sambrook *et al.*, 1989).

2.1.3.3 Effect of DNA concentration

With varying plasmid DNA concentration from 0 to 25 $\mu\text{g ml}^{-1}$, the number of green protoplasts

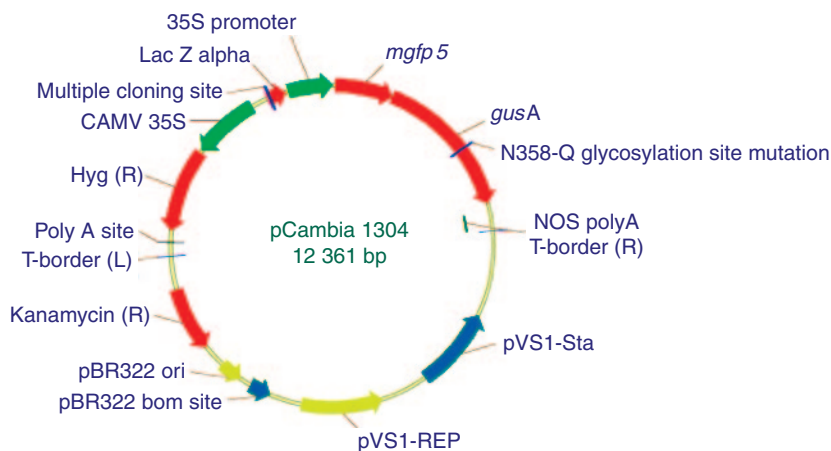


Figure 2 The map of transient expression assay vector pCambia 1304 (<http://www.cambia.org/daisy/cambia/2049/version/1/part/4/data/pCAMBIA1304.pdf?branch=main&language=default>)

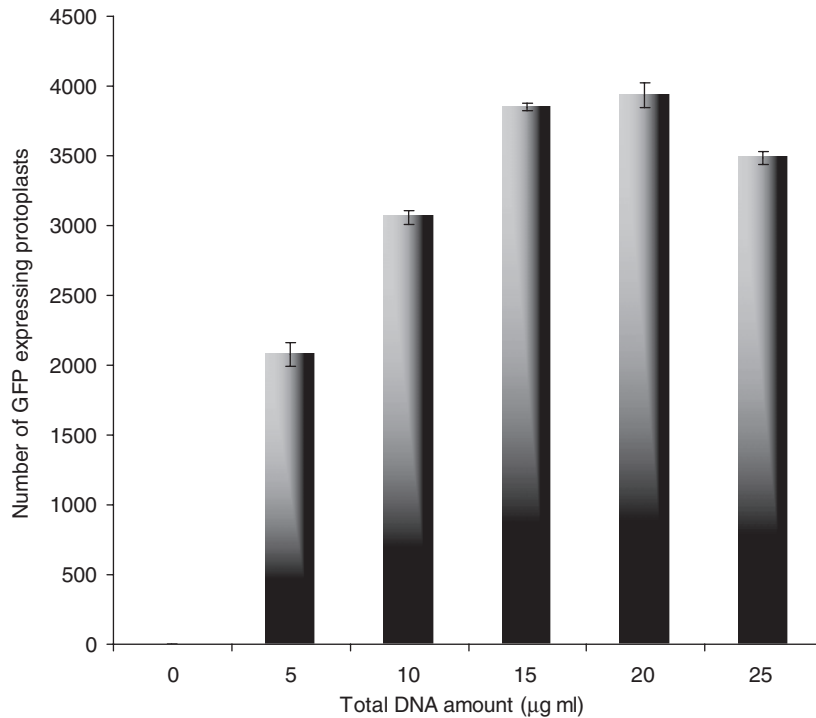


Figure 3 Influence of plasmid DNA concentration on the number of transformed (*GUS* gene expressing) protoplast cells of stevia; the electroporation was performed at 200 V

increased (Figure 3). With increasing the plasmid DNA concentration ($50\text{--}100\ \mu\text{g ml}^{-1}$), the number of blue protoplasts declined. Plasmid DNA of 15 to $20\ \mu\text{g ml}^{-1}$ was found to be optimal for efficient expression. An increase in the DNA concentration would infer directly to an increase in gene expression. However, if the DNA concentration is too high, uptake might possibly be decreased due to competition with DNA strands for protoplast uptake (Dhir *et al.*, 1991).

2.1.3.4 Effect of field strength on protoplast viability

Transient expression of a gene coding for a GFP (Chalfie *et al.*, 1994) can be used for *in vivo* evaluation of transformation efficiency. To maximize the expression of introduced DNA into protoplast, it is important to test protoplast viability under different ranges of electric field. The viability of protoplasts prior to electroporation was approximately higher (95%). Two hours after a single

pulse electroporation, at voltages ranging from 50 to 400 V, the viability decreased from 95% to 27%. In order to test the number of pulse on protoplast viability, protoplasts were pulsed twice at voltage ranging from 50 to 400 V. Viability decreased from 75% to 6% with increasing voltage. However, at 100–200 V single pulse, the viability was 80–63% which is considered to be optimal for DNA delivery after electroporation (Dhir *et al.*, 1992). Such a combination induces more pores, larger pores, or longer lived pores in the plasma membrane, allowing more DNA uptake (He and Lazzeri, 1998).

2.1.3.5 Effect of field strength on transient gene expression

A known amplitude of 100 V field strength is reported to be an optimal value for electroporation of rice leaf base tissue (Dekeyser *et al.*, 1990). However, at this amplitude, the number of green fluorescent protoplasts was approximately 2500. By increasing the voltage from 100 to 250 V,

the number of green fluorescent protoplasts also increased but beyond 250 V the number of protoplasts expressing the *GFP* gene decreased due to decline in tissue viability. Maximum transient gene expression has been reported under electric field strengths causing more than 50% reduction in protoplast viability (Fromm *et al.*, 1985; Hauptmann *et al.*, 1987; Oard *et al.*, 1989). This indicates that the expression of *GFP* gene increased in proportion to the voltage, suggesting a correlation with number and/or size of pores in electroporated cells (Nagata, 1989). Almost complete death of tissues has been reported at 400 V.

2.1.3.6 Effect of different electroporation buffers

Different types of electroporation buffers with varying amounts of high or low ionic strength had significant effect on DNA uptake. EPR at pH 7.2 showed maximum green protoplasts, approximately 3300 per electroporated sample. Larkin *et al.* (1990) reported that the expression of DNA in tobacco protoplasts was positively correlated with pH up to 9.0, possibility due to charge modification and destabilization of the plasma membrane at high pH. Nonviable and damaged protoplasts were rarely seen after electroporation in buffer EPR, which gave almost 100% expression of *GFP*. However, the frequency of stable transformation was lower than that expected from transient gene expression in electroporated protoplasts (Hain *et al.*, 1985; Shillito *et al.*, 1985), probably due to some retention of transgene expression ability in damaged protoplasts for periods shorter than 72 h. The chloride ions in buffer HBS and HBM resulted in the production of toxic chlorine gas when electric pulses were applied, leading to a reduction in cellular viability.

2.1.4 Analysis of GFP expression

Electroporated protoplasts will finally be resuspended in culture medium (Kao (1977) modified by Gilmour *et al.* (1989)) to give 1.0×10^6 protoplasts per milliliter per liter and kept in the dark for 24 h at 4 °C. Nonelectroporated protoplasts in 800 ml of electroporation buffer with 10 mg of

plasmid DNA were ice incubated for 45 min. As a measure of transformation efficiency, the number of transgene-expressing and nonexpressing protoplasts will be scored using fluorescence microscopy at 395 nm (Figures 1 and 4). The viability of the tissue after electroporation can be measured with phenosafranine and by visual observation of cell damage (Dhir *et al.*, 1991). Putative transgenic plants of stevia can be obtained at high frequencies from electroporated protoplasts while an effective plant regeneration protocol is used.

2.2 Genetic Transformation and Plant Regeneration in Stevia Using Microprojectile Bombardment

Microprojectile or particle bombardment, also called biolistic particle delivery, can introduce DNA into many cells (including cell-walled plant cells) simultaneously. In this procedure, DNA-coated microparticles (composed of metals such as gold or tungsten) are accelerated to high velocity to penetrate cell membranes or cell walls (Yang *et al.*, 1990; Ye *et al.*, 1990; Yang and Sun, 1995). Because of the difficulty in controlling the DNA entry pathway, this procedure is applied mainly in adherent cell cultures and has yet to be widely used systemically. Stevia is generally self-incompatible, therefore it is difficult to increase the commercial scale production through conventional sexual means. Limited or no work has been done on stevia transformation through particle bombardment. Dhir *et al.* (unpublished) has successfully performed certain pilot experiments for genetic modification of stevia using such improvised technique. Rigorous testing is inevitable to establish a successful transient and stable transformation protocol. Numerous parameters affect the efficiency and success of gene transfer through particle bombardment; those include mainly distance between stopping plate and target tissue, helium pressure, number of bombardment, and type of particles (tungsten or gold). The results from various preliminary research projects showed that for stevia leaf-derived callus the optimum parameters would be 6 cm distance, 1100 psi He pressure, gold particles, and one bombardment. The expression of GFP in tissue can be observed after 12 h of bombardment

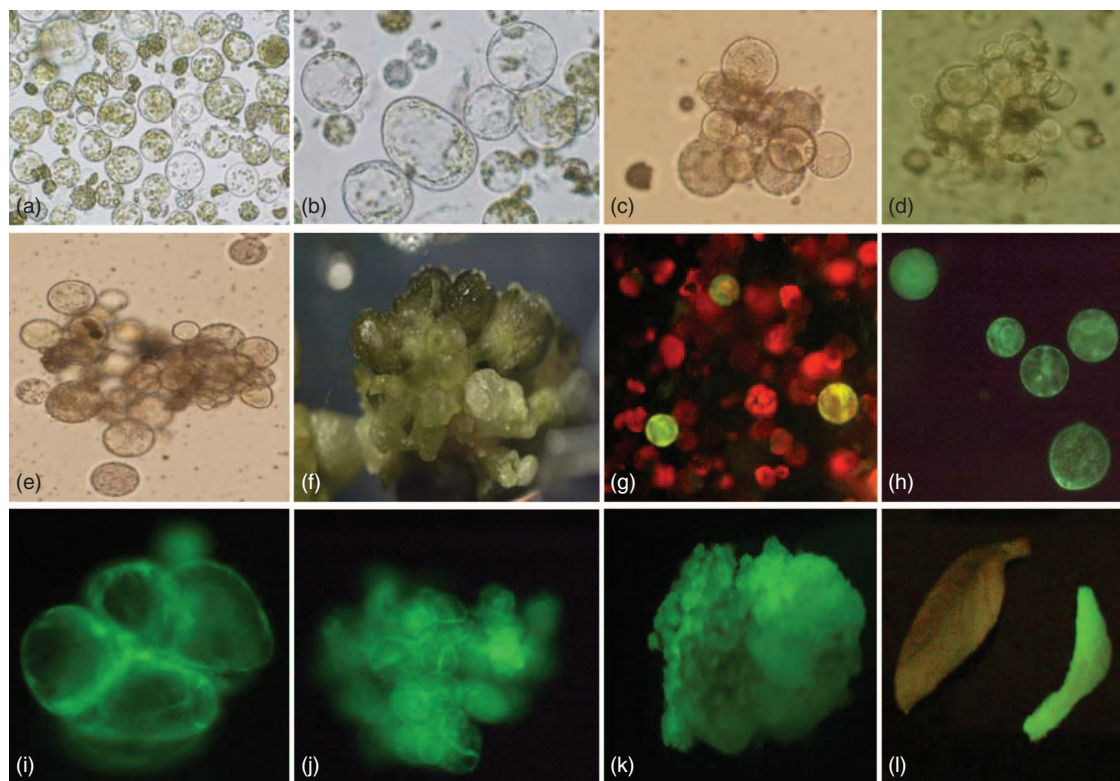


Figure 4 Electroporation-mediated stable genetic transformation of *Stevia rebaudiana* protoplasts: (a) freshly isolated mesophyll protoplast; (b) first cell division; (c) protoplast-derived cell colony; (d) clusters from protoplast-derived cells; (e) microcalli in agarose in bead; (f) somatic embryos formed on MS medium; (g) *GFP* gene expression in stevia mesophyll protoplast red fluorescence is nontransformed cell; (h) expression of *GFP* gene in protoplast; (i) *GFP* expressing protoplast in four-cell division stage; (j, k) *GFP* gene expressing microcolonies after 3–4 weeks of culture; and (l) nontransformed and transformed stevia leaf

and GFP expression has been found stable in the embryogenic callus.

2.3 Culture, Micropropagation, and Plant Regeneration in *Stevia* as Prerequisites for Development of Transgenics

2.3.1 Explants used and their preparation

Explants for culture can be obtained either from seeds or a mature plant itself. The seeds of stevia were surface sterilized (12 min in 15% bleach and washed three times with sterile distilled water) and germinated *in vitro* in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without growth regulators but containing 30 g l^{-1} (w/v) sucrose and solidified with 3 g l^{-1} (w/v) Gelrite (Phytotechnology) at $24 \pm 2^\circ \text{C}$ with a 16/8 h

light/dark photoperiod. Explants can also be obtained from plants already growing *in vivo*. These plants can be either in greenhouse, field, or in wild, but standard aseptic techniques should be followed. Typical explants used for tissue culture experiments are shoot tips (1–3 cm long with axillary bud), leaves, stems, and roots. On regular basis, the explants are generally surface sterilized with 70% ethanol for 10 s, followed by immersion in 15% Clorox solution for 15 min, and then rinsed with sterile water three to four times. The culture media typically contains 3% (w/v) sucrose or maltose and 0.3% (w/v) Phytogel. The pH of all media is adjusted to 5.8 prior to autoclaving at 121°C for 20 min at 15 psi. The cultures are maintained at $26 \pm 2^\circ \text{C}$ and a light/dark cycle of 16/8 h under a photon irradiance of $120 \mu \text{M m}^{-2} \text{s}^{-1}$. The explants are subcultured routinely onto fresh medium every

2–3 weeks. *In vitro* raised plants (Figure 4) can then be used for protoplast isolation and transformation experiments.

2.3.2 Micropropagation

The seeds of stevia show a very low germination percentage (Felippe *et al.*, 1971; Felipe and Lucas, 1971; Monteiro, 1980; Toffler and Orio, 1981). Propagation by seeds does not allow the production of homogeneous populations and results in high variability in important features like sweetening levels and composition (Tamura *et al.*, 1984; Nakamura and Tamura, 1985). Vegetative propagation is also limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982). Among various other parts, stevia's nodes are quite often used for micropropagation. It has been observed that cutting the shoot 0.5–1 cm above the node and 4–6 cm below the node is an appropriate size to use for propagation. Once the desired size of the cuttings is made, they can then be placed on MS media for normal regeneration and growth (Figure 5). In 2–3 weeks' time, the plants are regenerated. By this time the multiple axillary meristems were already developing in the shoot tip. The production of shoots is even greater during the second subculture, as observed in *Acacia nilotica* (Dewan, 1992). This fact can be explained as a result of the release of apical dominance imposed by the apical shoot. If the shoot tip or apical shoot had been excised at the time of first subculture, the frequency of shoot might eventually be greater. The rooting can be initiated in shoots longer than 1–2 cm using MS medium supplemented with 5.71–22.84 μM indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). Plantlets with roots were transferred to basal medium for 1 month and then to small pots filled with garden soil and sand (1:1). Standard statistical procedures should be followed in experimental design. Plant material was subcultured onto fresh medium at 4 weeks intervals.

2.3.3 Somatic embryo induction

Somatic embryogenesis has been described for more than a hundred plant species (Terzi and Loschiavo, 1990), but the number of reports of

somatic embryogenesis among members of the Asteraceae family is still low (May and Trigiano, 1991). The development of protocols for regeneration of stevia via somatic embryogenesis is important as this technique can be used in the clonal propagation of this plant, or as explant material for protoplast isolation and regeneration (Puite, 1992). For embryogenic culture induction, leaf segments (1–2 cm long) are removed from 5 to 6-week-old *in vitro* raised plants. Leaf segment will then be incubated (adaxial side down) on MS medium supplemented with B5 vitamins, 30 g sucrose and 3 g Gelrite. 2,4-dichlorophenoxyacetic acid (2,4-D) is used in combination with each of three cytokinins: 6-benzyl adenine (BA), kinetin (KN), and zeatin. The auxin 2,4-D used with the range of 1.35 and 4.52 μM in combination with BA (1.33–4.43 μM), KN (0.86, 2.28 μM) allowed a high percentage of culture induction in leaf segments (65–93% 2,4-D/BA; 53–85% 2,4-D/KN; and 49–65% 2,4-D) within 6 weeks (Dhir *et al.*, unpublished). The proembryogenic masses will appear after 8 weeks of subculture. Within 2–3 weeks of subcultures, somatic embryos will appear on the surface of cultures.

2.3.4 Embryo maturation

After 9 weeks (three subcultures), callus tissues (initiated from leaf explants) with somatic embryos at the globular state that were including different media were transferred onto embryo induction medium. Dhir *et al.* (unpublished) tested three auxins: 2,4-D (3.16 μM), α -naphthaleneacetic acid (NAA) (3.75 μM), and IBA (3.44 μM) in combination with one each of two cytokinins, BA (0.44 or 1.33 μM), KN (46 or 1.39 μM). Cultures on induction medium (at globular and heart-shaped embryos) supplemented with BA and 2,4-D were transferred for somatic embryo maturation and germination onto media supplemented with BA in combination with 2,4-D, NAA, or IBA. The temperature, photoperiod, and light intensity were same as described above. Numbers of callus-forming explants, calli-producing embryos, and mature/germinating embryos were recorded at the end of each subculture phase. Somatic embryo formation and development were performed after transferring cultures on embryo maturation and germination medium, MS medium supplemented

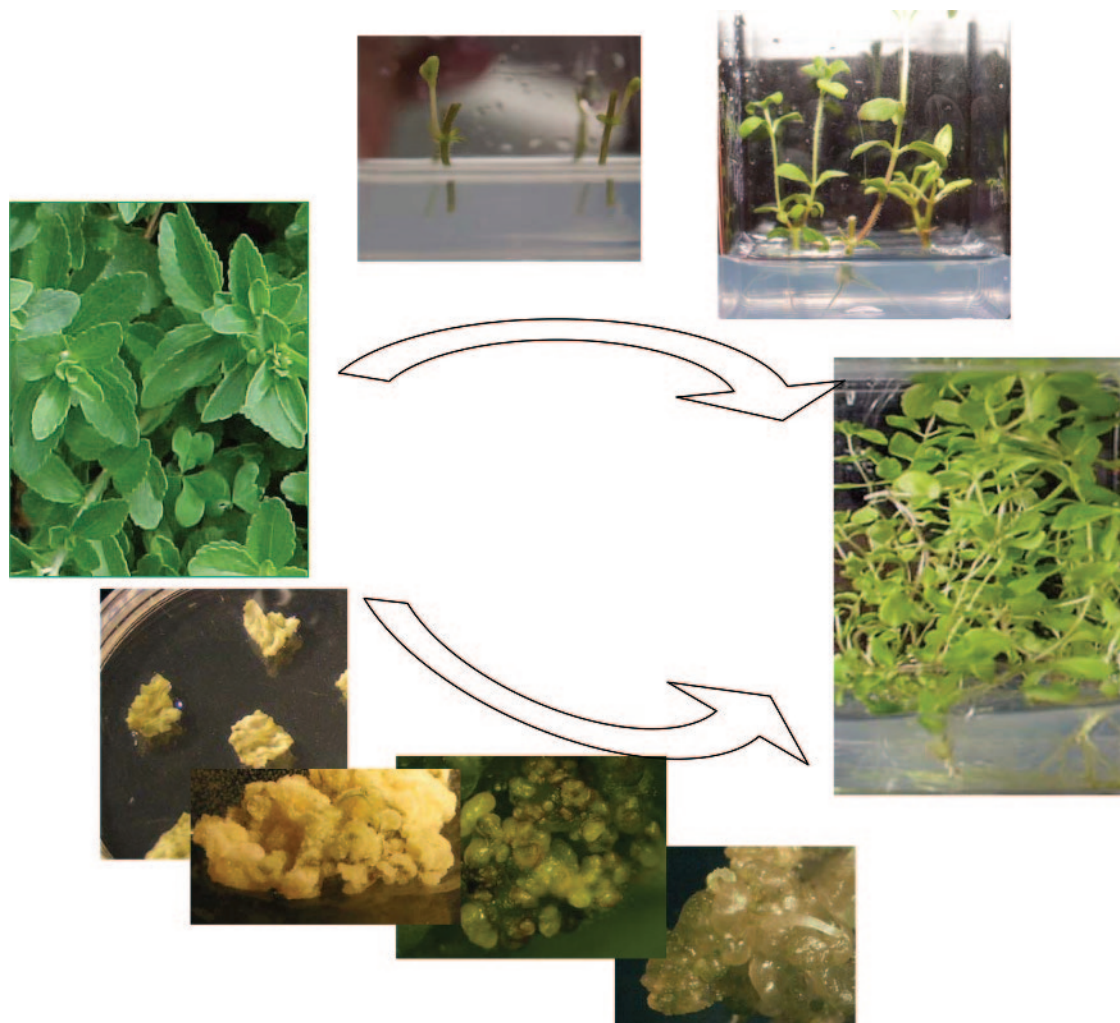


Figure 5 Micropropagation and plant regeneration of *Stevia rebaudiana*

with $3.44 \mu\text{M}$ IBA and $0.44 \mu\text{M}$ BA. Different stages of somatic embryo development (globular stage, heart- and torpedo-shaped somatic embryos, cotyledonary stage, germinating embryos, and embryo-desired plantlet) are shown on maturation and germination media (Figure 6).

2.3.5 Plant regeneration

Mature somatic embryos that had undergone germination to form plantlets were excised from calli. After the plantlets reached 3–4 weeks, they were transferred to pots with vermiculite and grown for 6–8 weeks in growth chambers for

acclimatization, after which they were transferred to bigger pots and greenhouse. Experiments were repeated three to five times. Plantlets transferred to Magenta boxes (four per boxes) containing rooting medium that contained double strength MS medium, with gibberellic acid A3 (GA3) $2.89 \mu\text{M}$ and NAA $5.37 \mu\text{M}$ (Figure 6).

3. FUTURE ROAD MAP

The great interest in stevia as a noncaloric, natural sweetener has fueled many studies on it, including toxicological ones. The main sweet chemical, stevioside, has been found to be nontoxic

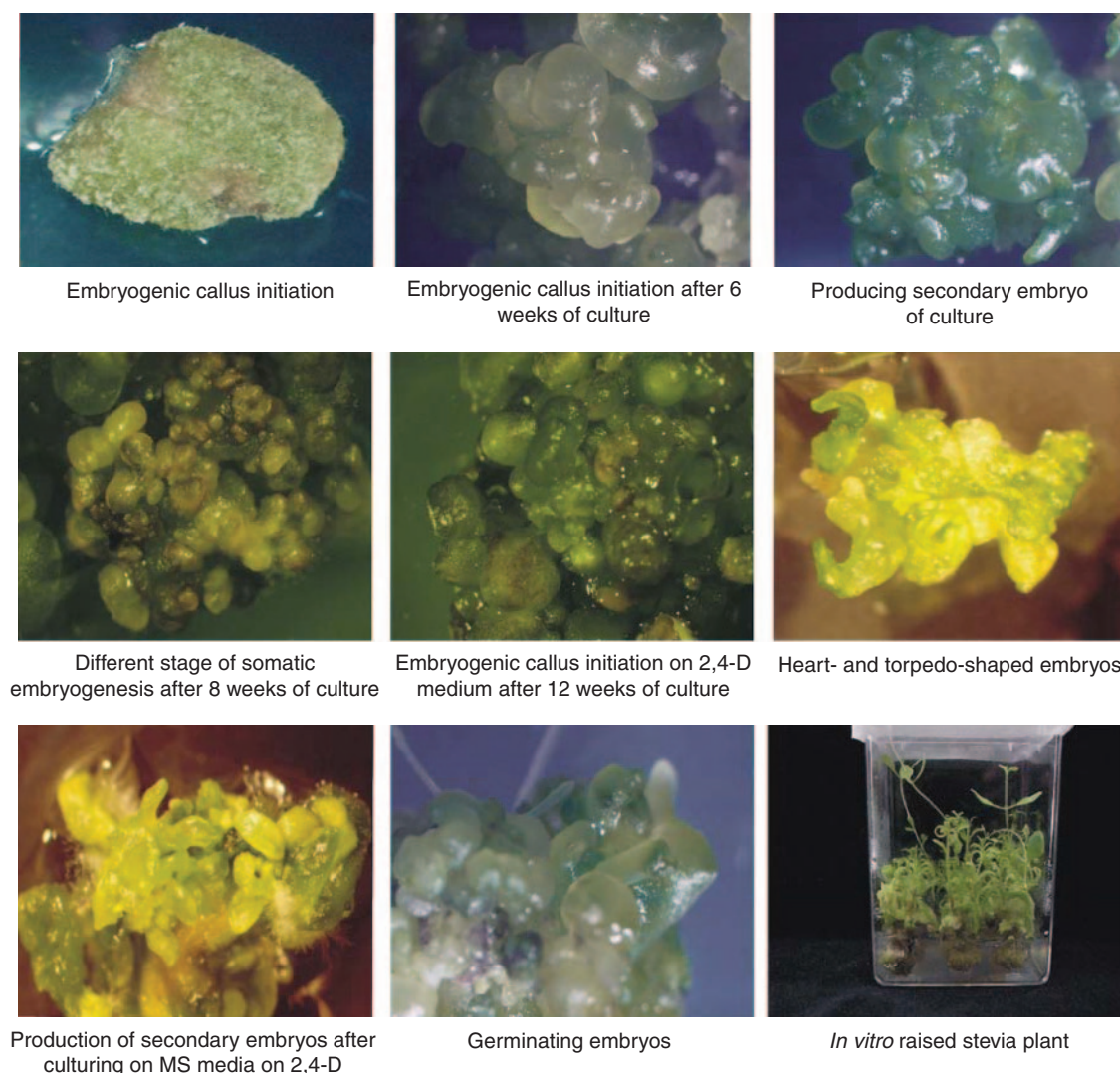


Figure 6 Callus induction, somatic embryogenesis, and plantlet regeneration of *Stevia rebaudiana*

in acute toxicity studies with rats, rabbits, guinea pigs, and birds. It also has been shown not to cause cellular changes (mutagenic) or to have any effect on fertility. However, studies conflict as to the effect of stevia leaf on fertility. The majority of clinical studies show stevia leaf to have no effect on fertility in both males and females. In one study, however, a water extract of the leaf was shown to reduce testosterone levels and sperm count in male rats.

In 1991, at the request of an anonymous complaint, the US Food and Drug Administration (FDA) labeled stevia as an “unsafe food additive” and restricted its import. The FDA’s stated reason was “toxicological information on stevia is

inadequate to demonstrate its safety.” This ruling was controversial, as stevia proponents pointed out that this designation violates the FDA’s own guidelines, under which any natural substance used prior to 1958 with no reported adverse effects should be recognized as safe.

Contraindications:

- Stevia leaf (at dosages higher than used for sweetening purposes) has been documented to have a hypoglycemic effect. Those with diabetes should use high amounts of stevia with caution and monitor their blood sugar levels as medications may need adjusting.

- Stevia leaf has been documented to have a hypotensive, or blood pressure lowering effect (at dosages higher than used for sweetening purposes). Persons with low blood pressure and those taking antihypertensive drugs should avoid using large amounts of stevia and monitor their blood pressure levels accordingly for these possible effects.

Drug Interactions. In large amounts, it may potentiate antihypertensive and antidiabetic medications.

Today, stevia leaves and leaf extracts are commonly found in most health food stores; however, they may only be sold in the United States as dietary/herbal supplements, not as food additives or sweeteners. In fact, in 1991 the FDA even banned all imports of stevia into the country. This political move was viewed by many to have monetary ties to the sweetener industry, which stood to lose a lot, and it created a huge public outcry in the natural products industry. The import ban was lifted in 1995 after much lobbying led by the American Herbal Products Association and other industry leaders. This allowed stevia to be sold as a dietary supplement under new legislation called the Dietary Supplement Health and Education Act of 1994.

A lot of efforts have been put forth by researchers in the last one decade for better understanding the stevia. The first linkage map of stevia has been constructed, which will lay a foundation for molecular breeding efforts (Yao *et al.*, 1999). Expressed sequence tags are providing a new approach to gene discovery in stevia's secondary metabolism. Transcripts involved in diterpene synthesis have been investigated to understand the steviol glycoside biosynthesis (Brandle *et al.*, 2002). Functional genomics has been successfully utilized in uncovering the three major glucosyltransferase involved in the synthesis of the major sweet glucosides of stevia (Richman *et al.*, 2005).

There is need for creating awareness among the people about stevia's availability and its potential as a low-calorie sweetener, which is of significant nutritional and therapeutic values. Growing demand for herbal foods might encourage stevia cultivation and production and would help enjoy the sweet taste with minimal calories for those who are on restricted diet for sugar/carbohydrate.

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Potato

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Potato (*Solanum tuberosum* L. subsp. *tuberosum*) is a crop of New World origin, but exactly when and where potato was first cultivated are subjects of debate. Evidence comes from archeological artifacts and plant remains, written accounts of early New World explorers, and molecular data.

Salaman (1937), Laufer (1938), Hawkes (1967, 1990), and Sauer (1993) reviewed archeological evidence for ancient consumption and possible cultivation of potato, including carbon dated plant remains and ceramics depicting potatoes. Ugent *et al.* (1987) reported preserved tuber skins collected from fire pits at a site inhabited 13 000 years ago at Monte Verde in southern Chile. Based on phase contrast and scanning electron microscopy analysis of starch grains, the authors identify the remains as *Solanum maglia*. However, Hawkes (1990) argues that the remains cannot be *S. maglia*, extant populations of which do not occur in the region. Grun (1990) raises similar concerns. Whatever the species identity of the remains, however, Hawkes (1990) agreed with Ugent *et al.* (1987) that the archeological context in which they were discovered points to human consumption of potato tubers predating the establishment of agriculture. Engel (1970)

reported fossilized potato tubers recovered from a central Peruvian cave inhabited 10 000 years ago. While Engel (1970) only identified the tubers as *Solanum* sp., Ugent *et al.* (1982) later concluded they may be *S. tuberosum* based on phase-contrast microscopy of starch grains. It remains unclear whether the tubers were cultivated or gathered from the wild. Similarly, Martins (1976) identified tuber remains from the Puna de Chilca Canyon of the Peruvian highlands as *S. tuberosum* based on light and scanning electron microscopy of gross morphology, starch grains, cork cells, and distribution and organization of xylem vessels. The samples are approximately 8000 years old. Archeological evidence of early human consumption of *S. tuberosum* was also reported by Ugent *et al.* (1982). A total of 20 tubers, recovered from four sites near Casma in the subtropical coastal desert of central Peru, were identified as *S. tuberosum* on the basis of surface features and phase contrast microscopy of starch grains. Carbon dating revealed these tubers to be 3200–4000 years old. While the arid conditions near Casma favored the preservation of the potato tubers, the climate is inhospitable to *Solanum* species. Pozorski and Pozorski (1979) reported evidence that the ancient inhabitants of coastal regions of Peru practiced irrigation agriculture. Thus, Ugent *et al.* (1982) argue that potato remains found at Casma may have

originated from intentional cultivation rather than foraging. But Sauer (1993) argues that the relative paucity of potato remains at Peruvian coastal archeological sites suggests that potatoes may have been harvested from the highlands and transported to the coast, rather than cultivated by coastal inhabitants themselves.

Clay phytomorphic representations of potato support archeological evidence that potato was a food crop known to the ancient people of South America. Potato ceramics first appeared about 2000 years ago in northern (Moche and Chimú) and southern (Nazca) coastal Peruvian cultures (Sauer, 1993). In some instances, clay pots depicted highly stylized potatoes, often adopting humanlike characteristics (Hawkes, 1967, 1990). Some potato pots depicted *chuño*, a freeze-dried potato product produced even today in the Andean highlands (Sauer, 1993). Presumably, *chuño* would have been known to the ancient coastal Peruvian potters through trade with highland people since it could not have been produced at lower altitudes. Hawkes (1990) further describes ceramic potato pots from coastal regions as “rare” and argues that, owing to poor potato production conditions along the coast, potters would have been familiar with all types of potato mainly through trade with highland people.

Archeological evidence clearly indicates that potato was first cultivated in South America. Consistently, Vavilov (1951) identified as the center of origin for potato a broadly defined New World region encompassing Mexico, Peru, and adjoining countries. Even before that time, scientists had debated both exactly where potatoes were first domesticated, with the Andean highlands of Peru and Chile/Argentina/Bolivia being leading contenders, and whether potato was domesticated once or multiple times. Spooner *et al.* (2005) examined relationships among 261 wild potato and 98 cultivated potato landrace accessions. Based on 438 amplified fragment length polymorphism (AFLP) markers, the wild accessions could be divided into “Northern” (Peru) and “Southern” (Bolivia and Argentina) clades. Relationships within these clades were poorly resolved and the authors conclude that further systematic study and possible taxonomic revisions are warranted. Significantly, the data also support a single domestication event for all cultivated potatoes. Cultivated potato is most closely related

to northern wild accessions, suggesting that modern-day cultivated potato originated from wild accessions in what is present-day southern Peru (Spooner *et al.*, 2005). The conclusions of Spooner *et al.* (2005) based on robust molecular analysis differ from those of earlier authors who argue for multiple domestication events possibly involving complex and cryptic interspecific hybridization (Ugent, 1970; Grun, 1990; Hosaka, 1995; van den Berg *et al.*, 1998; Huaman and Spooner, 2002) and a greater involvement of southern accessions (Bukasov, 1978; Hawkes, 1990; Ochoa, 1990) in the origin of cultivated materials.

Exploration of the New World by early European explorers led ultimately to the introduction of potato to the Old World. Salaman (1937), Laufer (1938), and Hawkes (1967, 1990) offer detailed analyses of this subject. The introduction of potato into Europe is steeped in folklore. By some accounts, potatoes were first transported from South America by Sir Francis Drake as a result of his famed circumnavigation of the globe (1577–1580). However, several authors have pointed out that potato tubers surely would not have survived the 2-year westward voyage from South America back to Europe (Hawkes, 1990; Sauer, 1993). Another legend attributes introduction of the potato into Ireland to Sir Walter Raleigh in the late 1580s, an assertion supported by Salaman (1937) but refuted by McNeill (1949). Alternatively, Salaman (1937) and Laufer (1938) estimate that potato may have first been introduced from South America into Spain around 1570 since it was not mentioned in a published account of the flora of the region by botanist Clusius (Charles de l’Ecluse), who visited Spain in 1564. Hawkes (1990) concurs with this estimate. From Spain potato traveled to Italy where it became a food crop of regional significance. Later, it spread throughout Europe and Clusius first reports seeing potato tubers in 1588 in Belgium, these having arrived from Italy in 1587 (detailed in Laufer, 1938). Around 1590, potato arrived in England, perhaps through direct shipment from the New World (Hawkes, 1990). Potato is described in the 1596 garden catalog of English botanist John Gerard (Salaman, 1937; Laufer, 1938; Glendinning, 1983). In the centuries that followed, potato became a staple crop throughout much of Europe, supporting the rapid population

expansion that accompanied the Industrial Revolution (Grun, 1990).

From Europe, the potato was transported to North America. Potatoes may first have been transported from England to Bermuda in 1613 and then from Bermuda to the North American mainland in 1621, a hypothesis favored by Laufer (1938) and Hawkes (1990). Potato was present in India by 1610 and mainland China by 1700 (Sauer, 1993). Potatoes were taken to New Zealand in 1769 by Captain Cook and gained agronomic significance for the native Maori by 1840 (Sauer, 1993). Missionaries may have played a crucial role in distribution of the potato from Europe throughout the world (Laufer, 1938; Sauer, 1993).

1.2 Taxonomy, Habit, Habitat, Genome Size, and Cytological Features

The Solanaceae, also known as the potato family or the nightshade family, is a large plant family distributed throughout the Old and New Worlds. The family encompasses many important agronomic and horticultural crops, with the genus *Solanum*, from which the Solanaceae gets its name, being the most economically significant genus. Depending on whose taxonomy one follows, *Solanum* includes 1000 (D'Arcy, 1991) to 1700 (Mabberley, 1997) species, making it one of the largest of all plant genera. *Solanum* includes three significant food crops, potato (*S. tuberosum*), tomato (*S. lycopersicum*), and eggplant (*S. melongena*). Other species in the genus include food crops of minor or regional significance, e.g., pepino (*S. muricatum*), scarlet eggplant (*S. aethiopicum*), and gboma eggplant (*S. macrocarpon*) and weed species, e.g., nightshade (*S. nigrum*) and *S. sisymbriifolium*. On a worldwide scale, potato is the most important food crop in the genus, with nearly 325 million metric tons produced in 2005, ranking potato as the world's fourth most important human food crop behind maize (702 million metric tons), wheat (630 million metric tons), and rice (618 million metric tons) (FAOSTAT data 2005).

Based on morphological and molecular data, *Solanum* may be divided into several subgenera. Subgenus *Potatoe* includes approximately 200 tuber-bearing and nontuber-bearing potato species. These species are exclusively of New World

origin and are found naturally distributed throughout the southwestern United States, Mexico and Central America, and South America. The slopes of the Andes Mountains are particularly rich in potato species. Crossability between these related potato species is mediated through the Endosperm Balance Number (EBN). According to the EBN hypothesis of Johnston *et al.* (1980), genetic factors within the potato genome determine the inherent EBN for the species. EBN values for potato species include EBN 1, 2, and 4. For an interspecific cross to be successful, the endosperm of the developing seed must carry a maternal:paternal EBN ratio of 2:1. EBN can be manipulated by altering parental ploidies through use of $2n$ gametes or artificial procedures (Carputo and Barone, 2005). Mismatched EBNs have limited gene flow between certain potato species, especially between the evolutionarily primitive 1EBN species and cultivated potato (4EBN). Hawkes (1990) provides the most recent complete taxonomic treatment of the potatoes, although subsequent research (e.g., Rodriguez and Spooner, 1997; Spooner and Castillo, 1997; Lara-Cabrera and Spooner, 2004; Spooner *et al.*, 2004) points to a clear need for further systematic study and taxonomic refinement. More recent treatments for species native to specific geographical regions are provided by Ochoa (1999), Contreras and Spooner (1999), and Spooner *et al.* (2004). In total, seven potato species are cultivated, but the cultivated potato of worldwide significance is *S. tuberosum* ssp. *tuberosum* and is the primary focus of this chapter. The taxonomy of the cultivated potato is summarized in Table 1.

Potato is a short-lived perennial species forming weak annual stems arising from underground

Table 1 Botanical classification of the cultivated potato

Family	Solanaceae
Subfamily	Solanoideae
Tribe	Solaneae
Genus	<i>Solanum</i> L.
Subgenus	<i>Potatoe</i> (G. Don) D'Arcy
Section	<i>Petota</i> Dumortier
Subsection	<i>Potatoe</i> G. Don
Superseries	<i>Rotata</i> Hawkes
Series	<i>Tuberosa</i> (Rydb.) Hawkes
Species	<i>Solanum tuberosum</i> L.
Subspecies	<i>tuberosum</i>

tubers. As an agricultural commodity, potatoes are raised as an annual crop. Plants are small with individual stems 1 m or less in length. Plant habit ranges from compact to sprawling. Leaves are pinnately compound, arising alternately from the aboveground stems. The leaves may be lightly covered with fine trichomes. Potato flowers are clustered in a primary cymose inflorescence. Flowers are hypogynous and actinomorphic, with fused corollas with five lobes. Corolla color ranges from purple to pinkish to white. Fertilized flowers form spherical berries high in toxic glycoalkaloids. The berries carry small, flat, oval or kidney-shaped seeds that are yellowish to tan in color. The tuber is a carbohydrate storage structure arising from stolons (short underground stems or rhizomes). The prominent “eyes” of the tuber are actually clusters of axillary buds from which aboveground stems may arise. The tuber is both the primary commercial propagule and the organ of economic significance. Rubatzky and Yamaguchi (1997) provide detailed discussion of tuber morphology.

The basic chromosome number for potato species is 12. Cultivated potato is an autotetraploid ($2n = 4x = 48$ chromosomes) with tetravalent chromosome pairing during meiosis. Potato chromosomes are relatively small ($1\text{--}3.5\ \mu\text{M}$ at metaphase) and predominantly metacentric (Dong *et al.*, 2000). Mohanty *et al.* (2004) examined the karyotype of 30 different potato cultivars and breeding lines. Based on chromosome size and relative positioning of restrictions, the authors described five different chromosome morphologies. Individual genotypes differed considerably in overall karyotype, with several genotypes possessing unique combinations of the five basic chromosome types (Mohanty *et al.*, 2004), suggesting that morphology is not fully predictive of a given chromosome. Consistently, Dong *et al.* (2000) indicate that individual chromosomes cannot be reliably identified based solely on morphological features. Mok *et al.* (1974), Lee and Hanneman (1976), Pijnacker and Ferwerda (1984), and Wagenvoort *et al.* (1994) utilized Giemsa-banding patterns to identify specific potato chromosomes. Yeh and Peloquin (1965), Ramanna and Wagenvoort (1976), and Wagenvoort (1988) analyzed potato chromosomes at pachytene. At this stage, the patterns of heterochromatin and euchromatin can distinguish every chromosome, although several chromosomes have very similar banding patterns. The method has not found

widespread application owing to its technical difficulty and suitability only for analysis of diploid materials (Dong *et al.*, 2000). Dong *et al.* (2000) developed an entire set of chromosome-specific bacterial artificial chromosome (BAC) probes for fluorescent *in situ* hybridization (FISH). Mohanty *et al.* (2004) demonstrated that a single somatic cell (4C) varies in DNA content from 7.28 pg to 15.83 pg (picogram), a statistically significant difference. The authors attributed variation in DNA content to expansion or contraction of repetitive DNA in certain potato lineages (Mohanty *et al.*, 2004).

1.3 Economic Importance

For the 41-year period from 1965 to 2005, worldwide potato production increased by 19.26% from 270 913 502 t in 1965 to 323 102 918 t in 2005. Gains were most notable in Asia (Figure 1a), with production in China increasing 356.02% from 16 016 136 t in 1965 to 73 036 500 t in 2005 and production in India increasing 593.48% from 3 605 000 t in 1965 to 25 000 000 t in 2005. China and India currently lead world production (Table 2).

During the same 41-year period, there was a worldwide decrease in the number of hectares of potatoes grown of 12.95% from 21 391 391 ha in 1965 to 18 622 188 ha in 2005 (Figure 1b). Production area losses were greatest in European countries including Poland (a loss of 2 170 892 ha or 78.51%), Germany (a loss of 1 231 533 ha or 81.64%), France (a loss of 417 118 ha or 72.53%), Italy (a loss of 278 391 ha or 79.93%), and Spain (a loss of 275 095 ha or 74.67%). Production area gains were greatest in China (a gain of 2 700 266 ha or 158.72%), India (a gain of 971 000 ha or 226.34%), Bangladesh (a gain of 270 738 ha or 488.33%), Nigeria (a gain of 175 000 ha or 8750.00%), and Iran (a gain of 162 000 ha or 490.91%).

Between 1965 and 2005, worldwide average potato yield increased 37% from 126 646 hg ha^{-1} to 173 504 hg ha^{-1} (Figure 1c). Increases were most notable for North America and Central America (an increase in average yield of 75.69% from 214 954 hg ha^{-1} in 1965 to 377 659 hg ha^{-1} in 2005) and Oceania (an increase in average yield of 131.14% from 164 437 hg ha^{-1} in 1965 to 380 084 hg ha^{-1} in 2005). Other major production

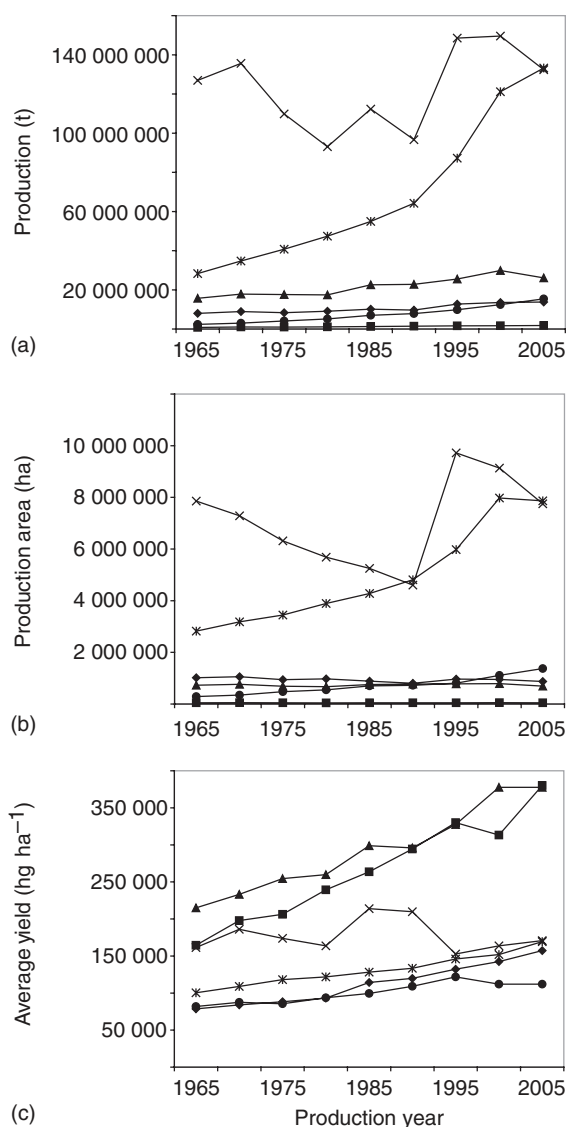


Figure 1 Trends in world potato production (1965–2005). Potato production statistics for Africa (●), Asia (*), Europe (x), North and Central America (▲), Oceania: Australia and New Zealand (■), and South America (◆) for the period 1965–2005. (a) Total annual potato production in metric tons. (b) Production area in ha. (c) Average yield in hg/ha. During the past four decades, total potato production in much of the world has remained constant. However, dramatic increases can be noted for Asia. European potato production during this period has fluctuated. Potato production area in much of the world has been steady with the exception of Asia, which has experienced large increases in potato production area, and Europe, where potato production area has fluctuated. Yield increases are particularly evident for North and Central America and Oceania, with only modest increases in potato yield noted in other regions of the world [Source: FAOSTAT.fao.org]

areas of the world experienced modest though predominantly steady gains in yield during this same period (Figure 1c).

Potato provides approximately 820 calories kg^{-1} , mostly in the form of carbohydrate, and has higher calorie per hectare yield potential than any grain (Rubatzky and Yamaguchi, 1997). Although potato provides only about 2.1 g of protein per 100 g fresh edible portion (Rubatzky and Yamaguchi, 1997), potato is a significant source of protein in the human diet in certain countries due to high consumption rates. In Belarus, average daily per capita consumption of potatoes in 2004 was 488.63 g, providing 15.17% of the United States Department of Agriculture (USDA) Recommended Daily Allowance for a 24 year's old (72 kg) male. Other top potato consuming nations in 2004 included Kyrgyzstan (385.59 g per day per capita), Rwanda (381.83 g per day per capita), Lithuania (373.76 g per day per capita), and Poland (373.73 g per day per capita) (FAOSTAT data 2004). Potato is a rich source of minerals, especially potassium (420 mg per 100 g fresh edible portion), and vitamin C (18 mg per 100 g fresh edible portion).

Potatoes are utilized in several ways. For culinary uses, tuber characteristics including water content, tuber shape and size, eye depth, sugar composition, and storability affect cultivar utility. As a direct food product, potatoes are commonly baked, boiled, or fried. Important processed potato foods include French fries and potato chips. Some South American communities still produce the freeze-dried potato product, *chuño*. Potatoes are the traditional source of the alcoholic vodka. Industrial uses for potato include the extraction of starch.

1.4 Traditional Breeding: Objectives, Tools and Strategies, and Achievements

Conventional strategies for potato breeding must address the complex genetic characteristics of this crop. Potato is a highly heterozygous polyploid with tetrasomic inheritance. Nonadditive gene interactions, e.g., intralocus (heterozygosity) and interlocus (epistasis) interactions, are important in determining potato phenotypes, thus offering the potential for exploiting heterosis in plant improvement since high levels of allelic diversity at loci of interest guarantee increased heterosis. The

Table 2 Leaders in world potato production (1965–2005)

Rank ^(b)	Production year									
	1965	1970	1975	1980	1985	1990	1995	2000	2005	
1	China	16016136	21 523 634	24 339 922	25 896 072	26 792 976	32 031 189	45 983 531	66 318 167	73 036 500
2	India	3 605 000	3 912 600	6 225 400	8 326 600	12 570 600	14 770 800	17 401 300	24 713 200	25 000 000
3	Ukraine	—	—	—	—	—	—	14 729 400	19 838 100	19 462 000
4	United States	13 210 568	14 774 200	14 604 600	13 785 000	18 443 008	18 239 008	20 122 000	23 297 460	19 090 750
5	Germany	30 951 200	29 303 808	18 525 616	17 146 192	21 053 840	14 471 000	10 888 100	13 694 283	11 624 000
6	Poland	42 665 008	50 301 408	46 429 040	26 390 544	36 546 144	36 312 784	24 891 330	24 232 376	11 009 392
7	Belarus	—	—	—	—	—	—	9 504 000	8 717 800	8 185 000
8	Netherlands	3 229 591	5 647 683	5 003 130	6 266 641	7 149 605	7 036 000	7 340 000	8 227 000	6 835 985
9	France	11 223 210	8 025 300	6 641 500	6 617 800	7 787 000	4 754 415	5 839 000	6 434 053	6 644 859
10	United Kingdom	8 006 000	7 821 000	4 787 000	7 105 000	6 892 000	6 467 000	6 404 000	6 636 000	5 815 000
	World	270 913 502	298 048 017	270 384 075	240 464 105	281 530 607	266 499 589	285 850 306	328 656 044	323 102 918

^(a)Source: FAOSTAT^(b)Rank based on 2005 production statistics

more diverse the alleles are within a specific locus, the higher the heterozygosity and the greater the number of increased interlocus and epistatic interactions. Up to four different alleles are possible at each locus and maximum allelic diversity at a given locus A (e.g., $a_1a_2a_3a_4$) guarantees first, second, and third order interactions. Potato breeding has generally focused on genetic improvement at two ploidy and EBN levels: the tetraploid (4EBN) level and the diploid (2EBN) level.

Genetic improvement at the tetraploid (4EBN) level is mostly based on phenotypic recurrent selection, involving crosses between tetraploid varieties and/or advanced clones, and then field evaluation and selection. Most varieties currently used were derived using this breeding approach. The procedure starts with the selection of desirable parents, with breeders commonly targeting parents of diverse genetic origin to increase the chances of pyramiding different desired alleles in the progeny genotypes. Following crossing, various progeny tests, data on general and specific combining ability, and information derived from molecular markers are used to select the best progeny. Due to the high levels of heterozygosity, segregation of traits is expected in the F_1 generation. Tubers obtained from each selected F_1 clone are grown for further evaluations and to increase the number of seed tubers. During the next season clones are cultivated and screened in larger plots. After several years of clonal selection, superior clones are tested in different locations to estimate the genotype \times environment interaction.

While the genetic base of the cultivated potato is considered to be narrow (Mendoza and Haynes, 1974) and yield stasis exists within the potato germplasm of North America (Douches *et al.*, 1996), the potato has an extremely rich gene pool with seven cultivated and approximately 200 wild species (Spooner and Hijmans, 2001). To broaden the genetic base of the cultivated potato and introduce new traits, North and South American potato species have been used as sources of new genes (Haynes, 1972; Hermundstad and Peloquin, 1985). To exploit the wealth in allelic diversity of these tuber and nontuber bearing species and to take advantage of disomic rather than tetrasomic inheritance, several breeding programs have focused on genetic improvement at the diploid (2EBN) level. These programs make use of sexual hybridization between $2x$ (2EBN) species and

$2x$ (2EBN) *S. tuberosum* haploids. Haploid-species hybrids are selected for traits of interest and also for a propensity for $2n$ gamete production. In Poland, haploid-species hybrids with multiple resistances and tuber qualities are developed and used in $4x \times 2x$ crossing schemes to produce parental lines and cultivars. The same holds true in Italy, Great Britain, Netherlands, and other European Union (EU) countries. These hybrids are then employed in sexual polyploidization crossing schemes with potato ($4x$ (4EBN)) to re-establish the tetraploid level of the cultivated *S. tuberosum*. $2n$ gametes not only allow the restoration of the tetraploid level, but also permit the transmission of large amounts of the parental heterozygosity and epistatic interactions without disruption by recombination (Peloquin *et al.*, 1999). Exploitation of $2n$ gametes also allows the development of various breeding schemes based on the manipulation of whole chromosome sets, permitting gene flow through interploid and bridge crosses (reviewed in Carputo and Barone, 2005).

The development and marketing of new varieties in Europe is generally the responsibility of private breeding companies. Public programs have been mostly discontinued and currently public institutions are involved in variety release only in a few countries (mainly in Eastern Europe). By contrast, in the United States, potato breeding is the concern of the public sector and only a few companies develop new varieties. As with many other crops, both in Europe and in the United States, traditional potato breeding is not concerned with only one objective, but with many. Priorities include resistance to biotic and abiotic stresses (diseases, pests, drought, heat, and cold) and quality requirements for either table use or processing (tuber yield, shape and size, dry matter and sugar content, sensitivity to cold sweetening, discoloration, bruising, and starch characteristics). Tarn *et al.* (1992) have identified 18 traits related to fresh and processing uses, 17 pathogen and six pest resistance traits, and numerous agronomic traits that need to be considered in a potato breeding program.

Resistance to late blight, caused by the oomycete *Phytophthora infestans*, is still a primary target in terms of biotic stresses. The disease is characterized by haulm destruction and decay of the tubers. Worldwide research has been brought together by the Global Initiative on Late

Blight (GILB), a network of researchers and technology developers from more than 70 countries. Europe, in particular, has a long history of resistance breeding against *P. infestans* and the European Union has funded the construction of a database (<http://www.Eucablight.com>) to provide tools for investigating variation in both the host and the pathogen. In the United States, due to conducive climatic conditions and growing practices, the Midwestern states are particularly vulnerable. Consistently, during recent years in North America, potato late blight has re-emerged as the most important pathogen of the potato crop. The US-8 genotype of *P. infestans*, the predominant genotype present in the United States, is characterized by reduced sensitivity to metalaxyl and the A2 mating type (Deahl *et al.*, 1993). One aspect of late blight disease management in the field is the use of resistant cultivars. It is important to draw upon many germplasm resources to develop a broad genetic base and identify differences in expression of single components of field resistance (i.e., resistance to infection, spread, and sporulation), then hybridize according to complementary components. Concurrently, the high standards demanded by the industry and consumers for yield, maturity, class, quality, and multiple resistances must be met.

The Colorado potato beetle, *Leptinotarsa decemlineata* is one of the most economically significant pests of potato in northern latitudes. The Colorado potato beetle led to the first large-scale use of insecticides in 1864 (Gauthier *et al.*, 1981) and insecticides remain the primary means of Colorado potato beetle control (Casagrande, 1987). About 1.3 million lbs of active ingredients of the insecticides are applied to potato crops to control Colorado potato beetle in the top eight potato-producing US states (Wiese *et al.*, 1998). However, the Colorado potato beetle has shown a remarkable ability to develop resistance to every insecticide used for its control (Bishop and Grafius, 1996) and has done so at an increasingly fast rate (Heim *et al.*, 1990; Ioannidis *et al.*, 1991). Resistance to nematodes is also another important breeding target. The potato is a major host for potato cyst nematodes *Globodera pallida* and *Globodera rostochiensis* and for root-knot nematodes (*Meloidogyne* spp.). In Europe, the availability of varieties resistant to *G. rostochiensis* has accentuated

the presence of *G. pallida*, and therefore breeding efforts are being directed toward the development of genotypes resistant to this cyst nematode.

Of the bacterial diseases, *Streptomyces scabies* is one of the major pathogens that infect potato and cause scab. The pathogen produces necrotic, corky-textured lesions on the outer surface of the potato. The lesions can vary in their appearance as being raised, surface, or pitted. Chip processors consider pitted lesions a chip defect because the pit will be apparent in the chip. Surface and raised lesions present less of a problem as potatoes are peeled before they are chipped, thereby removing the lesions. Even so, since the marketplace for potatoes is quality driven, the presence of scab lesions, especially those that are pitted, on the outer surface of the potato for both table and processing varieties significantly lessen their marketability. The best and most reliable solution to scab is through the use of host resistance (Ross, 1986; Loria *et al.*, 1997).

In recent years, in part due to the growing market for fresh, prepacked potatoes and to the expansion of EU to include eastern countries, resistance to other pests including *Colletotrichum coccodes*, *Rhizoctonia solani*, *Verticillium* spp., *Clavibacter michiganensis*, and *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) have also become major objectives for European potato breeders.

Among abiotic stresses, resistance to low temperatures is deemed very important in the Mediterranean area. Here the potato is grown "off-season" (starting from November), in a cycle that is much earlier than the typical spring-summer cycle. Due to the lack of resistant varieties, "off-season" production can be seriously damaged by frost events.

Breeding objectives related to quality requirements for processed potatoes are quite different from those for fresh use. The former are more precisely defined and are mainly related to dry matter content, level of reducing sugars after harvest, starch quality and, to some extent, tuber shape and size. The latter falls more in the category of "external traits" (e.g., skin and flesh color, eye depth), even though eating quality traits, such as after-cooking blackening are also important. A recent treatment by Renatus (2005) on the European potato trade pointed out that breeders need to consider that the fresh market is now segmented into two main categories. The first

includes the standard merchandise at a discount price level, where breeders are required to provide cost efficient, high yielding multiuse varieties. The second segment comprises the visually appealing high priced top quality potatoes. This high-end segment requires the release of varieties with superior taste and increased nutritional value. Due to growing awareness of the role of food quality on human health, new objectives of potato breeding have arisen in the last few years. Following discussion on acrylamide accumulation in fried products (Motttram *et al.*, 2002), a recent target for potato breeders is the development of varieties that do not accumulate asparagine, the precursor of acrylamide. Equally important has become the possibility to have varieties with long dormancy, in order to avoid the use of chemical sprout suppressants. Recent work to improve potato nutritional quality has also focused on modifying carotenoid, anthocyanin, or antioxidant content, as well as exploring the natural variation for important endogenous nutrients, such as vitamin C (Lu *et al.*, 2001; Brown *et al.*, 2003; Love *et al.*, 2004; Reyes *et al.*, 2004). Thus far, nutritional improvement in potato has been limited to designing potatoes for delivery of unique carotenoids shown to reduce the incidence of particular diseases, or increasing general antioxidant levels. For example, overexpression of phytoene synthase was shown to result in 19-fold higher levels of lutein, as well as higher β -carotene levels (Ducreux *et al.*, 2005), zeaxanthin contents were increased up to 130-fold through disruption of zeaxanthin catabolism (Römer *et al.*, 2002), and accumulation of astaxanthin was achieved through expression of a microbial β -carotene ketolase gene (Morris *et al.*, 2006). Crowell *et al.* (2008) overexpressed p-hydroxyphenylpyruvate dioxygenase (HPPD) and homogentisate phytyltransferase (HPT) to assess their impact on vitamin E accumulation in potato tubers. Overexpression of *At-HPPD* resulted in a 2.7-fold increase in α -tocopherol, while overexpression of *At-HPT* doubled the levels.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional potato breeding approaches have led to the development of new varieties suitable

for various uses. Following parental genotype selection and crosses, progeny are phenotypically evaluated over several years and sites. It may take a number of years (up to 15) to develop a new variety using these approaches. In the case of conventional breeding conducted at the tetraploid level, the tetrasomic inheritance of the potato and its high level of heterozygosity require that large numbers of progeny (i.e., large numbers of individual F_1 seedlings) must be evaluated to find useful segregants. Difficulties are also presented by the polygenic control of many traits of interest. Since they are not inherited in a simple Mendelian fashion, phenotypes cannot be grouped into a small number of easily distinguished classes, and the genotype \times environment interaction is usually high.

Breeding schemes to access genes possessed by wild germplasm require various cycles of backcrosses, evaluation, and selection to eliminate undesirable traits derived from the wild. This process is often expensive in terms of costs, time, and resources. In addition, there are some reproductive aspects of the potato (e.g., nuclear-cytoplasmic male-sterility and incompatibility barriers) that may hamper breeding when interspecific/interploidy hybridization is used to expand the cultivated gene pool. The use of molecular markers linked to target genes or quantitative trait loci (QTLs) may allow faster and more efficient identification of superior genotypes. However, despite its potential, relatively few examples of molecular marker-assisted selection are reported in potato. According to Mullins *et al.* (2006) this is mainly due to the outbreeding, tetraploid nature of the cultivated potato and to the fact that most traits are quantitatively inherited, with a large genotype \times environment interaction.

Transgenic approaches can help to overcome some of the limitations of conventional breeding strategies. It should be pointed out, however, that the transgenic approach is not, as often presented, simply a faster and more precise alternative to classical breeding. Rather, it is an additional, complementary way of accessing genetic diversity for genotype improvement. Like other breeding approaches, the creation and evaluation of transgenic potato lines can be time consuming and costly. In effect, transgenic approaches and conventional breeding are parallel processes in that variety development through a transgenic

approach may require that hundreds of individuals be screened to identify those possessing the most desirable expression of the transgene. Similarly, in conventional breeding, large populations with high genotypic and phenotypic variability are evaluated. Bradford *et al.* (2005) reviewed how genetic modifications caused by conventional breeding can be considered analogous to transgenic events. Mutation breeding, for example, is based on random genetic changes and selection is mainly based on the phenotype, without any knowledge of the underlying genetic cause. Wide crosses and ploidy manipulations produce various pleiotropic effects on gene structure and trait expression. Loss-of-function alleles, transposon and retroviral movements are additional events common to conventional and transgenic breeding.

The use of transgenic approaches, combined with conventional breeding, is emerging as a balanced approach to develop improved potato varieties with long- and short-term breeding strategies. Transgenic potato was first reported in 1986 (An *et al.*, 1986; Shahin and Simpson, 1986). These were created via *Agrobacterium tumefaciens*-mediated transformation. Since that time, various vectors and approaches have been used to insert transgenes into the potato genome. Of particular promise, the development of particle bombardment technologies for potato (Romano *et al.*, 2001) offers the potential for the simultaneous transfer of multiple genes. Also noteworthy is the possibility to perform plastid transformation (Nguyen *et al.*, 2005), which can be crucial for producing vaccines in potato plants. The use of non-*Agrobacterium* bacteria (e.g., *Rhizobium* and *Mesorhizobium*), methods for gene silencing, and plant-derived vectors hold promise for improving potato transformation methods and approaches (see Section 2). In potato, the transgenic approach seems particularly feasible to access genes from wild species. Many of the wild potato species possess an enormous wealth of noteworthy genes whose introgression through conventional breeding may have various limitations in terms of time and resources. *Agrobacterium*-mediated transformation, for example, allowed the introgression from the incongruent species *Solanum bulbocastanum* of genes conferring resistance to *P. infestans* (Song *et al.*, 2003a; Van Der Vossen *et al.*, 2003, 2005). The transgenic

approach may also circumvent genetic problems related to backcrossing including reduction in heterotic effect and loss of desired phenotypic characteristics through meiotic reassortment.

The transgenic approach is probably the best system to exploit recent advances in the field of structural and functional potato genomics. These studies have contributed to an understanding of the complex multistep processes related to potato quality and to elucidation of the genetics of traits of interest. For example, the biosynthetic networks involved in carbohydrate metabolism, vitamin C, and glycoalkaloid production have been described, and the genetic basis of quality traits such as starch content, chip color, anthocyanin pigmentation, and tuber shape have been determined. Subsequently, genes controlling expression of many important traits have been isolated. The transfer of these genes as transgenes into potato genotypes of interest can improve cultivars lacking in quality-related traits or can lead to production of novel metabolites of industrial, pharmaceutical, and veterinarian use.

2. DEVELOPMENT OF TRANSGENIC POTATOES

2.1 Donor Genes

Research efforts in recent years have led to the genetic characterization, mapping, and cloning of several genes of agricultural relevance to potato. Of particular note are genes imparting improved resistance to diseases, pests, and abiotic stresses, better processing qualities, and novel industrial characteristics. These genes originate from a variety of organisms, including cultivated and wild potato species, nonpotato plant species, and even bacteria and viruses. Genes cloned from potato species and showing particular promise for improvement of cultivated potato through transgenic technologies are summarized in Table 3. De Jong (2005) reviewed important molecular approaches used to clone potato genes, including map-based cloning and candidate gene identification. The identification and cloning of agriculturally relevant genes opens the door to improvement of important potato cultivars using transgenic approaches.

Table 3 Transgenes from potato (*Solanum*) species with agricultural potential

Gene	Function	Origin	Transgene				Reference
			Vector	Promoter	Selectable marker ^(a)	Transformation method	
<i>GBSS</i> (antisense)	<i>GBSS</i> disruption	<i>S. tuberosum</i>	pROK-1	<i>CaMV</i> 35S	Kan	<i>Agrobacterium</i>	Visser <i>et al.</i> , 1991
<i>GBSS</i> (antisense)	<i>GBSS</i> disruption	<i>S. tuberosum</i>	pPGB-1	Native	Kan	<i>Agrobacterium</i>	Kuipers <i>et al.</i> , 1994
<i>Gpa2</i>	Nematode resistance	<i>S. tuberosum</i>	pBINPLUS	Native	Kan	<i>Agrobacterium</i>	Van Der Vossen <i>et al.</i> , 2000
<i>Gro1</i>	Nematode resistance	<i>S. spegazzinii</i>	pCLD04541	Native	Kan	<i>Agrobacterium</i>	Paal <i>et al.</i> , 2004
<i>Im-19</i> (antisense)	Acid invertase disruption	<i>S. tuberosum</i>	BINAR	<i>CaMV</i> 35S	Kan	<i>Agrobacterium</i>	Zrenner <i>et al.</i> , 1996
<i>R1</i>	Late blight resistance	<i>S. demissum</i>	pCLD04541	Native	Kan	<i>Agrobacterium</i>	Ballvora <i>et al.</i> , 2002
<i>R3a</i>	Late blight resistance	<i>S. demissum</i>	pBINPLUS	Native	Kan	<i>Agrobacterium</i>	Huang <i>et al.</i> , 2005
<i>RB (Rpi-blb1)</i>	Late blight resistance	<i>S. bulbocastanum</i>	pCLD04541	Native	Kan	<i>Agrobacterium</i>	Song <i>et al.</i> , 2003b
<i>Rpi-blb2</i>	Late blight resistance	<i>S. bulbocastanum</i>	pBINPLUS	Native	Kan	<i>Agrobacterium</i>	Van Der Vossen <i>et al.</i> , 2005
<i>Rx1</i>	Potato Virus X resistance	<i>S. tuberosum</i>	pSLJ7292	Native	Kan	<i>Agrobacterium</i>	Bendahmane <i>et al.</i> , 1999
<i>SBE A</i> (antisense)	Amylopectin disruption	<i>S. tuberosum</i>	pGPTV-HYG	dbl <i>CaMV</i> 35S	Hyg	<i>Agrobacterium</i>	Schwall <i>et al.</i> , 2000
<i>SBE B</i> (antisense)	Amylopectin disruption	<i>S. tuberosum</i>	pBIN19	dbl <i>CaMV</i> 35S	Kan	<i>Agrobacterium</i>	Schwall <i>et al.</i> , 2000
<i>UGPase</i> (antisense)	<i>UGPase</i> disruption	<i>S. tuberosum</i>	pBIN19	<i>CaMV</i> 35S	Kan	<i>Agrobacterium</i>	Zrenner <i>et al.</i> , 1993
<i>UGPase</i> (antisense)	<i>UGPase</i> disruption	<i>S. tuberosum</i>	pBIN440	Patatin	Kan	<i>Agrobacterium</i>	Spychalla <i>et al.</i> , 1994

^(a)Hyg, hygromycin B; Kan, kanamycin

2.1.1 Transgenes for resistance to biotic and abiotic stresses

Potato is host to more than 60 diseases of current economic significance (Stevenson *et al.*, 2001) and commercial potato production in most parts of the world relies heavily upon chemical inputs, including fungicides. Genetic resistance to significant potato diseases offers the potential to reduce grower costs and environmental risks associated with chemical inputs. Chief among potato diseases worldwide is late blight, a disease of both foliage and tuber caused by the oomycete *P. infestans*. Late blight has a notorious past for its role in the Irish Potato Famine of the mid 1800s, during which a million people starved to death and a million more emigrated from Europe to the United States. Today, the disease causes an estimated annual loss of US\$5 billion worldwide, both through yield losses of potato and tomato and through increased production costs associated with chemical controls (Kamoun, 2001). Wild potato species, especially those indigenous to central Mexico and certain regions of the Andes, areas ideal for late blight epidemics, are sources of resistance genes for improvement of cultivated potato. Not surprisingly, considerable research effort has been devoted to the molecular cloning of resistance genes imparting late blight resistance, with particular emphasis on wild potato species.

Among the earliest examples of resistance breeding, the Mexican species *Solanum demissum* was crossed with potato nearly 100 years ago (Salaman, 1911; Umaerus and Umaerus, 1994). Eleven late blight resistance genes have been genetically described from this species (Black and Gallegly, 1957; Malcolmson and Black, 1966; Umaerus and Umaerus, 1994). These genes are referred to as the “R series” and are numbered from *R1* to *R11*. Each is associated with a pathogen race-specific, hypersensitive response (HR) characterized by controlled cell death surrounding the site of infection. Several have been mapped: *R1* on chromosome 5 (Leonards-Schippers *et al.*, 1992), *R2* on chromosome 4 (Li *et al.*, 1998), and *R3*, *R6*, and *R7* on chromosome 11 (El-Kharbotly *et al.*, 1996). Initially promising in the agricultural setting, these genes proved to be of short durability as the pathogen quickly circumvented their defenses (Toxopeus, 1956; Black and Gallegly, 1957).

Using BAC-based positional cloning in combination with a candidate gene approach, Ballvora *et al.* (2002) reported the cloning of *R1*. The gene was first fine mapped using populations generated from diploid *S. tuberosum* lines into which the *S. demissum* *R1* gene was introgressed (Gebhardt *et al.*, 1989; Leonards-Schippers *et al.*, 1992; Meksem *et al.*, 1995). Once corresponding BAC clones were isolated, BAC-end sequence analysis revealed similarity to a previously cloned plant disease resistance gene. Subsequent further BAC isolation and subcloning into binary cosmid vector pCDL04541 led the authors to a 10 kb fragment containing the candidate gene along with 2 kb upstream and 4 kb downstream of the predicted coding region. The binary cosmid vector pCDL04541 was derived from vector SLJ1711 (Jones *et al.*, 1992) by the addition of a cos site for lambda bacteriophage packaging. In turn, SLJ1711 was derived from the broad host range vector pRK290 (Ditta *et al.*, 1980) by the addition of an insert that carries the *NPTII* gene for resistance to the antibiotic kanamycin driven by the constitutive cauliflower mosaic virus promoter, CaMV 35S and a polylinker site associated with dark Bluescript (dBS) blue/white selection. pCDL04541, like pRK290, also carries a gene for resistance to the antibiotic tetracycline. pCDL04541 has been widely used in plant transformation research. By transforming late blight susceptible potato cultivar Désirée with the pCDL04541 vector containing a 10 kb genomic insert, Ballvora *et al.* (2002) demonstrated that the late blight resistance gene *R1* including the endogenous promoter and any downstream elements was carried in the subclone. *R1* was the first late blight resistance gene to be cloned.

S. demissum is also source of the late blight resistance gene *R3a*. The locus maps to a resistance gene “hot spot” on chromosome 11. Using fine-mapping and functional testing, Huang *et al.* (2004) demonstrated that the previously defined *R3* locus is actually two tightly linked loci with distinct specificities, *R3a* and *R3b*. Subsequently, the authors reported the cloning of *R3a* (Huang *et al.*, 2005). *R3a* was cloned using a comparative genomics approach. Significantly, microsynteny was noted between the potato *R3a* locus (harboring the late blight resistance allele derived from *S. demissum*) and the corresponding

region in tomato. The tomato region encompassed a portion of the *I2* gene complex responsible for resistance to *Fusarium* wilt. Huang *et al.* (2005) screened potato BAC DNA pools encompassing 0.05 genome equivalents for the presence of markers mapped in close proximity to *R3a*. The same pools were also screened for the presence of *I2*-like sequences using primers developed from tomato sequence. In total, the authors identified 11 overlapping BAC clones encompassing a 700 kb genomic region that included both *R3a* markers and *I2*-like sequences. Two BACs were sequenced, revealing five *I2*-like sequences, each a candidate for the functional *R3a* locus. The candidate genes were subcloned as 10 kb fragments into the pBINPLUS vector. pBINPLUS was developed from pBIN19 (van Engelen *et al.*, 1995). The vector carries the *NPTII* gene for resistance to the antibiotic tetramycin and the *lacZ'* gene for blue/white selection. Each *R3a* candidate gene cloned into the pBINPLUS vector included 2–3 kb of genomic sequence before and after the putative transcript, presumably incorporating the endogenous promoter and downstream elements. Transformation revealed that one of the candidate genes imparted late blight resistance to susceptible potato cultivars (Huang *et al.*, 2005).

Other *S. demissum* late blight resistance genes are also likely candidates for cloning in the near future, including the *R7* gene of chromosome 11. While isolation of *R1-R11* is historically and scientifically significant, the early defeat of these genes in the field suggests they may be of limited agricultural application as transgenes. In contrast, the Mexican diploid wild potato *S. bulbocastanum* is a source of broad-spectrum late blight resistance genes that may be more durable in an agricultural setting. *S. bulbocastanum* has long been recognized as a source of late blight resistance (Reddick, 1939; Neiderhauser and Mills, 1953; Graham *et al.*, 1959). However, direct sexual crosses between potato and *S. bulbocastanum* have never been reported. Researchers have attempted to access resistance from *S. bulbocastanum* using a series of bridge crosses (Hermesen and Ramanna, 1973) or somatic hybridization (Helgeson *et al.*, 1998) approaches that incorporate undesirable characteristics along with resistance, complicating cultivar development. Late blight resistance in *S. bulbocastanum* is functionally distinct from that of *S. demissum*. Examining somatic hybrid

(cultivated potato + *S. bulbocastanum*)-derived lines in the Toluca Valley, Helgeson *et al.* (1998) concluded resistance from *S. bulbocastanum* lacks pathogen race specificity and a discernible HR phenotype. Instead, resistance from *S. bulbocastanum* suppresses but does not prevent disease development (Helgeson *et al.*, 1998). Suppression of the rate of symptom development may alone impart agriculturally meaningful, durable resistance.

The first late blight resistance gene cloned from *S. bulbocastanum* was *RB* (Song *et al.*, 2003b), also referred to as *Rpi-blb1* (Van Der Vossen *et al.*, 2003). *RB* was first mapped to *S. bulbocastanum* chromosome 8 using a somatic hybrid (potato + *S. bulbocastanum*)-derived mapping population (Naess *et al.*, 2000). Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers associated with *RB* were starting points for BAC-based positional cloning while concomitant fine-mapping using BAC-end-derived markers further defined the resistance region genetically (Bradeen *et al.*, 2003). A single BAC clone encompassing the resistance region was sequenced, revealing a cluster of four putative genes belonging to the nucleotide binding site-leucine rich repeat (NBS-LRR) family. Approximately 75% of all cloned plant disease resistance genes belong to this family and the four *S. bulbocastanum* sequences were each considered as candidates for the functional *RB* gene. The *S. bulbocastanum* genotype used to map *RB* and for BAC library construction was heterozygous for *RB*, with one haplotype carrying an allele at the *RB* locus for late blight resistance and the other haplotype carrying an allele at the *RB* locus for late blight susceptibility. The BAC clone sequenced by Song *et al.* (2003b) was shown through genetic mapping to carry the susceptibility allele; the resistance haplotype was never recovered from the 12× genome equivalents BAC library, despite exhaustive library screening. This was later explained by instability of BAC clones carrying the *RB* resistance allele (Song *et al.*, 2003a) and necessitated that positional cloning be supplemented with a long-range (LR)-polymerase chain reaction (PCR) approach (Song *et al.*, 2003b). Briefly, genomic DNA from a late blight resistant *S. bulbocastanum* genotype was used as a template for LR-PCR amplification of each of the candidate genes using primers designed

from BAC sequence from the susceptibility allele haplotype. To ensure recovery of the endogenous promoter and terminator elements, LR-PCR primers were designed such that the amplicons would encompass several kilobytes of genomic sequence upstream and downstream of the putative transcripts. Each of the four candidate genes was targeted using this approach and in each case, the amplicon included DNA copies derived from both *RB* haplotypes (Song *et al.*, 2003b). The LR-PCR amplicons were cloned into vector pCLD04541. Transformation of the late blight susceptible potato cultivar Katahdin revealed that one of the candidate genes was *RB*. *RB* imparts a broad-spectrum, nonrace-specific resistance to transformed potato and tomato lines.

A second late blight resistance gene, *Rpi-blb2*, has also been cloned from *S. bulbocastanum* (Van Der Vossen *et al.*, 2005). The gene was first mapped to a single region of *S. bulbocastanum* chromosome 6 in tetraploid populations derived from interspecific crosses involving four different species. Subsequent fine-mapping was done in a diploid *S. bulbocastanum* population and involved the use of a resistance gene homolog-fingerprinting technique. Results of these efforts suggested that *Rpi-blb2* shared DNA sequence homology to the tomato *Mi-1* gene. BAC-based positional cloning ensued. The authors ultimately developed a BAC contig encompassing approximately 12 *Mi-1* like sequences and the entire genetically defined resistance region. A subclone library of approximately 10 kb fragments was generated from each BAC clone using vector pBINPLUS. Subclones were subjected to a PCR screen and subclones containing nine different *Mi-1* like candidate genes were identified. Transformation of susceptible potato cultivars revealed that one of the candidate genes imparted late blight resistance and was, therefore, *Rpi-blb2*. *Rpi-blb2*, like *RB*, imparts broad-spectrum, nonrace-specific late blight resistance.

S. bulbocastanum is a likely source of other late blight resistance genes (Park *et al.*, 2005) that may be of agricultural significance. Other potato species including *Solanum berthaultii* (Ewing *et al.*, 2000; Rauscher *et al.*, 2006), *Solanum mochiquense* (Smilde *et al.*, 2005), *Solanum phureja* (Sliwka *et al.*, 2006), *Solanum pinnatisectum* (Kuhl *et al.*, 2001; Chen *et al.*, 2003), and *Solanum polyadenium* (Bradeen, unpublished results) are sources of novel

late blight resistance genes that may be future subjects of cloning efforts.

Genes imparting late blight resistance have not been the only targets for molecular biologists. Several genes imparting resistance to other significant potato disease have also been cloned. Two genes, the *Rx1* gene for resistance to potato virus X and the *Gpa2* gene for resistance to the potato cyst nematode (*G. pallida*) were cloned from a common region of chromosome 12 of *S. tuberosum* subsp. *andigena* (Bendahmane *et al.*, 1999; Kanyuka *et al.*, 1999; Rouppe Van Der Voort *et al.*, 1999; Van Der Vossen *et al.*, 2000). The genes were cloned using a BAC-based positional cloning approach that included fine-mapping of the traits in several different populations (Rouppe Van Der Voort *et al.*, 1999). *Rx1* was cloned first (Bendahmane *et al.*, 1999). The authors verified that a single BAC clone encompassed the genetically defined *Rx1* region through genetic mapping (Kanyuka *et al.*, 1999) and functionally confirmed this by biolistic-mediated transient expression assays of the BAC clone and BAC subclones in potato and the related *Nicotiana benthamiana* (Bendahmane *et al.*, 1999). Ultimately, the functional *Rx1* transcript was shown to reside within an 11 kb BAC subclone fragment. A transgene for stable integration was developed by subcloning an 18 kb BAC fragment (which encompassed the 11 kb fragment and provided enough additional genomic sequence to ensure recovery of native regulatory regions) into the binary vector pSLJ7292. pSLJ7292 was developed from vector pRK290 and carries the *NPTI* gene for kanamycin resistance and the *Tet* gene for tetracycline resistance. Bendahmane *et al.* (1999) transformed potato virus X-susceptible potato cultivar Maris Bard using the putative *Rx1* transgene. Subsequent functional tests confirmed resistance of transgenic lines. To isolate *Gpa2*, four BAC clones spanning the genetically defined region were sequenced (Van Der Vossen *et al.*, 2000). Four NBS-LRR gene copies were identified, one of which corresponded to *Rx1*. The remaining three sequences were considered as candidates for *Gpa2*. Subclones containing 6.5–11 kb BAC fragments encompassing the candidate genes were inserted into vector pBINPLUS. Each included at least 2 kb of genomic sequence before and 0.5 kb of genomic sequence after the putative transcript. Transformation of the nematode-susceptible potato line V and phenotypic testing

confirmed that a 10.3 kb genomic fragment imparted resistance and thus encoded *Gpa2*. Importantly, *Gpa2* and *Rx1* share greater than 88% amino acid identity, indicating that highly similar genes may condition resistance to very different pathogens (Van Der Vossen *et al.*, 2000).

Paal *et al.* (2004) used a candidate gene approach to clone a second nematode resistance gene. *Gro1* confers resistance to the root cyst nematode (*G. rostochiensis*) and originated from the wild potato *Solanum spegazzinii* but was introgressed into cultivated potato via sexual crosses. First, resistance was mapped to a 1.4 cM region of potato chromosome 7 (Barone *et al.*, 1990; Ballvora *et al.*, 1995). Next, Leister *et al.* (1996) used degenerate primer PCR targeting conserved motifs common to NBS-LRR genes to amplify fragments from the potato genome. The authors identified two resistance genelike (RGL) sequences that co-segregated with *Gro1* and defined a multigene family. Paal *et al.* (2004) used restriction fragment length patterns generated from diverse root cyst nematode resistant and susceptible genotypes to identify a series of candidate genes for *Gro1* based on sequence similarity to RGL markers generated by Leister *et al.* (1996). Various molecular approaches including fine-mapping and segregation analyses allowed the authors to narrow the candidate gene pool down to three gene copies. BAC clones corresponding to these candidate genes were identified. Subclones for each, ranging in size from 10.5 kb to 22.0 kb and including 2.3 kb to 4.0 kb before and 1.1 kb to 3.4 kb after the putative transcript, were generated in vector pCLD04541. Transformation of susceptible cultivar Desiree revealed that one of the candidate genes imparted nematode resistance.

In addition to disease resistance genes isolated from potato species, several resistance genes with relevance to potato have been isolated from other plant species. Especially relevant examples cloned from tomato include the *Cf-9* gene conferring resistance to *Cladosporium fulvum* (Jones *et al.*, 1994), *Hero*, conferring resistance to *G. rostochiensis* (Ernst *et al.*, 2002), *I2*, conferring resistance to *Fusarium oxysporum* (Ori *et al.*, 1997), *Mi-1*, conferring resistance to root-knot nematode (*Meloidogyne javanica*) (Milligan *et al.*, 1998) and the insect whitefly (*Bemisia tabaci*) (Nombela *et al.*, 2003), the *Prf* (Salmeron *et al.*, 1996) and *Pto* (Martin *et al.*, 1993) loci conferring resistance

against *Pseudomonas syringae*, *Sw5d*, conferring resistance to tomato spotted wilt virus (Brommonschenkel *et al.*, 2000), and *Ve1* and *Ve2*, closely linked genes conferring resistance against *Verticillium albo-atrum* (Kawchuk *et al.*, 2001). These tomato genes have been cloned using a variety of methods, especially positional cloning facilitated by fine-scale molecular mapping, and candidate gene approaches. The close genetic relationship between potato and tomato suggests that these tomato disease resistance genes may be of agricultural relevance to both species. Additionally, functional potato homologs of these genes may be identified. A functional potato homolog of the tomato *Ve1* gene, *StVe1*, has been reported (Simko *et al.*, 2004) and *Rpi-blb2*, cloned from *S. bulbocastanum*, is a functional homolog of the tomato *Mi-1* gene (Van Der Vossen *et al.*, 2005). Improvement of disease resistance in potato using transgenes from more distantly related plants species also shows promise (reviewed in Mullins *et al.*, 2006).

Researchers have also utilized transgenic strategies to limit the impact of Colorado potato beetle on potato production. *Bacillus thuringiensis* (*Bt*) is an aerobic, gram-positive, soil bacterium that accumulates high levels of insecticidal crystal proteins during sporulation (McGaughey and Whalon, 1992; Barton and Miller, 1993). The *Bt* bacteria produce insecticidal crystal proteins that are encoded by single genes. To date, more than 300 *Bt* proteins associated with insecticidal crystals have been identified. These have been classified into 51 groups based on amino acid homologies (Ruiz de Escudero *et al.*, 2006). Most of these proteins are known as Cry proteins and many have been successfully used in biological insecticides. Cry proteins are effective against a variety of insect species, including the Colorado potato beetle. Schnepf *et al.* (1998) reviewed the salient molecular properties and modes of action of insecticidal *Bt* crystal proteins. Several of the insecticidal *Bt* genes have also been used as transgenes and the generation of transgenic plants may be the most effective means to deliver *Bt*-based insecticides.

Relative to those of most plant species, the *Bt* genome is A-T rich and *Bt* genes used directly as transgenes in plants can perform poorly due to nonplant codon bias and the introduction of unintentional splice sites, polyadenylation sites, messenger-RNA (mRNA) degradation sites, and transcription

termination sites (Schnepf *et al.*, 1998). Accordingly, researchers have modified native *Bt* genes to more closely mimic plant genes. The efficacy of codon-modified *Bt* genes such as *Bt-cry1* and *Bt-cry3A* has been demonstrated to be greater than the wild type *Bt* genes in crop plants (Perlak *et al.*, 1991; Wünn *et al.*, 1996). Adang *et al.* (1993) transformed potato with a *Bt-cry3A* gene modified to reflect dicot codon bias. The transgene consisted of the 1.8 kb modified coding region under the control of the Mac promoter, a chimeric promoter constructed from the CaMV 35S promoter and the mannopine synthetase (*mas*) promoter. The transgene also included a *mas*-derived termination signal. The resulting cassette was cloned into the binary vector pCGN1558, which carries the *NPT* gene for kanamycin resistance (McBride and Summerfelt, 1990). Analysis of the resulting transgenic potatoes revealed that 58 of 63 lines displayed enhanced tolerance to feeding by Colorado potato beetle (Adang *et al.*, 1993). Perlak *et al.* (1993) also modified the *Bt-cry3A* gene, without modifying the resultant amino acid sequence, to enhance expression in plants. Cloned into vector pMON10547 and driven by the CaMV 35S promoter, the transgene imparted Colorado potato beetle resistance to the susceptible cultivar Russet Burbank. The transgene was effective in both the laboratory and the field and had no apparent impact on agronomic traits (Perlak *et al.*, 1993). Other *Bt-cry3A* potato lines have been developed in the public sector that combine *Bt-cry3A* with natural resistance (Coombs *et al.*, 2003). NewLeaf[®] potatoes (from Monsanto Technology LLC) containing a *Bt-cry3A* transgene were registered and made available for commercial production from 1995–2001. NewLeaf potatoes were marketed under the name “NatureMark”. First available for cv. Russet Burbank, the NewLeaf technology was later applied to cvs. Atlantic and Superior. NewLeaf Plus[®] potatoes followed, combining *Bt-cry3A*-mediated resistance to Colorado potato beetle and resistance to potato leaf roll virus (PLRV) conferred, presumably through RNA silencing, by transformation with the PLRV replicase gene (Lawson *et al.*, 2001). A third NatureMark product, NewLeaf Y combined *Bt-cry3A*-mediated resistance to Colorado potato beetle and resistance to potato virus Y (PVY). NatureMark products were eventually taken off

the market because of processors’ concerns about genetically modified foods in international sales.

A key tactic to resistance management is the development of alternative modes of action in the *Bt*-transgenic potato. No single form of resistance, either genetically engineered or classically bred, is likely to provide long-term control to highly adaptable insects, like the Colorado potato beetle, particularly if other mortality factors, such as crop rotation or biological control, are not included in the management system. More durable strategies than single factor *Bt*-based host plant resistance must be developed.

Minimizing an insect’s access to biotin using genetic engineering approaches may compliment *Bt*-mediated crop protection. Biotin is an essential co-enzyme required for all insect growth and development. Without this co-enzyme, an insect’s growth is severely stunted, eventually leading to death (Markwick *et al.*, 2001). Avidin is a protein found in chicken egg whites; this protein protects the chicken embryo by sequestering biotin from disease-causing organisms (Hood *et al.*, 1997). The gene for avidin production has been cloned (Hood *et al.*, 1997). The avidin gene has also been inserted and expressed in a few crops, including maize and tobacco and has demonstrated resistance to a wide spectrum of insect pests (Kramer *et al.*, 2000; Markwick *et al.*, 2001; Burgess *et al.*, 2002). All insects need biotin; therefore, avidin is a more broad-spectrum toxin than *Bt* crystal proteins. Avidin could be used not only to delay resistance of Colorado potato beetle to *Bt-cry3A*, but it may also be useful to control other pests such as potato leafhopper, aphids, and European corn borer.

Another approach to delaying or preventing an insect’s resistance to *Bt* insecticidal proteins is to combine *Bt* transgenes with insect resistance genes of different modes of action acquired through traditional breeding strategies. Host plant resistance management combining genetically engineered resistance with traditionally bred host plant resistance has the potential to be much more sustainable and easily implemented. Combining host plant resistance factors as a resistance management strategy does not require grower cooperation or regulatory monitoring or enforcement. Incorporation of host plant resistance into an integrated pest management system involving multiple biological, cultural, and

chemical controls will further increase the sustainability of a pest management system.

Glycoalkaloids are compounds expressed in the leaves and tubers of certain potato species that deter insect feeding. When expressed at high levels in the tubers, glycoalkaloids may be toxic to mammals. The wild potato *Solanum chacoense* produces novel glycoalkaloids known as leptines in the leaves but not the tubers (Sinden *et al.*, 1986a, b; Sanford *et al.*, 1997). It may be possible to incorporate high foliar leptine production from *S. chacoense* into cultivated potato using sexual crosses and somatic hybridization (Cheng *et al.*, 1995; Veilleux and Miller, 1998; Rangarajan *et al.*, 2000). Studies by Cooper *et al.* (2004) suggests that the mode of action of leptines may be different from that of *Bt*, leading to the hypothesis that pyramiding *Bt* and leptine-based insect resistance may be more effective than either resistance alone. High leptine expression has been combined with high *Bt-cry3A* expression in a single potato genotype (Coombs *et al.*, 2002). This line, along with lines that have either *Bt-cry3A* or leptine expression alone, allow researchers to test the effectiveness of combined resistance mechanisms versus individual mechanisms. The primary effort of the potato-breeding project is to combine these two resistance mechanisms into one cultivar. Such breeding efforts have been initiated to develop advanced breeding lines that express leptine-based insect resistance for the chip processing and tablestock industry. These lines have been crossed to advanced breeding lines that have good agronomic performance along with either chip processing or tablestock qualities. The superior individuals from each cross were selected for specific gravity, tuber appearance rating, chip processing, leptine/TGA (total glycoalkaloid) content, and Colorado potato beetle resistance. High performance liquid chromatography (HPLC) procedures are being used to measure leptine/TGA concentration in the potato foliage (Sinden *et al.*, 1986a). The superior selections from this breeding effort will be candidates for combining with the *Bt-cry3A* gene via *Agrobacterium*-mediated transformation (Douches *et al.*, 1998). The same strategy of combining natural and engineered traits can be applied to glandular trichome-mediated resistance. The combination of *Bt-cry3A* gene and glandular trichomes may provide a broader-based

insect resistance providing control of small-bodied insects, such as leafhoppers and aphids, along with the Colorado potato beetle.

Douches and Grafius (2005) provide a recent review of the use of transformation technologies for improving insect resistance in potato, including discussions on management of insect resistance.

Tolerance to abiotic stresses, in addition to biotic stresses, may be a target for enhancement using genetic transformation technologies. Of particular note among abiotic stresses of potato is cold. Potato is grown as a cool-season crop, yet is susceptible to frost damage, resulting in yield losses or complete crop failure. Frost is a major limiting factor in potato production in certain parts of the world (Sukumaran and Weiser, 1972; Chen and Li, 1980; Chen *et al.*, 1999), leading potato breeders worldwide to target improved cold tolerance, in addition to other important traits, in selection criteria. The cold tolerance phenotype is actually the culmination of two distinct traits, nonacclimated freezing capacity and acclimation capacity, which are under differential genetic control (Stone *et al.*, 1993). Wild potato species may be good sources of frost tolerance for improvement of cultivated potato (Hijmans *et al.*, 2003). The D. Carputo laboratory at the University of Naples has extensively used *Solanum commersonii* as a source of frost tolerance (Carputo *et al.*, 2000; Carputo *et al.*, 2003). From a complementary DNA (cDNA) library of *S. commersonii*, De Palma *et al.* (2008) identified cDNAs encoding various desaturases. The *Delta-9* desaturase gene was overexpressed in *S. tuberosum* and resulted in an increased tolerance upon cold acclimation of about 2 °C. Additional genes responsible for frost tolerance in wild species are likely targets of eventual mapping and cloning efforts.

2.1.2 Transgenes for improvement of quality traits

Agriculturally significant quality traits of potato include the level of starch and sugars in the tuber. Of particular note, the accumulation of the hexose reducing sugars glucose and fructose in the potato tuber is undesirable since subsequent frying at high temperatures can cause darkening through a nonenzymatic Maillard reaction,

leading to commercially unacceptable processed food products. Kumar *et al.* (2004) reviewed factors that can affect sugar concentration in the potato tuber, including genetic background and environmental factors in the field and in storage. Storage of potato at cold temperatures reduces tuber loss due to disease and extends storage life by suppressing sprouting. Unfortunately, when stored below 10 °C, the potato tuber converts starch to sucrose and sucrose to glucose and fructose (Coffin *et al.*, 1987; Scheffler *et al.*, 1992), a phenomenon known as “cold sweetening”. Cold sweetening requires additional poststorage, preprocessing tuber treatment, adding expense. Sowokinos (2001) reviewed the benefits of a potato cultivar that can be processed directly from cold storage without additional conditioning, including reduced disease and reduced expense associated with chemical sprout inhibitors and heating potato storage facilities in cold climates. In an attempt to reduce cold sweetening in potato tubers, researchers have applied transgenic approaches to disrupt both the conversion of starch to sucrose and the conversion of sucrose to glucose and fructose.

Uridine-5'-diphosphoglucose pyrophosphorylase (UGPase) catalyzes a rate-limiting step in the conversion of starch to sucrose (Sowokinos, 2001; Kumar *et al.*, 2004). Sowokinos *et al.* (2004) demonstrated sequence polymorphism in UGPases from a collection of six potato cultivars differing in sensitivity to cold sweetening. The authors concluded that UGPase activity might be tightly regulated in the plant in response to varying environmental conditions. Consistently, several researchers have targeted disruption of UGPase activity using transgenic approaches in an attempt to minimize the cold sweetening.

Zrenner *et al.* (1993) examined UGPase transcript levels in different potato plant tissues throughout development and in mature tubers stored at varying temperatures, demonstrating that transcription is highest during tuber expansion and in mature tubers stored for 2 weeks or more at 4 °C. Next, the authors used antisense RNA to disrupt UGPase activity. The coding region of UGPase was isolated from potato cDNA via PCR using primers generated from a previously reported potato UGPase gene. The PCR product was ligated to the CaMV 35S promoter and the octopine-synthase gene polyadenylation signal and the entire construct cloned into vector

pBin19. Digestion patterns were used to identify a plasmid containing the UGPase coding region cloned in an antisense direction. Tubers from plants transformed with the construct showed significant reduction in UGPase transcript levels. Semi-quantitative Western analysis confirmed that UGPase protein had been reduced to as low as 2–5% of that of nontransformed control tubers, correlating well with observed UGPase activity. Importantly, the authors noted no significant difference between transformed and control tubers in total yield, total carbohydrate, sucrose:starch ratio, or hexose sugars. Zrenner *et al.* (1993) concluded that 4% of wild type UGPase activity was sufficient for accumulation of normal levels of carbohydrate in the potato tuber. Although the authors did not specifically look at the phenomenon of cold sweetening, their results may suggest that modifying UGPase activity will not lead to decreased sensitivity to cold sweetening.

Spychalla *et al.* (1994) also used an antisense RNA approach to disrupt UGPase activity in the potato tuber and specifically looked at the effect of UGPase disruption on cold sweetening. The authors identified a potato UGPase clone from a λ gt11 cDNA expression library by probing with polyclonal anti-UGPase sera. A construct consisting of the patatin promoter, the 5' most 0.5 kb of the UGPase cDNA in antisense orientation, and the polyadenylation signal from the nopaline synthase gene was cloned into binary vector pBIN440. Tubers carrying the construct had significantly lower levels of UGPase activity at harvest and throughout 6 weeks of storage at 4 °C. In the most extreme case, UGPase activity in one transgenic line was reduced to 30% that of nontransformed controls. Consistent with the conclusions of Zrenner *et al.* (1993), Spychalla *et al.* (1994) found that partial disruption of UGPase activity did not alter hexose sugar concentrations in the tuber during 6 weeks of storage. However, in contrast to Zrenner *et al.* (1993), Spychalla *et al.* (1994) did note a significant reduction in tuber sucrose in weeks three through six that weakly correlated with UGPase activity. The authors argued that UGPase may play an important role in cold sweetening, especially during long periods (greater than 3 months) of tuber storage (Spychalla *et al.*, 1994).

Borovkov *et al.* (1996) used two RNA silencing approaches to disrupt UGPase: transformation of

potato with a UGPase ribozyme and antisense expression of UGPase. The authors took advantage of a GUC trinucleotide naturally occurring in the UGPase transcript to develop a hammerhead ribozyme (Haseloff and Gerlach, 1988) that would catalyze specific cleavage of UGPase RNA. The ribozyme consisted of the hammerhead catalytic domain flanked by UGPase-derived sequence. The ribozyme was cloned between a CaMV 35S promoter and a functional β -glucuronidase gene (*gus*) followed by the nopaline synthase polyadenylation signal in the binary pBI121 plasmid. The authors also created an antisense UGPase construct consisting of a 1.2 kb genomic fragment from a cloned UGPase gene (containing approximately one-third of the coding region of the gene) cloned in antisense orientation between the CaMV 35S promoter and the functional *gus* gene in pBI121. In total, the authors examined 103 plants transformed with either the ribozyme or the antisense construct. Only 13 plants showed significantly reduced UGPase activity, relative to untransformed controls. In the most extreme cases, UGPase activity was reduced to 50–60% that of control plants. Using field-grown tubers stored for 2 months at 6 °C, the authors identified two transformed lines with significantly lower sucrose accumulation, relative to controls. The authors argued that their results indicate that UGPase plays a critical role in the regulation of carbohydrates in the tuber during storage and predicted manipulation of UGPase levels in the tuber can lead to improved tuber quality (Borovkov *et al.*, 1996).

In plants, enzymes known as invertases play a crucial role in regulating carbon metabolism throughout plant development. Tymowska-Lalanne and Kreis (1998) provide extensive review of the cellular roles of invertases. In potato, invertases are involved in cold sweetening, specifically catalyzing the conversion of sucrose into glucose and fructose. McKenzie *et al.* (2005) examined invertase and UGPase activities in a collection of 15 potato clones with differing sensitivities to cold sweetening. The authors concluded that, for that specific collection of materials, invertase activity was a stronger determinant of cold sweetening sensitivity than was UGPase activity, with stronger invertase activity correlating with increased sensitivity to cold sweetening. Li *et al.* (2006) demonstrated

through an association mapping approach that potato *Invap-b* loci *invGE* and *invGF* play an important role in determining tuber starch and sugar content. These and other findings have led researchers to hypothesize that disruption of invertase activity in the tuber may result in reduced accumulation of glucose and fructose.

Zrenner *et al.* (1996) disrupted invertase activity using an antisense RNA approach. Using a collection of 24 potato genotypes differing in sensitivity to cold sweetening, the authors demonstrated correlation between the amount of hexose sugars in stored tubers and the amount of soluble acid invertases. Next, the authors isolated a potato extracellular acid invertase cDNA by screening a potato library with a homologous probe from carrot. The potato cDNA was cloned in reverse orientation into binary vector BinAR between the CaMV 35S promoter and the octopine-synthase polyadenylation signal. Potato plants transformed with the construct produced tubers showing 12–58% reduction in acid invertases relative to untransformed controls. While transgenic and control tubers stored for 20 weeks at 4 °C had similar levels of total sugars, tubers of transgenic plants displayed reduced hexose and increased sucrose concentrations. The authors concluded that acid invertases are major determiners of hexose:sucrose ratios in cold stored tubers.

Greiner *et al.* (1998) disrupted invertase activity using a different approach. The authors isolated an inhibitor of invertase from tobacco by first purifying and partially sequencing the functional protein, Nt-inh1, and then isolating the corresponding cDNA using reverse transcription (RT)-PCR. Next, the authors transformed potato with a homolog of *Nt-inh1*, *Nt-inhh*, under the control of the CaMV 35S promoter (Greiner *et al.*, 1999). For transformation, the authors used the binary vector BinAR. Transformed plants developed tubers that appeared phenotypically normal and the authors noted no adverse effect on tuber weight or overall yield. Significantly, after 6 weeks of cold storage, the tubers of transformed plants had reduced glucose (up to 83% reduction relative to wild type) and fructose (up to 73% reduction relative to wild type) and increased sucrose (up to 323% increase relative to wild type). The reduction of glucose and fructose in the transgenic tubers was associated with improved processing quality (Greiner *et al.*, 1998).

Sowokinos (2001) indicated that functional redundancy in carbohydrate metabolic pathways of potato can complicate transgenic attempts to modify tuber starch:hexose ratios. Nevertheless, continued characterization of the role of specific invertases in cold sweetening, the isolation of inhibitors of invertase activity, and the transformation of agriculturally important potato cultivars to inhibit cold sweetening seem to be likely future research directions.

2.1.3 Transgenes for modification of industrial characteristics

Transgenic approaches have also been used to modify tuber starch composition for industrial applications. Tuber starch is comprised largely of amylose, a linear $\alpha(1,4)$ -D-glucan polymer, and amylopectin, an $\alpha(1,4)$ -linked, $\alpha(1,6)$ -branched D-glucan polymer. The ratio of linear amylose to branched amylopectin is an important factor for industrial applications and transgenic approaches have been used to alter this ratio. Both amylose-free and amylose-rich potatoes have resulted.

Granule-bound starch synthase (GBSS) catalyzes the synthesis of amylose from ADP-glucose. Visser *et al.* (1991) demonstrated that an antisense approach targeting *GBSS* reduces or even eliminates amylose production in the tuber. In that study, the authors transformed diploid ($2n = 2x = 24$) potato lines with a construct consisting of a full-length potato *GBSS* cDNA in antisense orientation between a CaMV 35S promoter and the nopaline synthase terminator. The construct was cloned into the binary vector pROK-1, which carries the *NPTII* gene for resistance to kanamycin, and was transferred to potato via *Agrobacterium rhizogenes*. Using a rapid iodine staining procedure, the authors demonstrated that the construct disrupted amylose accumulation in most roots but that the effect was reversible and unstable. Next, the authors regenerated 16 clones, from which they produced tubers. Despite the results from the analysis of roots, 11 of the 16 clones produced tubers that were amylose free, 2 of the 16 clones produced tubers composed of a mixture of cells, some being amylose free and some being amylose rich, and three of the 16 clones produced tubers that were amylose rich. Tubers from all clones, regardless of starch

composition, displayed reduced GBSS activity, relative to the untransformed controls. Inhibition ranged from 70% to 100%. Subsequently, Kuipers *et al.* (1994) generated tetraploid antisense-GBSS potato lines. The authors used two different constructs, including the construct used by Visser *et al.* (1991), described above, and a similar construct in which the full length *GBSS* gene, in antisense orientation, was under the control of the native *GBSS* promoter, rather than the CaMV 35S promoter. The latter construct was cloned into vector pPGB-1, which is derived from pBI121 and includes the *NPTII* gene for kanamycin resistance. Of 66 transgenic clones analyzed, 10 bore tubers displaying no GBSS activity and no amylose accumulation. A general correlation between transgene copy numbers and degree of *GBSS* silencing was noted and the authors concluded that a minimum of two transgene copy numbers was needed to completely silence *GBSS*. As might be expected, the authors also noted a correlation between the amount of accumulated *GBSS* transcript and accumulation of amylose. Taken together, these studies demonstrate that antisense technology can be successfully utilized to alter potato tuber starch composition.

While the efforts described above were aimed at the creation of amylose-free tubers, other researchers have used transgenic approaches to create amylose-rich tubers. Schwall *et al.* (2000) achieved high amylose production by simultaneously suppressing two genes, *starch branching enzyme A* (*SBE A*) and *starch branching enzyme B* (*SBE B*). These genes encode proteins involved in the synthesis of branched amylopectin. Gene silencing was achieved by two separate rounds of transformation. First, a potato line was transformed with an *SBE B* construct consisting of a 2.3 kb partial cDNA fragment of the potato *SBE B* gene in antisense orientation between double CaMV 35S promoters and the CaMV 35S polyadenylation signal. This construct, cloned into binary vector pBIN19, was delivered via *A. tumefaciens* (Safford *et al.*, 1998). Four transformed lines showing low levels of *SBE B* activity were subsequently retransformed with the antisense *SBE A* construct of Jobling *et al.* (1999). This construct consisted of a 1.2 kb partial cDNA fragment of the potato *SBE A* gene in antisense orientation between two CaMV 35S promoters and the nopaline synthase terminator.

The construct was cloned into vector pGPTV-HYG, which encodes resistance to hygromycin B, and delivered via *A. tumefaciens*. The authors surveyed three independent double transformation lines and demonstrated that SBE A and SBE B activity had been reduced to nearly undetectable levels. Nineteen of 71 double transformed lines surveyed had $\geq 40\%$ amylose content, with the best having amylose content of 75–89%. Significantly, double transformed lines had an altered starch structure and some lines completely lacked wild type amylopectin. Potato starch from transformed lines also showed up to sixfold increases in phosphorous content. The authors concluded that the high-amylose, low-amylopectin, high-phosphorous potato starch generated through transformation technology offered novel industrial and food utilities (Schwall *et al.*, 2000).

2.2 Transformation Methods

Efficient methods for *A. tumefaciens*-mediated transformation of potato leaf, stem, tuber, and microtuber tissues were developed long ago (see for example Shahin and Simpson, 1986; De Block, 1988; Sheerman and Bevan, 1988; Tavazza *et al.*, 1988; Ishida *et al.*, 1989; Szychalla and Bevan, 1991; Visser, 1991; Kumar, 1995). As reflected in Table 3, *Agrobacterium*-mediated transformation has been widely employed. Alternative approaches, including polyethylene glycol (PEG)-mediated transformation of protoplasts (Feher *et al.*, 1991; Craig *et al.*, 2005) and microprojectile bombardment or biolistics (Romano *et al.*, 2001; Romano *et al.*, 2003; Craig *et al.*, 2005; Romano *et al.*, 2005) have been reported for potato. Ercolano *et al.* (2004) demonstrated successful biolistic transformation of potato with a 106 kb BAC fragment and advocated for the approach as a means of speeding positional cloning efforts. However, to date, PEG-mediated and biolistic methods for transformation of potato have been of limited application. Protocols for the transformation of the potato plastid genome have also been reported (Sidorov *et al.*, 1999; Nguyen *et al.*, 2005).

2.3 Selection of Transformed Tissue

Antibiotic resistance, and especially resistance to kanamycin, has been the primary means of

selecting transformed potato tissues (Table 3). Selection of transgenic potato based on resistance to herbicide has been reported (Andersson *et al.*, 2003) but has not been widely employed. Transformation methods yielding transformed plants lacking a selectable marker have also been developed. de Vetten *et al.* (2003) used a PCR-based screen of regenerants to identify transformed potato genotypes lacking both a selectable marker gene and vector backbone DNA. Wang *et al.* (2005) developed a binary vector utilizing the Cre/lox system. The authors demonstrated the successful simultaneous heat shock-mediated elimination of both Cre and antibiotic resistance genes from transgenic tobacco and speculated that the system could be adapted for potato. Rommens *et al.* (2004) replaced the *A. tumefaciens* transfer DNA with sequences derived from the potato genome. Paired with different strategies for marker-free selection or selectable marker eradication, the approach yielded up to 29% of regenerants carrying the transgene and lacking a selectable marker. Transformation strategies that do not require the stable integration of selectable marker genes may become increasingly important in addressing consumer and processor concerns associated with genetically modified potatoes.

2.4 Regeneration of Whole Plant

Potato, like several other species in the Solanaceae, is fairly responsive to *in vitro* manipulation, including regeneration of whole plants from callus tissues. Multiple successful regeneration protocols (see for example, Shahin and Simpson, 1986; Edwards *et al.*, 1991; Visser *et al.*, 1991; Cearley and Bolyard, 1997) and numerous modifications have been reported. However, potato cultivars differ substantially in response to regeneration efforts. Heeres *et al.* (2002) attempted *Agrobacterium*-mediated transformation of 16 different potato cultivars using two different transformation protocols that differed in regeneration conditions. Considerable genotype-to-genotype variation was noted, with regeneration efficiency ranging from 0.3% to 55.7%. The authors speculated based on known pedigrees of the genotypes studied that regeneration efficiency may be under the control of specific genetic factors. Consistent with the results of Heeres *et al.* (2002),

transformation protocols optimized for specific potato cultivars have been reported (Cearley and Bolyard, 1997; Gustafson *et al.*, 2006).

2.5 Testing of Transgenic Lines

The transformation and regeneration process can sometimes have unintended effects on plant phenotypes. Phenotypic changes may result from pleiotropic effects of the transgene or from somaclonal disruption of normal genome organization or function (Kumar, 1994). Variation in expression of the transtrait is also common. Consequently, testing of transgenic potato clones has focused simultaneously on successful expression of the transtrait and recovery of an otherwise characteristic cultivar phenotype. Commercially, potato is clonally propagated, so sexual transmission of the transgene may not be of primary concern. However, clonal generation-to-clonal generation variation in transgene effect can occur, necessitating testing across several generations.

Heeres *et al.* (2002) noted visible morphological deviation of transgenic plants from the untransformed controls for 10.3% of regenerants in greenhouse tests and 19.6% of regenerants in field tests. Deviations included changes in tuber appearance and leaf color and shape. Interestingly, the authors noted that independent regenerants that deviated from wild type did so in a cultivar-specific fashion. Thus, for example, deviations for regenerants of cultivar Indira tended to have an irregular leaf surface and characteristic discolorations (Heeres *et al.*, 2002). Transformation and regeneration may also affect traits of agronomic significance. Schwall *et al.* (2000) reported that disruption of the potato *SBE B* gene reduced overall yield relative to untransformed controls and that high amylose potato lines, achieved through simultaneous silencing of *SBE A* and *SBE B* yielded elongated tubers, relative to controls. Kuipers *et al.* (1992) examined four diploid amylose-free potato clones generated through antisense disruption of *GBSS*, comparing yield with that of untransformed control. The authors demonstrated that each of the four lines produced fewer tubers than the control, but that total yield of two of the lines was statistically identical to that of the control

and two of the transformed lines produced tubers that were significantly heavier than those of the control. The authors concluded that there is significant potential for modification of traits of potato through transgenic methods without sacrificing yield. Similarly, Heeres *et al.* (2002) examined yield in several independent lines of two tetraploid amylose-free transgenic potato cultivars. The authors note that the average yield of one cultivar was 95% of that of the untransformed control and that one transformed line of the second cultivar actually yielded 5% more than the corresponding untransformed control. Taken together, these studies demonstrate that, while the transformation and regeneration processes may induce genetic changes and alter agronomic traits, transformants that are substantially similar to wild type (with the exception of the transtrait) can be readily identified in many cases.

Large-scale deployment of transgenic plants warrants special attention to transgene function under varying environmental conditions and in different genetic backgrounds. As a case study, consider current efforts to deploy the late blight resistance gene *RB*. Toward devising a strategy for successful gene deployment, researchers have combined field- and greenhouse-based and laboratory-based assays.

Field and greenhouse assays have demonstrated the broad-spectrum nature of *RB* and have tested effects of transformation on plant fitness. The map-based cloning of *RB* was initiated by generating interspecific somatic hybrids between the wild *S. bulbocastanum* and cultivated potato (Helgeson *et al.*, 1998). Analysis of late blight resistance in the somatic hybrids and backcrosses to potato in greenhouse tests and in field trials in Wisconsin, USA, demonstrated that *RB* imparts resistance to *P. infestans* race US8, the predominant pathotype in the United States today (Helgeson *et al.*, 1998). Subsequent testing of backcross materials in the Toluca Valley of Mexico, an area of the world with ideal late blight epidemic conditions and an extremely diverse, sexually mating pathogen population, revealed that *RB* also imparts broad-spectrum, pathogen race nonspecific resistance (Naess *et al.*, 2000). Fine molecular mapping and eventual gene cloning ensued (Naess *et al.*, 2000; Bradeen *et al.*, 2003; Song *et al.*, 2003b), enabling the *RB* transgene to be tested in a variety of cultivated

potato genetic backgrounds and in a variety of different locations. Since its cloning, *RB* has been transformed into several important potato cultivars (e.g., ‘Russet Burbank’, ‘Superior’, ‘Dark Red Norland’) and has been tested in replicated greenhouse trials at the University of Wisconsin, USA, and the University of Minnesota, USA, and in replicated field trials in the US states of Minnesota, Michigan, and Oregon. Field trials have also been conducted in Europe and India. Genetic background-dependent variation in *RB* function has been noted (Bradeen, unpublished) but in every trial, the intact transgene has imparted the rate-limiting, broad-spectrum resistance reported by Song *et al.* (2003b) (Figure 2). In greenhouse tests, Halterman *et al.* (2008) examined the effects of varying *P. infestans* inoculum concentration on disease development in transgenic potato. Using two different *P. infestans*

isolates at concentrations ranging from 258 300 to 391 665 sporangia per plant, the authors demonstrated no significant difference in disease scores. In field plots, the same authors demonstrated for four different potato cultivars that addition of the *RB* transgene does not alter plant fitness, as measured by tuber yield (Halterman *et al.*, 2008).

Laboratory-based characterization of *RB* has also been helpful in determining how best to utilize this gene in a production setting. Significant contributions have included the development of PCR markers for marker-aided selection and transgene expression. Colton *et al.* (2006) developed PCR markers for selection of somatic hybrid-derived breeding lines. The authors demonstrate that *RB* markers can be used to accurately predict late blight resistance. It is expected that the approach will be useful



Figure 2 Transgene *RB* imparts resistance to foliar late blight. A photograph of a late blight screening nursery at the University of Minnesota. Potato cv. Katahdin, a normally late blight susceptible cultivar, was transformed with the *RB* late blight resistance gene from the wild potato *Solanum bulbocastanum* (Song *et al.*, 2003b). Rows of transgenic materials (green plants) were planted between rows of the late blight susceptible potato cv. Norchip (dead, brown plants). Approximately 60 days after planting, the field was inoculated with *Phytophthora infestans* race US8. Frequent overhead irrigation kept humidity levels high, favoring disease development. Photograph was taken 21 days after inoculation. While untransformed potato was killed by disease, the *RB* transgenic lines remain uninfected [Reproduced from Millett and Bradeen (2007). © Springer]

to breeding programs aimed at accessing late blight resistance using somatic hybrid, instead of transgenic methods. Millett and Bradeen (2007) developed complementary PCR and RT-PCR assays that are specific for the *RB* transgene. Their approach took advantage of single nucleotide polymorphisms that were introduced into the transgene as an artifact of cloning (Song *et al.*, 2003b). Using mismatch amplification mutation assay (MAMA)-PCR (Cha *et al.*, 1992), the authors developed primers that are so specific for the *RB* transgene that they can differentiate the transgene from the *S. bulbocastanum* endogenous gene from which it was cloned (Millett and Bradeen, 2007). The method has been recently adapted for real time quantification (Bradeen, unpublished). The J. Bradeen laboratory at the University of Minnesota, USA, is now using the assay to examine the expression of transgene *RB* in different genetic backgrounds, at different stages in plant development, in different plant tissues, and under different environmental conditions (Figure 3). Paired with phenotypic tests for disease resistance, this research promises to provide guidelines on how best to integrate transgene *RB* into disease management practices.

Other research is aimed at understanding the evolution of *RB* and the isolation of novel, functional *RB* alleles. Song *et al.* (2003b) demonstrated that LR-PCR can be used to isolate full-length, intact resistance gene copies. *RB*, like many other plant resistance genes, exists in the genome as one functional copy within a larger cluster of related copies (paralogs) of differential function. The *RB* locus is 79.6–85.6% similar to its paralogs at the DNA sequence level (Song *et al.*, 2003b). Sanchez and Bradeen (2006), aiming to use LR-PCR to isolate *RB* alleles from a broad survey of *S. bulbocastanum* genotypes, optimized LR-PCR, specifically seeking reaction conditions and appropriate primers that could distinguish between *RB* alleles and paralogs. Subsequently, the authors demonstrated the utility of the approach by isolating *RB* alleles from 17 different *S. bulbocastanum* genotypes (Sanchez, 2005). DNA sequence analysis revealed broad distribution of *RB* alleles within the species, implying extensive gene flow. The authors also isolated a novel *RB* allele that putatively arose through an interlocus, *in planta* recombination event (Sanchez, 2005). Functional tests of the newly recovered allele are ongoing. The isolation of additional functional *RB*

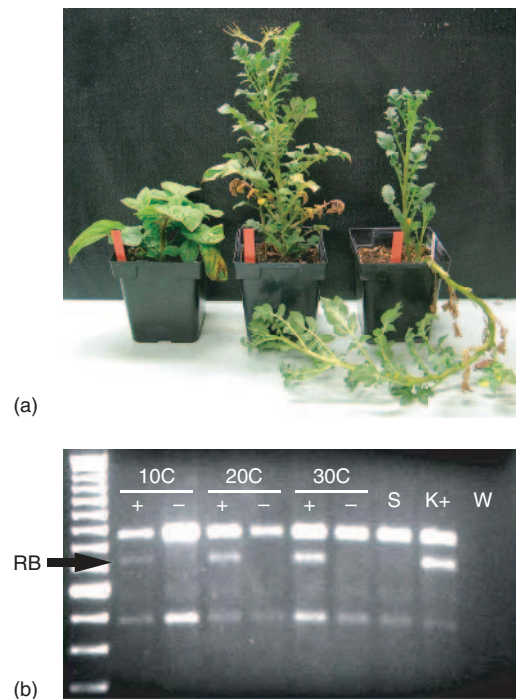


Figure 3 Exploring how environment affects transgene *RB*. (a) A photograph demonstrating the effects temperature has on plant morphology. Shown are three plants of the transgenic line SP2577 (cv. Dark Red Norland + the *RB* transgene) at 64 days after planting from greenhouse-produced tubers. The plants were grown in growth chambers at constant temperatures; left: 10°C, middle: 20°C, right: 30°C. Temperature substantially altered plant morphology and growth rates. Note stunted morphology of the 10°C plant and leggy appearance of the 30°C plant. The plant grown at 20°C is postflowering; plants grown at other temperatures have not yet flowered. (b) A photograph of an agarose gel showing results of RT-PCRs using an assay specific for the *RB* transgene (Millett and Bradeen, 2007). Lanes are (left to right): DNA size standard; six RT-PCRs using RNA extracted from transformed line SP2577 (+) or cv. Dark Red Norland (-; untransformed control) grown at 10°C, 20°C, or 30°C, as labeled; S: RT-PCR using RNA extracted from cv. Superior (independent untransformed control); K+: RT-PCR using RNA extracted from cv. Katahdin transformed with *RB* (independent positive control); and W: RT-PCR using water (no RNA) as a template (negative control). Upper fragment, present in all lanes except water, is an amplicon from the *RNA Polymerase II* gene and serves as an internal positive control for each reaction. The second fragment, indicated with an arrow, is an amplicon specific for the *RB* transgene. Note that the fragment is present in the positive control lane (K+) and absent in the negative control lane (S). The fragment is detected in transformed line SP2577 at 10°C, 20°C, and 30°C but is absent in the untransformed cv. Dark Red Norland at all temperatures. The lower fragment, present in all lanes except water, results from amplification of sequence not associated with *RB* and serves as an additional internal control [Reproduced from Millett and Bradeen (2007). © Springer]

alleles may allow improved transgene durability through allele pyramiding.

Consistent with research predicting *RB* to be of broad utility for the protection of potato against the late blight pathogen, there is current commercial interest from US and European companies in developing and releasing late blight resistance potatoes carrying the *RB* transgene.

2.6 Regulatory Measures for Field Release of Transgenic Potato

Regulatory measures related to the environmental testing, transport, commercial or industrial use of transgenic potato have been developed to ameliorate potential risks to the environment and the consumer. Risk evaluation factors may include the origin of the transgene, the components of the construct, including the promoter and terminator sequences, the presence of selectable markers, the potential for the transgene to escape including the crossability of potato with wild species indigenous to an area and the likelihood of transgenic potato survival from one season to the next, and likely health risks associated with the production of the transprotein. In some cases, laboratory or clinical tests demonstrating the health effects of the transgene may be warranted. Studies demonstrating that transformation of potato with specific transgenes resulted in less variation in the tuber proteome (Lehesranta *et al.*, 2005) and metabolome (Catchpole *et al.*, 2005) than has been achieved through conventional breeding may have effects on current regulations. Regulations and regulatory agencies are developed by individual nations although some transnational agreements have also been established. Here the regulation in the United States of transgenic crops, with emphasis on potato, is discussed as an illustration of the complex nature of this field.

The field release of transgenic potato either for research purposes or for commercial production may involve regulations established by three different US agencies: USDA Animal and Plant Health Inspection Service (APHIS), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). The three agencies jointly maintain the “U.S. Regulatory Agencies Unified Biotechnology Website” (<http://usbiotechreg.nbii.gov>), a useful portal for those interested in up-to-date US regulations on

transgenic food crops. APHIS, EPA, and FDA differ in terms of the scope of their missions and each agency has established rules and guidelines for the safe release of transgenic crops. Although guidelines continue to evolve, all agencies provide substantial advice on safe handling and testing procedures.

APHIS is charged with protecting agriculture from diseases and pests. The agency regulates the field release of transgenic crops in order to minimize unintentional negative effects on the environment or agriculture. APHIS has established both a “notification” and a “permit” process for the interstate transport and field release of transgenic plants for experimental purposes. Currently, qualification for the notification process requires that the transgene originates from an organism other than a human or animal pathogen, the functions of the transgene construct and product are known, the transgene product does not have adverse human, animal, or plant health effects, the transgene product is not a virus particle, the transgene does not result in a product intended for industrial or pharmaceutical use, and the transgene is stably integrated into the plant genome. The APHIS notification process is less stringent than the permits application process, which requires more detailed scientific evaluation. Applications requiring an APHIS permit include testing of transgenic potatoes producing pharmaceutical products and value-added components intended for human consumption. In either case, APHIS has established a series of compliance guidelines. For potato, these include isolation of transgenic plots from nontransgenic potato by at least 0.25 mile (0.4 km) or establishment of a pollen trap crop consisting of at least 30 ft. (9.14 m) of nontransgenic potato planted on all sides of the transgenic crop. Field monitoring and destruction of volunteer plants in the year following a transgenic potato field test are also required. APHIS specifies the timing and content of reports detailing the results of field trials. On-site assessments by USDA and/or state regulatory agencies may be conducted.

Among other activities, the EPA regulates the use of pesticides to minimize environmental and human health effects. In this capacity, EPA also regulates products produced by transgenic plants that protect against insects and pathogens. While the EPA specifically regulates these pesticidal compounds produced by transgenic plants, termed

“Plant Incorporated Protectants” (PIPs), it does not regulate the transgenic plant *per se*. EPA approval of a PIP requires evaluation of risk factors including unintentional effects on human health, nontarget organisms and the environment, the likelihood of transgene escape, and the need of a pest resistance management plan. EPA issues both PIP registrations and PIP experimental use permits. Registration of a PIP is required before the product can be marketed. The registration process entails careful review by EPA of the pesticidal component(s), the crop and location where it is to be used, frequency, amount, and timing of use, and disposal practices, as warranted. Like other pesticides, PIPs are evaluated in terms of human health and environmental risks. Registration of a PIP may take as little as 1 year or up to several years. Once registered, a PIP can be safely used for crop protection, provided registration label guidelines are followed. A PIP experimental use permit is required if the PIP in question is not registered or if a nonregistered application of a PIP is to be tested on more than 10 acres (4.05 ha) of land.

The FDA is charged with maintaining food safety and accurate labeling and has established guidelines for the regulation of transgenic crops intended for human consumption. These guidelines were first adopted in 1992. An individual or company interested in releasing a transgenic crop for commercial production must notify the FDA of intention to do so. Discussions on the food safety of the product ensue and generally require that the person or company intending to release the transgenic product demonstrate its safety through relevant research. This research may focus on the source of the transgene, comparison of transformed and nontransformed food products in terms of known allergens, toxins, and antinutrients, and simulated digestive tests of the transmodified protein. Occasionally, controlled animal or serum testing is also required. The FDA recognizes that no food product, whether transgenic or not, is completely risk free in terms of allergens, toxins, and antinutrients. Accordingly, transgenic food products that do not differ substantially from their traditionally bred counterparts in terms of these components are generally regarded as safe. In addition to mandated regulations, those developing transgenic foods may apply additional safety standards. For

example, companies may opt to not use transgenes from plants known to harbor allergens. In a US General Accounting Office conducted survey of scientists from the FDA, US academic and research institutions, biotechnology companies, and the EU, scientists agreed that current testing standards were adequate to ensure the comparable safety of transgenic foods (GAO, 2002). The same report, however, also points out that more sophisticated applications of transformation technology in the future (e.g., compositional and nutritive modification of crop plants) may require improved or altered testing procedures.

3. FUTURE ROAD MAP

3.1 Expected Products

Future efforts will likely yield commercially important potato cultivars carrying transgenes for improved disease resistance. Late blight, nematodes, and viruses are likely early targets of these efforts. The complete sequencing of the potato genome, an ongoing effort, will provide researchers with bioinformatics tools to speed map-based isolation of genes, including disease resistance genes. The development of improved molecular methods for rapid gene cloning (Sanchez and Bradeen, 2006) may have similar effect. As a result, the number of available resistance transgenes is certain to increase rapidly. Genes from more distantly related plant species may also be more widely utilized to achieve disease resistance in potato. Resistance transgenes against the same pathogen or different pathogens are likely to be pyramided in a common line. These efforts will require continued testing of transgenes in different genetic backgrounds and in different environments. Continued development and optimization of molecular tools to study transgene function and behavior are likely.

Cold sweetening is a costly processing issue. Researchers are likely to continue to elucidate the *in planta* regulation of this phenomenon and devise transgenic strategies for its obviation. As more efficient and reliable RNA silencing technologies emerge, they are likely to be used to target key genes involved in the cold sweetening phenotype, especially UGPases and invertases. RNA silencing technologies are also

likely to continue to be used to manipulate tuber starch composition, with continued research effort focusing on starch synthase and starch branching enzyme genes. More sophisticated refinement of starch composition is likely.

Transgenic technologies will also be used to improve the nutritional quality of potato. Potatoes high in carotenoid, anthocyanin, antioxidant, and vitamin E content may result. Researchers may also use transformation technologies to enhance the already notable levels of vitamin C in the potato tuber. Recent efforts to characterize flavor components in potato may yield an improved understanding of the genetics of flavor, opening the potential for eventual gene isolation and transformation.

Because potato can be easily and cheaply produced in most growing regions of the world, this crop plant holds considerable promise for the *in planta* production of vaccines. Toward this effort, potato has been transformed to synthesize the capsid protein of Norwalk virus (Tacket *et al.*, 1998) and a fusion protein including a HIV-1 envelope glycoprotein (Kim *et al.*, 2004). In a double blind trial, Thanavala *et al.* (2005) demonstrated an increase in blood serum, antihepatitis antibody titer in up to 62.5% of previously vaccinated participants who consumed raw potato tubers from plants transformed to produce hepatitis B antigen. Significantly, this research demonstrates the potential for developing an oral-administered plant-based vaccine that can be produced locally and inexpensively. Research toward development of potato as a factory for vaccines and other pharmaceutical compounds is likely to continue.

Mullins *et al.* (2006) reviews other potential attributes of potato that may be achieved or enhanced through transformation, including the improvement of tuber-derived flour, reduced tendency of tubers to sprout in storage, and the *in planta* production of biodegradable plastics.

3.2 Addressing Risks and Concerns

For transgenic potato, as for most other transgenic crops, assessment of human health risks associated with a transformation event has included careful research to explore the global effects of transgenes on the crop. Of particular note, recent research

has explored the effects of transformation on the potato tuber proteome and metabolome.

Lehesranta *et al.* (2005) reported proteomic comparison of tubers from 32 nontransgenic potato genotypes and from transformed lines originating from three different transformation experiments, along with tubers from relevant controls. On 2-D gels, nine protein spots out of a total of 730 spots differed significantly in intensity between transformed tubers and nontransformed controls. Seven of the differential spots were identified and included defense related proteins, proteins involved in protein destination and storage, and proteins of unknown function. The differences between transformed and untransformed tuber proteomes were insufficient to statistically separate the protein sets. Additionally, variation between transformed tubers and their untransformed counterparts was much lower than that observed between the 32 nontransgenic genotypes (Lehesranta *et al.*, 2005).

Catchpole *et al.* (2005) compared the metabolomes of transgenic potato tubers and their untransformed controls. The researchers used transgenic lines generated in two independent studies. Both groups of lines were engineered to produce high levels of inulin-type fructans, but through the activity of two distinct transgenes. For reference, the researchers also characterized the tuber metabolomes of a small collection of disparate, untransformed potato cultivars. The research utilized a hierarchical strategy for comprehensive metabolome characterization. This strategy was designed to provide (a) a general overview of the tuber metabolome via nonselective metabolite fingerprinting, revealing any gross distinctions between transgenic and nontransgenic metabolomes, (b) a detailed global view of specific metabolites, and (c) a targeted analysis of metabolites differing between transgenic and nontransgenic tubers. Using flow injection electrospray ionization, gas chromatography/ time of flight mass spectrometry, and liquid chromatography mass spectrometry, the researchers identified a total of 15 ions that significantly discriminated transgenic tubers from their nontransgenic controls. Each of these ions was fructans related, consistent with the goal of the original transformation effort. Importantly, the researchers reported that only anticipated metabolites, including those found in the untransformed controls and those conditioned

by the transgenes, were identified in transgenic tubers. Furthermore, variation observed in the metabolomes of untransformed potato cultivars was considerably greater than that observed in the transgenic lines. The researchers concluded that transformation of potato with the transgenes tested did not substantially alter the metabolite composition of the tubers (Catchpole *et al.*, 2005).

Testing for food safety is not a black and white issue. But the “-omics” approaches utilized by Lehesranta *et al.* (2005) and Catchpole *et al.* (2005) are among the first attempts to globally characterize intentional and unintentional changes induced by transformation of a food crop. Interestingly, both studies concluded that the variation inherently present in conventionally bred potato cultivars far exceeds that generated through transformation technologies.

In addition to human health and safety issues, unintentional escape of a transgene into wild relatives of crop plants is an issue of concern when deploying transgenic crops. The cultivated potato is a tetraploid ($2n = 4x = 48$), 4EBN species. The approximately 200 wild potato species comprise primary, secondary, and tertiary gene pools for cultivated potato. These species are distributed from the southwestern United States, throughout Mexico and Central America, into southern South America. In other regions of the world, the risk of transgene escape appears to be minimal owing to the lack of species sexually compatible with cultivated potato (Eijlander and Stiekema, 1994; Love, 1994; McPartlan and Dale, 1994). Where wild potato species do occur, the potential for gene flow between cultivated transgenic potato and wild potato species, and especially the potential for pollen transfer from transgenic potato to the wild species, warrants careful consideration prior to transgene deployment.

The wild potato species exist as a ploidy series from diploid, to tetraploid, to hexaploid. Observed EBNs include 1, 2, and 4. Theoretically, species with 1EBN or 2EBN are sexually incompatible with cultivated potato. However, naturally occurring polyploidization or the production of 2N gametes, both documented events in wild potato species, can circumvent the mismatched EBN-imposed crossing barrier. Jackson and Hanneman (1999) examined the frequency of $2n$ pollen production in wild potato and the crossability

of cultivated and wild potatoes under controlled conditions. In total the authors examined 121 species representing 17 of the 19 potato series. The researchers found $2n$ pollen production in every series and every species examined, noting that frequency of $2n$ pollen varied considerably. In general, genetic distance between cultivated potato and wild potato correlated with crossability. Genetically primitive, 1EBN diploid species from North and Central America could not be crossed with cultivated potato. From 1003 crosses between these species, no seeds were set. Crosses were conducted using the wild species both as the male (831 crosses) and as the female (172 crosses). More advanced 2EBN and 4EBN wild species from these same regions, however, could be crossed with cultivated potato. For example, species belonging to series *Demissa*, including *S. demissum*, the source of the *R1-R11* late blight resistance genes that were long ago introgressed into cultivated potato, produced an average of 0.2 seeds per pollination when used as a male parent and an average of 6.6 seeds per pollination when used as a female parent (Jackson and Hanneman, 1999). Furthermore, crosses between cultivated potato and South American wild potatoes were frequently successful. Genetic distance from cultivated potato dictated whether these wild species were successful as only a male parent or as both a female and a male parent. In general, the more distantly related species functioned successfully only as a male parent. Seeds from successful crosses varied considerably in viability and F_1 sterility was commonly noted (Jackson and Hanneman, 1999). While this study underscores the potential for gene flow between cultivated potato and wild species, it must be interpreted in light of the ideal controlled pollination conditions employed, including the use of a cut-stem pollination method with no parallel in the wild. Clearly, further refined study of the fate of interspecific hybrids and of introgressed chromosome regions in the wild is warranted.

Transgenes may also move from one cropped field to another. To assess the risk of transgene escape, Flannery *et al.* (2005) employed a numerical model incorporating the frequency of sexually compatible wild species in a region, the likelihood the transgenic crop will flower and produce viable pollen, the average distance pollen is likely to travel from the field, the mating system

of the crop plant, and the probability an F_1 hybrid would become established in a region. Applied to Ireland, a region lacking sexually compatible wild species, the researchers concluded the risk for transgene flow from transformed potato was mainly from one cultivated potato to another. While many commercial potato varieties produce low levels of viable pollen and 95% of potato pollen travels 10 m or less from the source, the researchers concluded there is some low-level risk of unintentional transgene flow. This risk is most notable for regions of Ireland engaging in true seed production of potato (Flannery *et al.*, 2005). This research is the first of its kind and serves to provide a baseline assessment for developing effective management practices for transgenic crops, including recommendations on physical isolation of transformed and nontransformed crops.

Mullins *et al.* (2006) detailed the need to integrate effective pest resistance management strategies into transgenic potato production systems. The authors advocated for the use of nontransgenic “refuges” near transgenic crops, providing a low or no selection pressure area where resistance to the transgene imparts no survival benefit. The use of refuges theoretically expands the effective life of a transgene. Mullins *et al.* (2006) also advocated for pyramiding different pest resistance transgenes in a common line. Combining different disease or insect resistance genes is thought to present the pest simultaneously with several different strategies of defense, requiring a pest to perish or overcome the multiple resistances. This too is thought to extend the effective life of a transgene. With the recent cloning of several resistance genes effective against the late blight pathogen (Table 3), it is not surprising that there is current commercial interest in pyramiding late blight resistance genes in a common line (Mullins *et al.*, 2006). As additional genes for resistance against pathogens and insects are cloned, pyramiding efforts, in an attempt to extend transgene functional life, are likely.

3.3 Expected Technologies

Major potato genomics efforts in recent years in Europe, Canada, and the United States are shedding light on the genetic control of many important

potato traits. Ongoing efforts to sequence the entire potato genome will provide researchers with new tools for gene pathway characterization and isolation. To match an anticipated increase in the number of potato transgenes emerging from these and other research efforts, transformation systems for potato are also likely to continue to improve. While *Agrobacterium*-mediated transformation of potato has been widely used (Table 3), the potential to develop alternative systems based on other bacteria (Broothaerts *et al.*, 2005) may provide enhanced transformation efficiencies. Other improvements in transformation technologies for potato will focus on methods for multigene insertions, reduction of transgene variability, and development and optimization of selectable marker-free approaches.

As more is learned about the control of complex traits in potato, there is an emerging need for efficient transformation approaches for the simultaneous insertion of multiple transgenes, enabling, for example, insertion of entire metabolic pathways or QTLs. Toward this goal, Chen *et al.* (2006) developed an *Agrobacterium* vector allowing simultaneous transformation with multiple genes. In a proof-of-concept experiment, the authors demonstrated transformation of tobacco with seven target DNA sequences. This and similar vectors with multigene capabilities may be essential for the routine polygenic transformation of potato. Other researchers are using biolistic approaches to insert large fragment DNAs into potato (Ercolano *et al.*, 2004). This practice may be increasingly utilized both for multigene insertions and to speed map-based cloning efforts.

Variation in the expression and function of transgenes currently necessitates the screening of a large number of transformants to identify lines of maximal effect. Butaye *et al.* (2005) reviewed causes in variation in transgene expression that are observed from one transformation event to the next. These include variation in transgene copy number, site of insertion, and RNA silencing. Variation in the number of transgenes inserted has been widely reported. When multiple copies of a transgene are inserted, the frequency of gene silencing or even loss of the transgene through chromosomal rearrangements increases, leading to gross variation from transformant to transformant in trait expression. For this reason,

researchers are exploring methods of assuring single copy insertions. Srivastava *et al.* (1999) utilized the Cre/*lox* system to achieve single insertion transgenic wheat. The researchers developed a construct in which the transgene was sandwiched between inverted *lox* sites. In wheat, as in many plants, the simultaneous insertion of multiple, tandem transgenes is commonplace. Srivastava *et al.* (1999) demonstrated that transformed wheat lines carried several copies of their construct and, presumably, some of these existed as a tandem array. In the presence of Cre recombinase, direct *lox* sites recombine, eliminating intervening DNA from the genome. The researchers reasoned that if two or more transgenes and their bordering inverted *lox* sites were arrayed in tandem, recombination between direct orientation *lox* sites from adjacent constructs would result in elimination of all but one copy of the transgene. Accordingly, the researchers crossed Cre into the genome of the multicopy transformed wheat lines, demonstrating that the enzyme did indeed catalyze resolution of arrayed multitransgenes into a single copy. Whether or not the Cre/*lox* system will become an important tool for manipulating transgene copy number in the potato genome remains to be seen.

Related to transgene copy number is RNA silencing through endogenous processes that are thought to have evolved to target mutant or “foreign” RNA molecules. Much has been learned about RNA silencing in plants and other organisms in recent years (reviewed in Tijsterman *et al.*, 2002; Matzke and Birchler, 2005). Current understanding of the RNA silencing phenomenon predicts that tight regulation of transgene copy numbers may reduce the occurrence of the phenomenon. Thus, utilizing the Cre/*lox* system or other approaches of manipulating transgene copy numbers may ameliorate the effects of RNA silencing.

The Cre/*lox* system has also been used to achieve quasi site-specific transgene insertions (reviewed in Ow, 2002). Other systems for site-directed transgene insertion in plants have been reported (reviewed in Butaye *et al.*, 2005), but efficiencies remain very low. The development of efficient site-directed insertion technologies appears to have significant potential for improving potato transformation technologies and is likely to remain an active research pursuit.

The development of selectable marker-free transgenic potato may be an important approach to addressing consumer concerns with current transformation technologies (detailed below) and is an area of current research emphasis. The use of PCR-based screening (de Vetten *et al.*, 2003), the Cre/*lox* system (Wang *et al.*, 2005), or other technologies to identify or generate potato transformants lacking selectable marker genes is likely to continue. Also likely to continue is the development of transformation vectors derived from plant sequences (Rommens *et al.*, 2004).

3.4 Public and Industrial Perceptions of Transgenic Potato

In the United States, processor resistance to genetically modified potato represents a social barrier that may limit commercialization of transgenic potato in the near future. In Europe, Japan, and other parts of the world, public opinion against genetically modified foods in general is likely to continue to have a similar effect. Education efforts aimed to achieve public awareness of the origin of transgenes and both the benefits and drawbacks of transformation technologies continue to be important.

Recognizing that the source of a transgene can have significant impact on how the gene is regulated by governments and perceived by consumers, researchers have begun to adopt naming conventions and classification schemes that reflect the relatedness of donor and recipient species. Nielsen (2003) proposed such a system, advocating for five categories of relatedness between donor and recipient based on species classification. These same categories are ranked in terms of the potential for the target gene to be transferred or arise through conventional breeding technologies. For example, the “intragenic” category would encompass instances in which a gene is moved within a single genome or within a species. Genetic distance between donor and recipient in this case would be low and the target gene could be accessed through conventional breeding. At the other extreme, the “xenogenic” category would encompass instances in which the target gene is designed by researchers in a laboratory. Genetic distance between the donor and the recipient would be ranked as high and the target gene could

not be accessed through conventional breeding. The categories proposed by Nielsen (2003) are intended to provide a framework to discuss transgenic technologies for risk assessment at the scientific, regulatory, and ethical levels.

Along similar lines, the concept of the “*cisgene*” (as opposed to *transgene*) has emerged. Schouten *et al.* (2006a) defined a *cisgene* as a gene, including its native promoter, terminator, and intron(s), all in normal sense orientation, that is transferred through transformation technology from one plant to a close relative. Critically, the distinction these authors made between a *cisgene* and a *transgene* is that the former can be accessed by plant breeders through conventional breeding approaches, while that latter cannot. Thus, Schouten *et al.* (2006a) defined the genetic relatedness of donor and recipient solely on the basis of crossability rather than on a traditional species concept as proposed by Nielsen (2003). Among the genes isolated from potato species and used to transform cultivated potato (Table 3) disease resistance genes *Gpa2*, *Gro1*, *R1*, *R3a*, *RB*, *Rpi-blb2*, and *Rx1* fit the proposed definition of *cisgene*. Schouten *et al.* (2006a) argued that *cisgenes* are fundamentally different from *transgenes* and that plants produced through *cisgenesis* are conceptually and biologically similar to plants produced through conventional breeding, despite the use of transformation technology. In fact, the authors opined that the use of *cisgenes* may be safer than conventional breeding approaches since linkage drag associated with the introgression of genes from unadapted germplasm through conventional approaches may result in unintended and frequently negative effects. Consequently, these authors have advocated for recognition of *cisgenesis* as a process distinct from *transgenesis* and for relevant alteration of regulatory restrictions (Schouten *et al.*, 2006a, b).

Defining the risk associated with genetically modified plants on the basis of genetic relatedness of donor and recipient, whether defined based on species classification or crossability, may resonate with the informed consumer and those opposed to transformation-based genetic modification of plants on ethical grounds. Results from a US consumer survey conducted by Lusk and Sullivan (2002) indicate that consumers are more willing to consume transformed foods if the *transgene* originated from a closely related species or from

within the same species. When asked if they would eat a vegetable transformed with a gene originating from a bacterium, only 23% of respondents said they would. Sixty percent of respondents indicated they would eat a vegetable transformed with a gene originating from a different vegetable. But favorable responses rose to 80% when a vegetable was transformed with a gene originating from the same vegetable (Lusk and Sullivan, 2002), corresponding to a *cisgenic* approach. Myskja (2006) explored moral arguments for the use of *cisgenes* (referred to in that study as “*intragenes*”) for crop improvement, advocating for increased scientific priority on *cisgenesis*, as opposed to *transgenesis*, both to reduce scientific uncertainty and potential risks associated with broad gene transfer and to respect the opinions of consumers opposed to broad gene transfer. However, the proposed distinction between *cisgene* and *transgenes* is not without debate, even among researchers (de Cock Buning *et al.*, 2006; Giddings, 2006; Schubert and Williams, 2006). Whether public perception will be significantly swayed by the argument that many of the genes inserted into cultivated potato do in fact originate from a potato species remains to be seen.

The results of the survey of Lusk and Sullivan (2002) also suggest that open evaluation of the costs and benefits associated with transgenic potato may further convince some skeptics that the approach has merit. Specifically, the researchers found that respondents were more accepting of transformation technologies when they perceived a direct health benefit to the consumer or the environment (Lusk and Sullivan, 2002). Participants were asked about their acceptance of a range of transformed crop plants including high carotene “golden” rice, corn transformed to reduce pesticide usage, corn transformed to reduce on-farm production costs, and bananas transformed to produce vaccines. Among these hypothetical transformed crops, respondents were most accepting of golden rice (86.9%) and bananas transformed to produce vaccines (86.2%). Fewer respondents accepted corn transformed to reduce pesticide usage (78.3%) or production costs (76.9%). Lusk and Sullivan (2002) concluded that transformation applications geared toward improvement of crops with obvious consumer or environmental benefits is more palatable to consumers.

The worldwide significance of potato as a food crop and the potential for transformation technologies using genes isolated from potato species to reduce the massive chemical inputs currently associated with potato production might be an effective counterbalance to resistance against genetically modified potato. Consider, for example, late blight. The disease is estimated to cause US\$5 billion annual losses worldwide both in yield losses and in chemical controls in potato and tomato (Kamoun, 2001). The recent emergence of *P. infestans* races resistant to the systemic herbicide metalaxyl (Fry and Goodwin, 1997) has necessitated the release of millions of tons of preventative fungicides into the environment each year. The late blight resistance genes *RB* and *Rpi-blb2* cloned from wild potato *S. bulbocastanum* impart broad-spectrum, pathogen race nonspecific foliar blight resistance (Song *et al.*, 2003b; Van Der Vossen *et al.*, 2003, 2005). These genes have been transferred to cultivated potato using bridge crosses (Hermsen and Ramanna, 1973) and somatic hybridization (Helgeson *et al.*, 1998), but commercially important potato cultivars from these efforts have not yet emerged. Using transgenic methods, researchers are now transferring these genes into commercially prominent potato cultivars. These efforts include development of selectable marker-free lines that carry only the transgene under control of its native promoter and lack antibiotic resistance genes or other nonpotato-derived sequence (i.e., *cisgenesis*). Transformed lines developed through these efforts have the potential to significantly reduce worldwide chemical inputs for potato production, but those opposed to genetically modified foods might reject transformed potato varieties carrying these genes solely on the basis of the method used to create them. The deployment of late blight resistant transgenic potatoes is not without risk and careful evaluation of the human health and environmental impacts of such activities are clearly warranted. But evaluation of transgenic technologies to combat late blight or otherwise genetically improved potato should also assess the risk of *not* using the technology. In the case of late blight, these risks include crop failure and potential starvation in areas of the world dependent on potato as a food source and lacking sufficient funds or infrastructure for routine preventative fungicide applications, and

financial impact on the grower and health impact to the consumer and the environment in regions of the world where fungicide applications for the prevention of late blight are commonplace. Such balanced assessment of transgenic technologies for potato improvement may play an important role in expanding consumer acceptance.

Potato is among the most important food crops in the world. Recent advances in molecular biology have yielded insights into the genetics of potato disease and insect resistance, nutritional and starch qualities of tubers, and genetic and physiological responses to environmental conditions. Several genes with potential to improve potato have been cloned from a variety of species. With ongoing efforts to sequence the entire potato genome, the near future is likely to see the cloning of many more genes from the approximately 200 potato species. Improved transformation technologies, including approaches for potato transformation without introducing selectable marker genes, are emerging. While current processor and consumer resistance to transformed potato represents a social barrier for commercialization of transgenic potato in the near future, research on transgenic potato is likely to continue and the potential for using transformation technologies for the improvement of potato remains high.

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Sweet Potato

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The sweet potato, *Ipomoea batatas* (L.) Lam, is an important root crop supporting millions of people throughout the tropical regions of the world. Originally, sweet potato was domesticated in tropical South America from where it had spread to the South Pacific in prehistoric times. However, it is a relatively recent migrant in the countries where it now has the greatest importance. European traders and colonists brought the crop to Africa, Asia (Austin, 1988), and the western Pacific. Spanish explorers are believed to have taken the sweet potato to the Philippines and East Indies, from where it was soon carried to India, China, and Malaya by the Portuguese voyagers. It reached China, the world's number one sweet potato producer (88.9% of the world production) (FAO, 2004), in 1594 and Papua New Guinea (PNG), where it is a staple crop, sometime between 1600 and 1700. From China, it was introduced around 1700 to Japan, where it is now the third most important food crop. Rwanda, now one of the highest per capita consumers of sweet potato, began cultivating it only in the 18th century. Recently, the use of molecular markers provided evidence that Central America is the primary center of diversity and most likely the center of origin of sweet potato based on the richness of its wild relatives (Haung and Sun, 2000). One of the studies on the assessment of genetic diversity by

using amplified fragment length polymorphism (AFLP) marker advocated natural dispersal to be one of the key factors for the existing diversity of sweet potato (Rossel *et al.*, 1999–2000). Sweet potato is the seventh most important food crop worldwide (Jabsson and Raman, 1991) and is grown in 111 countries, particularly in Asia, Africa, and South America.

1.2 Taxonomy, Habit, Habitat, Genome Size, and Cytological Feature

Sweet potato, *I. batatas* (L.) Lam., belongs to the morning glory (Convolvulaceae) family. *Ipomoea* is derived from the Greek meaning worm bindweed. *Batatas* is the aboriginal American word for potato. Sweet potato is the only member of the genus *Ipomoea* whose roots are edible. Despite its name, sweet potato is not related to potato. Potato (*Solanum tuberosum*) is a member of the Solanaceae family, which also includes tomatoes, peppers, and eggplant. Unlike potato, which is a tuber, or thickened stem, sweet potato is a storage root. Sweet potato is an herbaceous and perennial plant. However, it is grown as an annual plant by vegetative propagation using either storage roots or stem cuttings. Its growth habit is predominantly prostrate with a vine system that expands rapidly horizontally on the ground. Also, depending on the length of internodes and frequency of branching, cultivars may be described as erect, semierect, spreading, and very spreading.

The most common type of sweet potato found in the US markets is the “moist-fleshed” type, red-skinned with dark-orange flesh. Dry-fleshed types of *I. batatas* (yellow, ivory, or white flesh) are popular among both Caribbean and Asian shoppers, especially on the US East Coast, and are sometimes sold under the Cuban name of *boniato*. “Boniato” is also the name of a specific cultivar.

Family	Convolvulaceae
Tribe	Ipomoeae
Genus	<i>Ipomoea</i>
Subgenus	<i>Quamoclit</i>
Section	Batatas
Species	<i>Ipomoea batatas</i> (L.) Lam.

Some consumers call sweet potato as “yams”. “Yam” (from the Yoruba word *iyama* (to eat)) is a term applied colloquially to the moist, orange-fleshed type of sweet potato. The true yam (*Dioscorea* spp.), a tuberous climbing vine not grown commercially on the US mainland, is naturalized in parts of the Upper South (chiefly *D. japonica*).

1.3 Karyotype and Cytology

The cultivated varieties of *I. batatas* display an important morphological polymorphism (Sihachakr and Ducreux, 1987). Among the species within the genus *Ipomoea* series Batatas, 13 are considered to be closely related to sweet potato (Austin, 1987), but the wild ancestor of this plant is still not identified. Several hypotheses have been put forward to explain the origin of sweet potato. It was thought to be originated from the diploid *I. leucantha* Jacq., from which tetraploid *I. littoralis* Blume was derived by polyploidization (Nishiyama, 1971).

Recently, Srisuwan *et al.* (2006) have studied the origin and evolution of sweet potato and its wild relatives through cytogenetic approaches. They studied the distribution and organization of 5S and 18S-5.8S-26S rDNA (ribosomal DNA) in ten varieties of hexaploid *I. batatas*, five accessions of tetraploid *I. trifida*, and six related species (five diploids, *I. trifida*, *I. triloba*, *I. tiliacea*, *I. leucantha*, and *I. setosa* and one tetraploid, *I. tabascanana*), by using fluorescence *in situ* hybridization. They concluded, based on chromosome morphology, that tetraploid *I. trifida* might be the progenitor

of *I. batatas*, and *I. tabascanana* might be the interspecific hybrid between these two species.

1.4 Economic Importance

Versatility and adaptability of sweet potato have given a rank to it as the world's seventh most important food crop following wheat, rice, maize, potato, barley, and cassava. It is adaptable to a wide range of climatic and soil regimes and is normally grown from 40° N to 32° S; from sea level to 9000-ft. (2743 m) elevation in areas where average temperature is 23.8°C or more with a well-distributed annual rainfall of 30–50 in. (0.76–1.27 m) and an abundance of sunshine. The more diversified utilization of sweet potato in Asia and Africa surely contribute to the improvement of local economies. Aside from being a subsistence crop, sweet potato makes a large contribution to livestock production in many areas. Nearly half of the sweet potato produced in Asia is used for animal feed, with the remainder being primarily used for human consumption, either as fresh or processed products. In contrast, although African farmers produce only about 7 million tons of sweet potatoes annually, most of the crop is cultivated for human consumption. African yields are quite low, about a third of Asian yields, indicating huge potential for future growth. Latin America, the original home of sweet potato, produces a mere 1.9 million tons annually. In the densely populated, semi-arid plains of eastern Africa, sweet potato is called *cilera abana* meaning “protector of the children”. This title alludes to the vital role it fulfills in thousands of villages, where people depend on the crop to combat hunger. Six sweet potato varieties that tolerate salt and drought (Yarada, Nacional, Tacna, Caplina, Atacama, and Costanero) were released by the Universidad Nacional Jorge Basadre from Tacna, Peru in 1992. The International Potato Center (CIP) reported that the share used as feed in Asia jumped from 14.5% in 1961–1963 to 44% in 1993–1995. Over 95% of the global sweet potato crop is produced in the developing countries, where it is the fifth most important food crop in terms of fresh weight. According to FAO's 1998 State of the World's Plant Genetic Resources, there are nearly 32 000 accessions held in gene banks around the world. The largest and most diverse collection is held at CIP, Peru.

Sweet potato has high content of carbohydrates and can produce more edible energy per hectare per day than wheat, rice, or cassava. The yellow-orange fleshed varieties provide particularly high quantities of vitamin A and C. The leaves can also be eaten, providing additional protein, vitamins, and minerals. The roots are most frequently boiled, fried, or baked. They can also be processed to make starch and a partial flour substitute. Industrial use of sweet potato for starch and other processed goods (e.g., alcohol, etc.) is more localized, but expanding. Thus sweet potato provides an additional source of cash for poor.

Sweet potato also possesses potent antioxidant properties and considered as antidiabetic and anti-inflammatory food. Our food ranking system also showed sweet potato to be a strong performer in terms of traditional nutrients. This root vegetable qualified as an excellent source of vitamin A in the form of β -carotene (Yoshimoto *et al.*, 1999; Konczak-Islam *et al.*, 2003; Haskell *et al.*, 2004; van Jaarsveld *et al.*, 2005) is a very good source of vitamin C and manganese, and a good source of copper, dietary fiber, vitamin B6, potassium, and iron. Both β -carotene and vitamin C are very powerful antioxidants that work in the body to eliminate free radicals. Free radicals are chemicals that damage cells and cell membranes and are associated with the development of conditions like atherosclerosis, diabetic heart disease, and colon cancer. Since these nutrients are also anti-inflammatory, they can be helpful in reducing the severity of conditions where inflammation plays a role, such as asthma, osteoarthritis, and rheumatoid arthritis.

In addition, sweet potatoes are a good source of vitamin B6, which is needed to convert homocysteine, an interim product created during an important chemical process in cells called methylation, into other benign molecules. Since high homocysteine levels are associated with an increased risk of heart attack and stroke, having a little extra vitamin B6 on hand is a good idea.

In South America, the juice of red sweet potatoes is combined with lime juice to make a dye for cloth. By varying the proportions of the juices, every shade from pink to purple to black can be obtained. All parts of the plant are used for animal feed. Sweet potato also has ethnomedical uses. The aerial roots are used as a galactagogue;

the leaves are used to treat diabetes (Yoshimoto *et al.*, 1999; Kusano *et al.*, 2001; Matsui *et al.*, 2001; Konczak-Islam *et al.*, 2003), hookworm, hemorrhage, and abscesses while the tuber is used to treat asthma. Recently, sweet potato products have also been considered to be used successfully in menus developed for space (NASA's advanced life support program) with added benefit of increased nutrient value and dietary variety (Wilson *et al.*, 1998; Ruminsky and Hentges, 2000; Bovell-Benjamin, 2007).

1.5 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

Breeding of sweet potato through sexual means dates back to as early as 1904 (Tioutine, 1935), which then got momentum after inducing sweet potato to flower and set seed in 1937 (Miller, 1937). Since these early works, a vast amount of research has been carried out to improve overall sweet potato production, and the magnitude and ramifications of research has been astronomical.

Despite the fact that tremendous improvements have been made in sweet potatoes, very little progress has been made in terms of yield when compared to other crops. Worldwide losses of sweet potato include pre- and postharvest losses from insects, nematodes, and diseases, as well as a whole host of other factors (Jabsson and Raman, 1991). A poll of researchers in the developing countries found that the crop loss due to insects was the most important problem in the field. Although the importance of pest species varies regionally, weevils are the most important threat worldwide, particularly certain species of *Cyclas* (Wolfe, 1991). The sweet potato weevil, *C. formicarius*, is a major constraint to the production in many areas and is the single most important constraint in tropical regions (Horton and Ewell, 1991). Insect feeding can alone result in yield losses of 60–100% (Chalfant *et al.*, 1990). The level of natural resistance to insects is low and resistance does not seem effective at all under high weevil population pressure (Collins and Mendoza, 1991). Consequently, research into ways of reducing insect damage would be highly beneficial. Although sweet potato encompasses extensive genetic variability, it is difficult to

improve by conventional breeding because of being hexaploid ($2n = 6x = 90$), possessing cross-incompatible genes, and having low seed set (Lowe *et al.*, 1994). Furthermore, it is not possible to cross different lines in many cases owing to cross-incompatibility, as well as instability of hybrid offspring. It could, however, be possible to genetically modify sweet potato through the application of tissue culture and biotechnology and improve the crop in ways that cannot be achieved through conventional plant breeding programs based on sexual hybridization. Work is underway on the isolation and characterization of genes that will confer insect resistance. These genes are all derived from plants and include various types of protease inhibitors, α -amylase inhibitors, lectins, and other proteins with enzymatic functions. One of the main targets is to successfully introduce the cowpea trypsin inhibitor gene (*CpTI*) into sweet potato using an *Agrobacterium*-based approach, as *CpTI* has been demonstrated to confer tolerance to insect-pests when expressed in tobacco plants (Hilder *et al.*, 1987).

Conventional breeding methods in sweet potato have shown limited progress in the genetic improvement for, resistance to diseases (Clark, 1986), nematodes (Jones and Dukes, 1980), insects (Jones and Cuthbert, 1973), enhancement in protein contents, and nutritional quality (Water *et al.*, 1984). The success of plant genetic manipulation depends upon the effectiveness of *in vitro* regeneration system, which is highly genotype-specific in sweet potato (Gosukonda *et al.*, 1995). Considerable studies have been conducted in sweet potato tissue culture, but the lack of a reliable regeneration system has limited the application of biotechnology (Al-Juboory and Skirvin, 1991; Chee and Cantliffe, 1992; Sihachakr and Ducreux, 1993; Prakash, 1994; Lowe *et al.*, 1994). Thus, there is a critical need for screening of a large number of genotypes to test their regeneration potential and for their subsequent use in transformation studies. To overcome these limitations, breeders must incorporate novel approaches such as somatic hybridization and genetic transformation into conventional sweet potato breeding programs. Various gene delivery systems have been used to develop transgenic sweet potato concentrating on a specific trait. Three major gene delivery systems have been discussed in the following text, which demonstrates the efforts made in developing transgenic sweet potato

either by *Agrobacterium*-mediated transformation, electroporation, or biolistic approaches.

2. TRANSGENIC DEVELOPED AND DNA DELIVERY SYSTEMS

Several innovative methods have been developed to insert genes into the plant cell genome. However, electroporation and particle bombardment or biolistic gun are the most common techniques for direct DNA transfer. In addition, *Agrobacterium* plasmid is also a versatile vehicle to introduce genes into dicotyledonous plants. The above three approaches, for producing transgenic sweet potato (Dodds *et al.*, 1991; Prakash and Varadarajan, 1991; Newell *et al.*, 1995; Gama *et al.*, 1996; Murata *et al.*, 1997; Morán *et al.*, 1998), have been well documented. However, these methods had a limited success because of the lack of a reliable regeneration system and stable expression of introduced genes into the genome. The regeneration potential and prospects has been dealt in detail in the following text.

2.1 *Agrobacterium*-Mediated Transformation

Reports are available on transformation in sweet potato using *Agrobacterium*-mediated systems (Dodds *et al.*, 1991; Newell *et al.*, 1995; Gama *et al.*, 1996; Morán *et al.*, 1998; Song *et al.*, 2004; Luo *et al.*, 2006). The species of soil bacteria, such as *Agrobacterium tumefaciens* and *A. rhizogenes*, are capable of transferring discrete sequences of transfer-DNA (T-DNA) from their tumor-inducing (Ti) plasmid into the plant genome. The genes in the T-DNA are integrated into the plant genome and expressed, producing certain unique plant hormones called opines (Gasser and Fraley, 1989). These hormones cause an unregulated proliferation of callus tissue. The T-DNA is a specific segment in the Ti plasmid of *A. tumefaciens* and is delimited by borders (*cis*-acting sequences, T-DNA border and *trans*-acting function, *Vir* function) of repeated sequences. *Agrobacterium* is now frequently used as a vector for the introduction of foreign genes into plant cells. Among the available transformation methods reported in sweet potato, *A. tumefaciens*-mediated transformation is more successful and desirable

(Song and Yamaguchi, 2006). Since the presence of opine genes (*onc*-genes) in the T-DNA is incompatible with regeneration of the transformed cells into complete plants, preferably disarmed strains (devoid of Ti genes) are used as vectors. A major breakthrough in the development of Ti-plasmid binary vectors occurred as a result of deleting the phytohormone biosynthetic genes (Fraley *et al.*, 1985), and inserting marker genes from any organism, and introducing DNA cassette into plant genome using *A. tumefaciens* as a delivery system (Gasser and Fraley, 1989). In sweet potato, regeneration of *Agrobacterium*-mediated transformed callus has been reported (Al-Juboory and Skirvin, 1991; Carelli *et al.*, 1991). However, no regeneration in this callus was obtained. Also, the inherent problems of somaclonal variation associated with longer time in culture renders this approach less desirable than more direct methods of regeneration. Transformation in sweet potato, using *A. rhizogenes*-based vector, has been reported (Dodds *et al.*, 1991; Otani *et al.*, 1993). Hairy roots were induced on inoculated leaf discs, and shoots were subsequently regenerated from the hairy roots. However, this procedure involves the regeneration of plants from transformed hairy roots. It can be difficult to obtain phenotypically similar plants, due to the carryover of neoplastic genes from the root inducing-DNA (Ri-DNA) along with the gene of interest (Tepfer, 1984). Successful transformation of the cultivars Jewel and TIS-70357, using *Agrobacterium*-mediated transformation, has been reported by Prakash and Varadarajan (1991). But these experiments could not be repeated in other laboratories. However, Newell *et al.* (1995) reported successful regeneration of transgenic sweet potato plants through co-cultivation of storage root discs with *Agrobacterium*. Six regenerated plants expressed β -glucuronidase (GUS), cowpea trypsin inhibitor, and snowdrop lectin genes.

Sweet potato is more hardy and needs fewer nutrients from the soil than most other crops. However, increasing production is limited by the severe damage caused by pests and diseases. In many regions where sweet potatoes are produced in low-input agricultural systems, insect related losses may often reach 60–100% (Chalfant *et al.*, 1990). Sweet potato weevil (*Cylas formicarius*) is the major pest worldwide (Sutherland, 1986). Morán *et al.* (1998) cloned *cryIIIA* δ -endotoxin gene under the regulation of the cauliflower

mosaic virus (CaMV) 35S promoter and the tobacco mosaic virus (TMV) Omega fragment through *A. tumefaciens*-mediated transformation. Evaluations for the insecticidal capacity of transgenic plants were performed against sweet potato weevil both under controlled and field conditions. Another common problem with sweet potato is the attack of stem nematodes. It has been found that *oryzacystatin-I* (*OCI*) gene confers resistance to plant nematodes, such as *Meloidogyne incognita*, *Globodera pallida*, and *Heterodera glycines*. So far, nematode-resistant transgenic plants in rice, potato, and *Arabidopsis* have been produced (Urwin *et al.*, 1997; Atkinson *et al.*, 1998; Vain *et al.*, 1998). Scarce data are available on nematode resistance in sweet potato. Jiang *et al.* (2004) were able to produce transgenic sweet potato plants with *OCI* gene through *Agrobacterium*-mediated transformation by utilizing the strain LBA4404 with a binary vector PBinH and *neomycin phosphotransferase II* (*NPTII*) and *OCI* genes. Plant regeneration was successfully obtained from embryogenic calluses through somatic embryogenesis.

Among diseases, viral diseases of sweet potato are very prevalent and often seriously damaging to the plant. Efforts have been made to develop transgenic sweet potato with antiviral properties, lately by Min *et al.* (2006), where they have utilized a binary vector PGA748 under the control of CaMV 35S promoter, with *NPT* gene as a selectable marker, and introduced into *A. tumefaciens* LBA4404 for production of human lactoferrin is an iron-binding protein found in milk that plays an important role in the immune system response. It also prevents the growth of pathogens, exerts antibacterial, and antiviral properties and controls cell and tissue damage caused by oxidation, and facilitates iron transport (Arakawa *et al.*, 1999). One of the severe strains of viruses is sweet potato feathery mottle virus (SPFMV-S), which causes russet crack disease (Okada *et al.*, 2001). Resistance against SPFMV has been incorporated by Okada *et al.* (2002) where they were able to produce transgenic sweet potato, introducing hygromycin resistance (*hpt*) and SPFMV-S coat protein (*CP*) genes, which have shown a significant resistance to SPFMV-S. Such a production system for the virus-resistant transgenic sweet potato is useful in sweet potato breeding.

Efforts also have been made toward quality improvement by altering the expression of specific enzymes or enzymatic pathway. Kimura *et al.* (2001) successfully obtained transgenic sweet potato plants through *A. tumefaciens*-mediated transformation of full-length sense complementary DNA (cDNA) for sweet potato granule bound starch synthase I driven by the CaMV 35S promoter, demonstrating the possibility of altering starch composition in the tuberous root of sweet potato while increasing starch (amylose) content by RNA interference of the starch branching enzyme II (*IbSBEII*) gene. Similarly, Shimada *et al.* (2006a, b) constructed a double-stranded RNA (dsRNA)-interference vector on the basis of the registered cDNA of sweet potato *SBEII* encoding class A branching enzyme and introduced into sweet potato genome via *Agrobacterium*-mediated gene transformation for increased starch yield. In addition, Transgenic sweet potato plants were developed with increased content of linolenic acid through *A. tumefaciens*-mediated transformation by introducing a tobacco microsomal ω -3 fatty acid desaturase gene (*NtFAD3*) under the control of CaMV 35S/E12 ω promoter (Wakita *et al.*, 2001).

Another shift in trend has been observed for expression of pharmaceutical proteins. Plant-based expression of pharmaceutical proteins has proven as a cost-effective alternative to traditional large-scale production systems such as microbial fermentation, insect and mammalian cell cultures, and transgenic animals. Transgenic sweet potato has been developed by Berberich *et al.* (2005) with antidiabetic properties. Mouse *adiponectin* (encoding an antidiabetic protein) cDNA has been utilized for developing transgenic sweet potato via *Agrobacterium*-mediated transformation. Adiponectin is a 30-kDa protein exclusively produced and secreted from adipocytes and as a cytokine has been found to be linked to obesity, insulin resistance, and type-2 diabetes. Production of biologically active adiponectin in large scale is desirable for pharmaceutical applications.

2.2 Electroporation

Another approach for obtaining transgenic plants is via electroporation of naked plasmid DNA

in protoplasts/cells (Fromm *et al.*, 1985; Dhir *et al.*, 1991; Joersbo and Brunstedt, 1991). In electroporation, applying a controlled electric current to exposed membranes of protoplast, temporarily causes the membranes to become polarized. The effects of electric field on membrane permeability are relatively long lived (minutes) along with the duration of the applied pulse(s) (micromilliseconds). At a certain level (at electric current of 250–500 V cm⁻¹), the membranes show small pores, which allow entry or uptake of exogenous DNA, and these pores usually self-repair shortly after exposure to the current (Joersbo and Brunstedt, 1991). Okada *et al.* (2001) successfully obtained transgenic sweet potato lines resistant to the most serious disease of sweet potato called russet crack disease caused by SPFMV. They constructed an expression vector carrying the *CP* and hygromycin phosphotransferase (*hpt*) gene driven by CaMV 35S promoter and inserted into the sweet potato variety Chikei 682-11 by electroporation. The transgenic lines were shown to be highly resistant not only to the primary but also to the secondary infection of SPFMV-S. Most of the published works on electroporation was used to achieve transient (Mitchell *et al.*, 1998; Lawton *et al.*, 2000; Winfield *et al.*, 2001) and stable (Winfield *et al.*, 2001) transformation in protoplasts and to produce transgenic microcalli (Figure 1). But recovering transformed plants from these microcalli was a failure (Nishiguchi *et al.*, 1992). Successful example of plant regeneration, via somatic embryogenesis, and transient gene expression in sweet potato petiole protoplast has been demonstrated by Dhir *et al.* (1998) (Figure 2).

2.3 Particle Bombardment

Particle (microprojectile) bombardment of explants of sweet potato cultivars Jewel and TIS-70357 with a plasmid DNA containing GUS and *NPTII* genes, produced calli and roots which were positive for GUS (Prakash and Varadarajan, 1992). However, experiments to produce somatic embryos or to promote shoots from these transgenic calli has been unsuccessful so far (Prakash and Varadarajan, 1992; Prakash, 1994). However, recent studies have evidenced success in obtaining transgenic sweet potato plants. Among them is

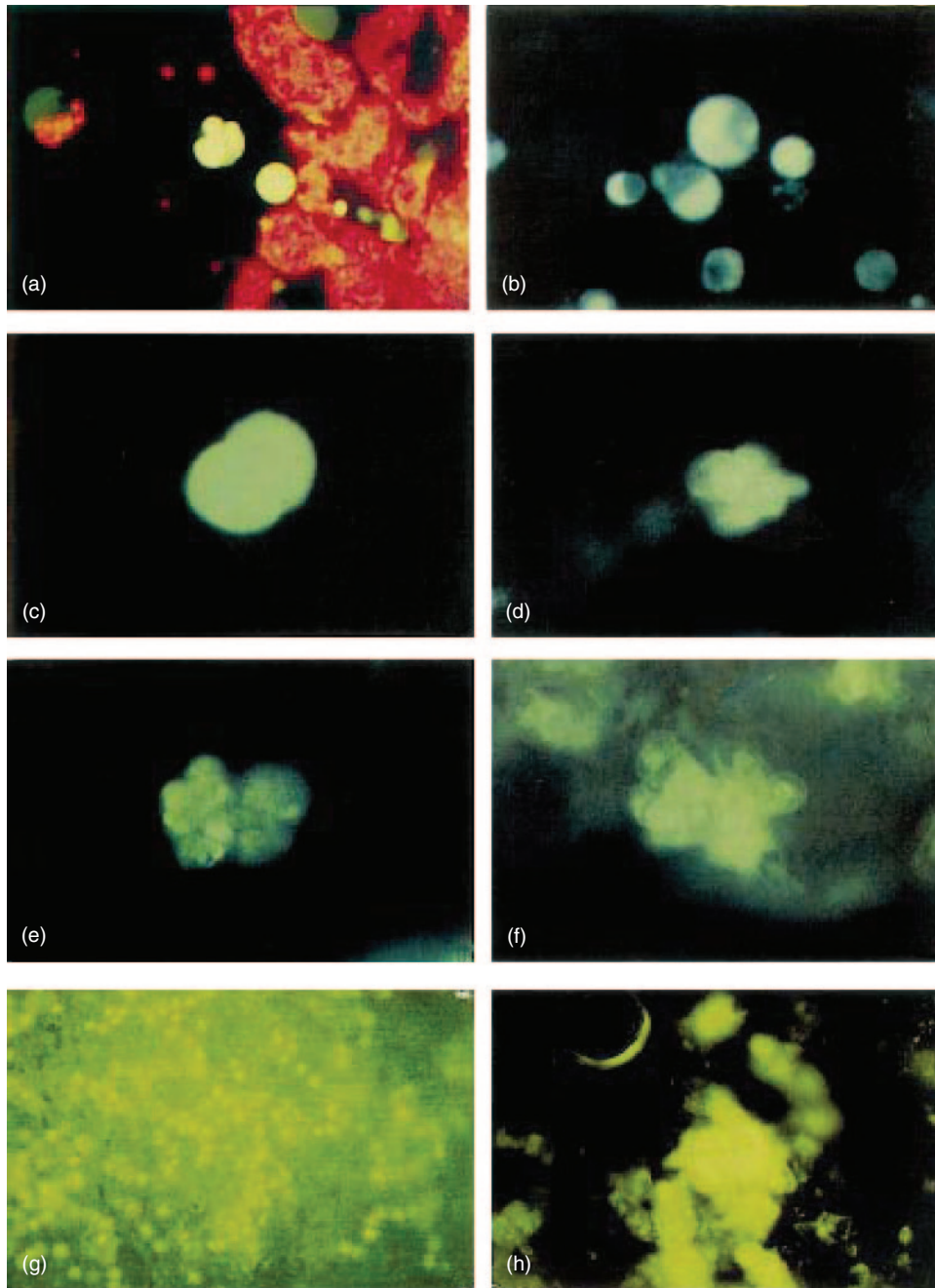


Figure 1 Transient and stable expression of green fluorescent protein (GFP) in sweet potato mesophyll and petiole protoplasts using electroporation: (a) the yellow-green fluorescence of GFP is visible in transfected mesophyll protoplasts and red autofluorescence from chlorophyll ($\times 250$); (b) GFP expression in mesophyll protoplasts (protoplasts were viewed with a U-MNIBA filter set to eliminate the autofluorescence of chlorophyll, 400); (c) first division in a protoplast showing expression of GFP gene after 4 days in mesophyll protoplasts (400); (d) six- to eight-celled mesophyll protoplast-derived individual colonies expressing GFP gene; (e) 12–16 microcolonies expressing GFP after 2 weeks of culture; (f) microcalluses expressing GFP gene after 4 weeks of culture; (g) each green patch represents an individual transient expression event 48 h after bombardment of leaf tissues using a U-MNIBA filter to eliminate the autofluorescence of chlorophyll ($\times 250$); (h) several stable events showing the expression of GFP in leaf-derived callus [Reproduced from Winfield *et al.* (2001). © Springer]

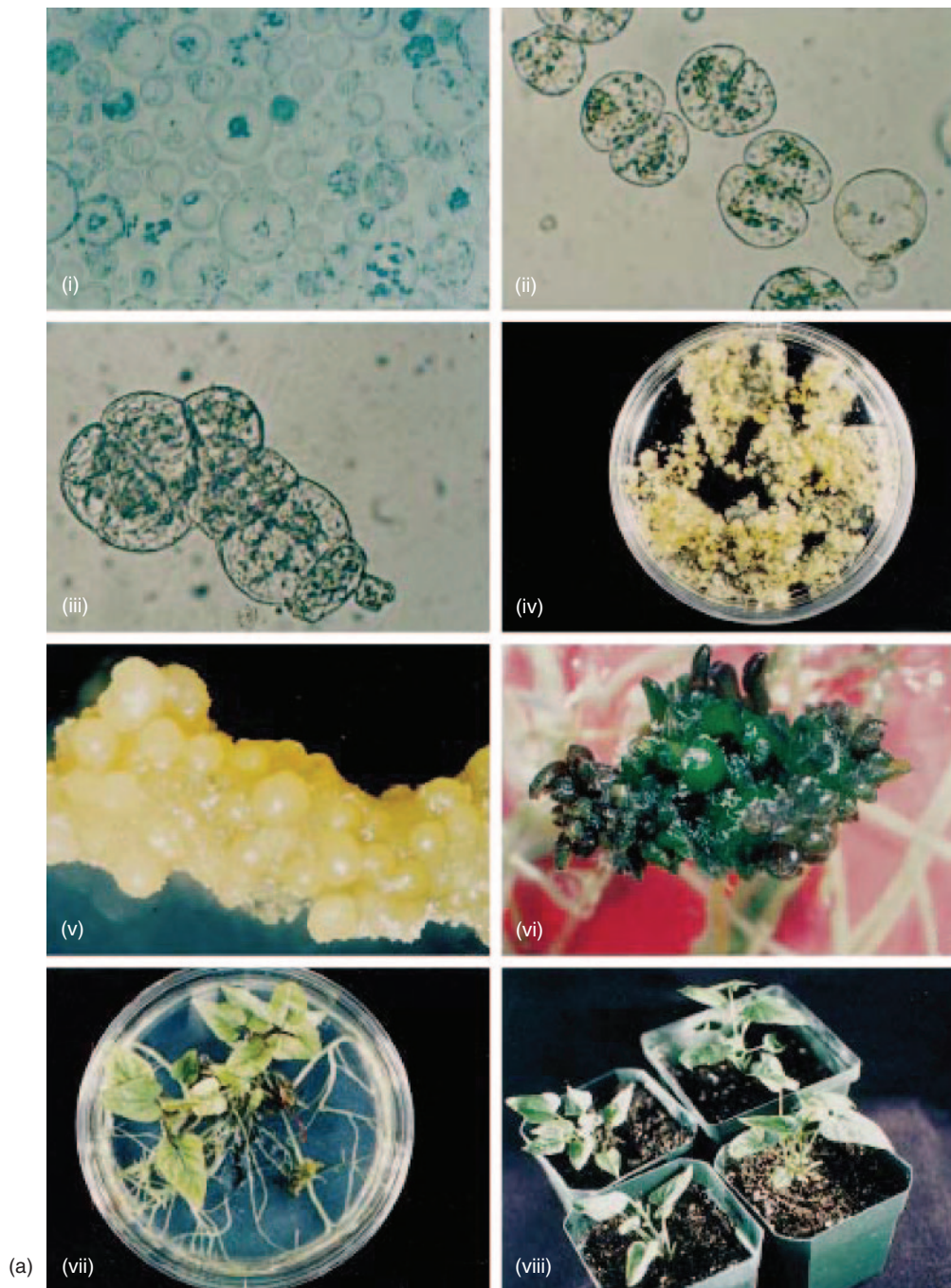


Figure 2 (a) Plant regeneration from petiole protoplasts via somatic embryogenesis: (i) freshly isolated intact purified petiole protoplasts; (ii) Protoplast division after 5–6 days of culture; (iii) eight cell divisions; (iv) protoplast-derived microcalli on agarose beads after 8 weeks of culture; (v) protoplast-derived calli showing somatic embryoids; (vi) mature somatic embryos at various stages of development; (vii) well-developed plantlets regenerated from somatic embryos; (viii) protoplast-derived plants established in pots [Reproduced from Dhir *et al.* (1998). © Springer]

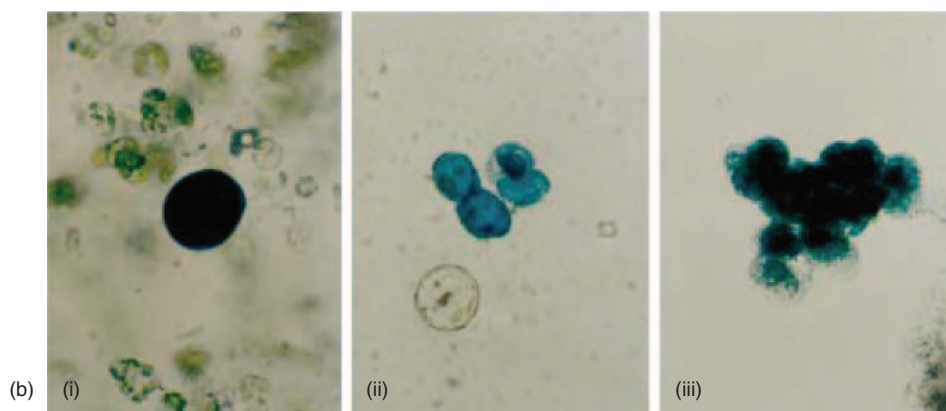


Figure 2 (b) transient gene expression in sweet potato protoplasts: (i) intense blue color indicating positive reaction of *gus* gene activity in electroporated protoplasts 24 h after X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) staining; (ii) expression of GUS in dividing protoplasts after 5–6 days of culture; (iii) cell colonies consisting of many cells showing intense blue color due to the expression of the *gus* reporter gene (protoplasts were electroporated and subcultured for 15 days and tested by a GUS histochemical assay)

herbicide-resistant sweet potato, where Yi *et al.* (2007) utilized biolistic method to introduce β -glucuronidase gene (*gusA*) and herbicide-resistant gene (*bar*) and reported successful transformation of the *bar* gene (Figure 3).

Besides insects, pests, and diseases, environmental stresses also play a significant role in crop production. Oxidative stress is one of the major factors causing injury to plants exposed to environmental stresses. Lim *et al.* (2007) have reported a remarkable achievement of enhancing the tolerance of transgenic sweet potato plants that express both CuZn superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX) in chloroplasts to methyl viologen-mediated oxidative stress and chilling. Particle bombardment has been used to express the genes of both CuZnSOD and APX under the control of an oxidative stress-inducible *SWAP2* promoter in the chloroplasts of sweet potato plants (referred to as SSA plants). Authors have advocated that SSA plants would be useful for commercial cultivation under unfavorable growth conditions. In addition, the manipulation of the oxidative mechanism in the chloroplasts can be applied to the development of various other transgenic crops with an increased tolerance to multiple environmental stresses. In recent experiments, Dhir *et al.* (unpublished) have also successfully expressed *GUS* gene in leaf explants of sweet potato through the use of particle bombardment (Figure 4).

2.4 Regeneration of Whole Plant

Sweet potato plant regeneration through tissue culture dates back to 1972 (see for review Henderson *et al.*, 1983) but is still considered to be difficult (Chee and Cantliffe, 1992; Chée *et al.*, 1992), especially from protoplast (Sihachakr and Ducreux, 1993), leaf (Wambugu and Henshaw, 1990), and somatic embryos. In order to improve the ultimate yield of regenerated whole plants via tissue culture, several investigators have utilized very young tissues as the explant source (Lowe *et al.*, 1994 and references therein). Various kinds of morphogenic responses reported for sweet potato tissues are described below.

2.4.1 Micropropagation: meristem and shoot tip culture

Meristem and shoot tips placed on Murashige and Skoog (MS) (Murashige and Skoog, 1962) culture medium allow formation of new shoots via axillary bud break, leading to the production of large number of shoots from a single explant in a short time. This technique has been applied in sweet potato to obtain virus-free clones for commercial purposes and also to obtain virus-free plant material in which viruses may be introduced to study the infection mechanism. Several researchers have reported establishment of

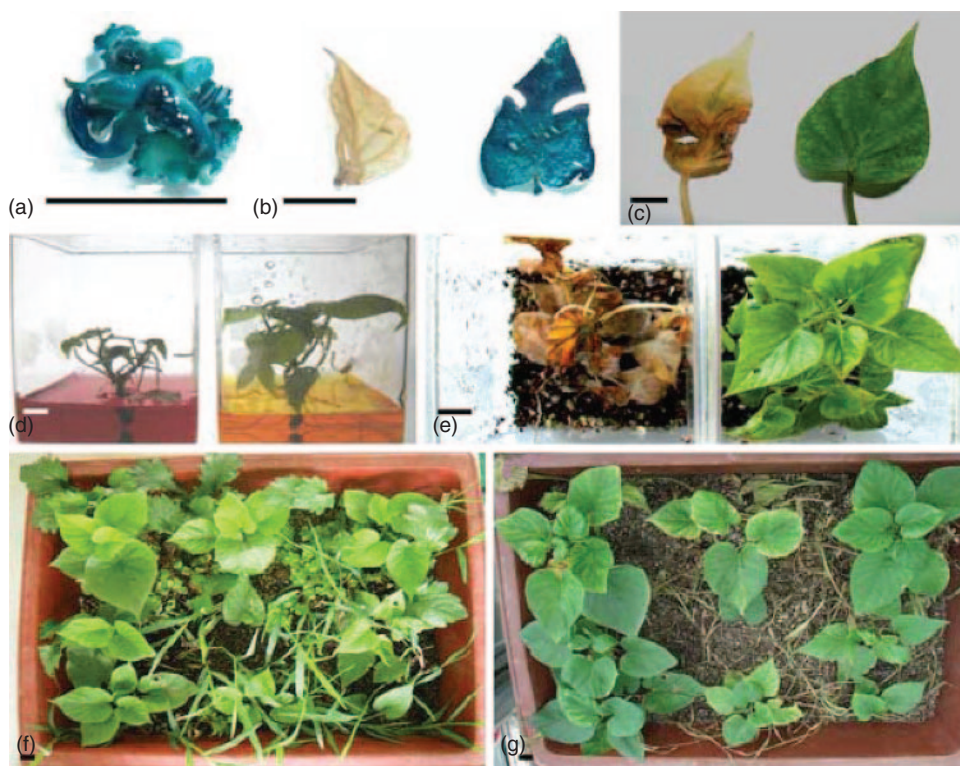


Figure 3 Sweet potato transformation, confirmation of transgenic events and transgenic plants growing in the greenhouse: (a) GUS expression in regenerating tissue; (b) GUS gene expression in leaf tissue (left, nontransformed; right, transformed); (c) Basta (0.3% v/v) painting on leaf tissue (left, nontransformed; right, transformed); (d) Chlorophenol Red assay on plantlets (left, nontransformed; right, transgenic plantlet) (the medium with red color indicates PPT sensitivity, whereas orange color indicates resistance); (e) 5 days after spraying Basta (0.3% v/v) to plants (left, nontransformed; right, transformed); (f) and (g) application of herbicide to sweet potato plants grown with a variety of weeds (f, before spray; g, 2 weeks after spray) (scale bars indicate 2 cm) [Reproduced from Yi *et al.* (2007). © Springer]

virus-free meristem cultures of many sweet potato cultivars (Elliott, 1969; Mori, 1971; Alconero *et al.*, 1975). Production of virus-free plants by a combination of heat treatment and shoot-tip culture has also been reported (Liao and Chung, 1979; Frison and Ng, 1981).

2.4.2 Organogenesis

Organogenesis is the formation and outgrowth of multiple shoots via callus or directly from explants and subsequent rooting to regenerate complete plantlets *in vitro* (Figure 5). Various studies have been conducted to optimize explant sources and a matching culture medium for sweet potato tissue culture (Hill, 1965; Henderson *et al.*, 1983). Gunckel *et al.* (1972) were the first to report the induction of roots and shoots

from storage root segments of the cultivar “Centennial” on MS medium. Similar finding were reported by other researchers (Hwang *et al.*, 1983). Shoot regeneration can be induced directly from adventitious roots either from shoot culture (Yamaguchi and Nakajima, 1972; Ozias-Akins and Perera, 1992) or from regenerated roots arising from tissue-cultured explants (Carswell and Lacy, 1984; Belarmino *et al.*, 1992). The level of regeneration in all cases was low, approximately one shoot per explant under optimal conditions.

Excised leaf tissue has also been used as an explant for producing callus, roots, and shoots (Sehgal, 1975). The capacity of leaves to produce callus was dependent on their maturity and the amount of callus was related to the degree of cuts given or the extent of injury caused (Sehgal, 1975). Also, Litz and Conover (1978) were able to induce callus from lateral buds and shoot apices,

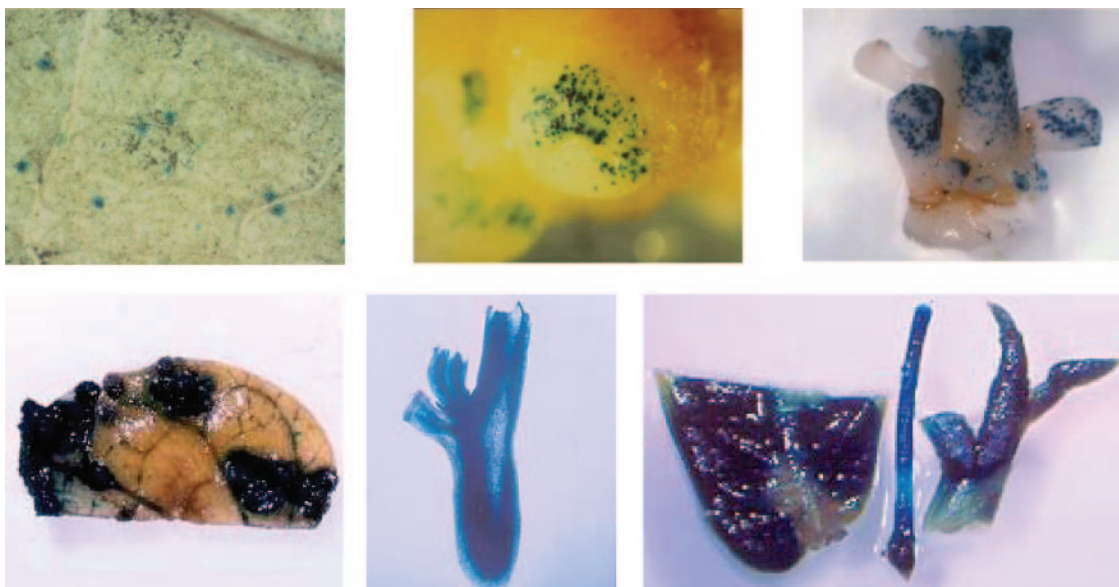


Figure 4 Expression of *GUS* gene into different explants of sweet potato using particle bombardment mediated gene transfer technique [Reproduced from Dhir *et al.*, unpublished data]

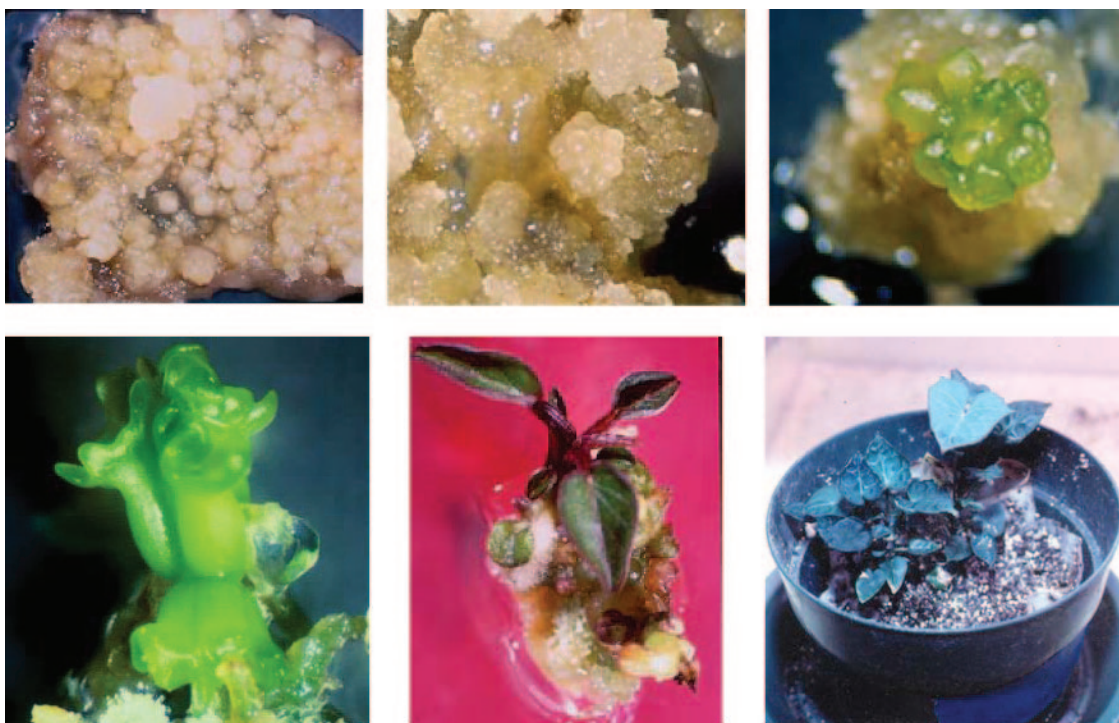


Figure 5 Somatic embryogenesis, organogenesis, and plant regeneration from sweet potato explants [Reproduced from S. Dhir *et al.*, unpublished data]

which then produced roots and shoots. Wambugu and Henshaw (1990) obtained shoot buds from 4-month-old leaf-derived callus. Shoot regeneration from petiole explant of sweet potato has also been reported (Hwang *et al.*, 1983; Templeton-Somers and Collins, 1986; Xin and Zhang, 1987; Suga and Irikura, 1988; Gosukonda *et al.*, 1995). However, in all reports the percentage of regeneration was reported to be very low.

2.4.3 Somatic embryogenesis

An alternative approach to regenerating plants involves the induction of somatic embryos from somatic cells. Somatic embryos (Figure 5), considered to be of single-cell origin, are independent bipolar structures and are not physically attached to the tissue of their origin. These structures can further develop and germinate into plantlets through the events that correspond with the zygotic embryogenesis (McKersie and VanAcker, 1994). Somatic embryos have potential use as *in vitro* germplasm and can be used as artificial seeds. Although somatic embryogenesis has been achieved in sweet potato (Hwang *et al.*, 1980; Jarret *et al.*, 1984), but the success in regenerating plants is restricted only to a few cultivars. Somatic embryos were most efficiently induced in shoot tips and in each case, embryos developed directly from callus induced by 2,4-dichlorophenoxy acetic acid. The whole plantlets regenerated from these embryos were phenotypically identical to the parent plants (Jarret *et al.*, 1984; Liu and Cantliffe, 1984; Harrell *et al.*, 1993). Histological studies demonstrated embryogenic callus initiation from the periphery of the apical dome and from the youngest pair of the leaf primordia (Liu *et al.*, 1989). In most cases, sweet potato somatic embryos were found arrested at all stages of development and showed great variability in developmental patterns and morphogenesis (Chée and Cantliffe, 1988a, b).

An improvement in the production of embryos in suspension cultures was achieved by using alginate beads or sieving methods, which led to the release of polarized individual cell aggregates during callus fragmentation (Chée and Cantliffe, 1988a). In these cultures, up to 53% of somatic embryos were capable of regenerating into whole plants (Chee *et al.*, 1990). Embryos produced in

suspension cultures differed from mature seed-derived embryos in morphology and apparent lack of dormancy (Chee *et al.*, 1990). Development of sweet potato somatic embryos is highly genotype dependent (Sonnino and Mini, 1993). The use of somatic embryos in sweet potato as synthetic seed is limited by the production of large number of high-quality, somatic embryos. To compete commercially with the true seed or for efficient micro- and macropopagation system, somatic embryos must be able to germinate rapidly with high frequencies (Bornman, 1993). Successful production of synthetic seed in sweet potato needs more research to study the effect of several factors on induction, growth, development, and maturation of somatic embryos.

2.4.4 Anther culture

The main advantage of using haploids in a breeding program is the production of reliable homozygous lines in a short period of time (Alejar *et al.*, 1995). To date, true haploids have not been reported in sweet potato, but there have been a number of experiments using anthers as the source for callus tissue from which plantlets were developed. Callus tissue derived from anthers of sweet potato flowers has been obtained (Tsai and Lin, 1973a, b; Sehgal, 1978; Tsai and Tseng, 1979); however, the developed plantlets were not haploid. Also, techniques to conserve sweet potato germplasm *in vitro* have been developed (Jarret, 1989; Jarret and Florkowski, 1990; Kuo, 1991).

2.4.5 Protoplast culture

The development of protoplast isolation and culture techniques probably offer the most promising strategy for evolving new plant hybrids and varieties, especially in vegetatively propagated plants like sweet potato (Perera and Ozias-Akins, 1991). Reports on development of fertile sweet potato plants from protoplasts are limited. The successful isolation of sweet potato protoplasts from stem callus was first reported by Wu and Ma (1979). Later, Bidney and Shepard (1980) were able to produce callus from petiole protoplasts and leaf mesophyll cells of the cultivars Jewel and Centennial, but no shoot morphogenesis or

embryogenesis was observed. Protoplasts have been isolated from various tissues including petioles, stems (Otani *et al.*, 1987; Sihachakr and Ducreux, 1987; Perera and Ozias-Akins, 1991), and calli (Nishimaki and Nozue, 1985). In contrast, mesophyll tissues are considered recalcitrant to enzyme digestion (Otani *et al.*, 1987). Recently, Perera and Ozias-Akins (1991) and Ozias-Akins and Perera (1992) isolated protoplasts from mesophyll tissue and petioles and observed sporadic shoot formation from the protoplast-derived callus. Protoplasts were also isolated from high anthocyanin-producing callus of sweet potato by Nishimaki and Nozue (1985). The first report of successful plantlet, shoot/root formation from protoplasts, was presented by Sihachakr and Ducreux (1987) and later by Dhir *et al.* (1998). Thus, it is possible to regenerate plants from protoplast. However, there is a need for refinement of the current procedures to make them applicable across various genotypes.

3. FUTURE ROAD MAP

The application of new techniques for improvement of sweet potato crops, particularly including the exploitation of somaclonal variation, gene transfer by genetic transformation and somatic hybridization, requires the control of plant regeneration from tissue culture (Sihachakr *et al.*, 1997). The success of transgenic effort can only be achieved by optimizing a successful regeneration protocol. Tremendous success has been achieved in the gene transfer and regeneration of transformed plants in the last one decade. With the trend of technology moving in such a fast pace in genetic modification, releasing genetically modified crops into the environment brings unforeseen threats and potential risk of gene flow via pollen from crops to related wild species (Sossey-Alaoui *et al.*, 1996) with consequences such as the potential for development of resistant pests and outcrossing to weedy relatives. However, sweet potato is an asexually propagated, cross-incompatible plant with morning glory the only known natural hexaploid in its family. Thus, chances of cross-pollination of transgenic sweet potato plants with wild plants are very remote. Sweet potato could be considered as an excellent novel source of natural health-promoting compounds, such as β -carotene

and anthocyanins, for the functional food market, also, the high concentration of anthocyanin and β -carotene in sweet potato, combined with the high stability of the color extract make it a promising and healthier alternative to synthetic coloring agents in food systems (Bovell-Benjamin, 2007). Development of insect-resistant transgenic cauliflower plants expressing the trypsin inhibitor gene isolated from local sweet potato was accomplished by Ding *et al.* (1998). Starch and flour processing from sweet potato can create new economic and employment activities for farmers and rural households, and can add nutritional value to food systems.

Besides, recent advances and availability of cutting-edge technology for the expression or manipulation of multiple genes in plants is still difficult to practice. Although a small proportion of commercial genetically modified (GM) crops present “stacked” or “pyramided” traits, only a handful products have been developed by introducing three or more novel genes (Haplin, 2005). A number of conventional and more novel techniques already exist for the stacking of genes and/or traits in transgenic plants. No single method is, as yet, ideal and individual methods may suit some purposes more than others.

In the postgenomic era, we have gained extraordinary information and understanding of various metabolic pathways and pinpointing the individual genes involved in a specific response or trait. This will provide foundation for developing resilient crops with potential of multiple stress tolerance, enhanced nutritive value, medicinal and nutraceutical importance.

It is quite often seen that transgenics with the potential of sustainable production capabilities fail in enabling a fully sustainable agriculture. Genetic traits that have a higher potential for promoting a sustainable agriculture have been precluded from development for a number of reasons. These include the lack of Environmental Protection Agency (EPA) and United States Department of Agriculture (USDA) regulatory policies that explicitly promote sustainable traits; the structure of the agricultural biotechnology industry, which is dominated by agricultural chemical companies; and patent law and industry policies that prohibits farm households from saving transgenic seed and tailoring transgenic crops to their local environmental

conditions—ecological, social, and economic (Hubbell and Welsh, 1998). There is need to develop sustainable system for potential transgenic technologies through regulatory approvals assuring the public about safety and stability of genetically modified products. The increasing world population and changes in consumption patterns may necessitate significant agricultural intensification in the next 50 years (Khush, 2001; Tilman *et al.*, 2001). Unless crop yield is improved and release of fertilizers and pesticides from crop lands is reduced, such intensification could aggravate contamination and perturbation of managed and natural ecosystems, ultimately harming biodiversity and public health (Pimentel and Raven, 2000; Khush, 2001; Tilman *et al.*, 2001; Huang *et al.*, 2002). It seems highly inevitable that future agricultural and, ultimately, also natural ecosystems will be challenged by the large-scale introduction of entirely novel genes and gene products in new combinations at high frequencies all of which will have unknown impacts on their associated complex of nontarget organisms, i.e., all organisms that are not targeted by the insecticidal protein. In times of severe global decline of biodiversity, proactive precaution is necessary and careful consideration of the likely expected effects of transgenic plants on biodiversity of plants and insects is mandatory.

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Cassava

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1. INTRODUCTION

1.1 Biology of Cassava

Cassava (*Manihot esculenta* Crantz) is a member of the dicotyledonous family Euphorbiaceae (Alves, 2002). The genus *Manihot* has been reported to have about 100 species of which *M. esculenta* is the only agronomically important species. Domesticated cassava is believed to have resulted from an interspecific cross of two wild species and to have occurred in southwestern Brazil (Olsen and Schaal, 1999). Genetically, cassava is an allotetraploid. The genome size is approximately 760 Mb (mega bases), distributed on 18 chromosomes with a GC content of 43.7%.

1.1.1 Plant architecture

Cassava grows between 1 and 4 m in height (Figure 1a). The branching pattern is characterized by sympodial growth wherein the main stem is typically divided into two to four branches with multiple subbranches. Cassava leaves are lobed with palmate veins. The number of leaf lobes ranges from three to nine. Leaves produced at the time of flowering are reduced in size and lobe number. The leaves have an alternate arrangement with a 2/5 phyllotaxy. Mature leaves are glabrous and are surrounded by two stipules at the base. The adaxial leaf surface is characterized by a

waxy epidermis that gives it a shiny appearance. The stomata are located mainly on the lower leaf surface (Cerqueira, 1989). Only 2% of the 1500 cultivars studied had stomata on their adaxial surface (El-Sharkawy and Cock, 1987). Stomata are functional on both sides of the leaf, but those on the upper surface of the leaf are larger (Cerqueira, 1989).

When present, flowers are typically located at the junctures between branches. This developmental series has been termed reproductive flowering (Alves, 2002). Cassava is monoecious producing both male (staminate) and female (pistillate) flowers on one plant. The staminate flower is located on the lower part of the branches opposed to the pistillate flower on the upper part. The pistillate flowers are more numerous than the staminate flowers. The female flowers open 1–2 weeks before the male flowers on the same inflorescence. The plants are, therefore, prone to cross-pollination, which might explain the high level of heterozygosity in cassava (Alves, 2002). The flowers lack a corolla or calyx but have a perianth with five sepals of varying color (yellow, red, purple) depending on cultivar. The male flower is about half the size of the female and has 10 stamens (Alves, 2002). The pollen is yellow or orange and ranges in size from 122 to 148 µm (Ghosh *et al.*, 1988). The female flower has a 10-lobed basal disk with a tricarpeal ovary having six ridges. The three locules each contain one ovule, which matures into carunculate seeds. The seed coat is smooth and dark brown mottled with gray.



Figure 1 (a) Cassava plants growing in the field, (b) cassava root freshly harvested from the field [Source: IITA website, <http://www.iita.org>]

Little is known about the control of flowering in cassava (Alves, 2002). Many cultivars are known not to flower. Flowering can start 6 weeks after planting but the time it takes to flower is a function of the cultivar and the environment. Moderate temperatures (approximately 24 °C) are known to be conducive for flower initiation. Photoperiod also is known to affect flowering. Flower initiation occurs best at photoperiods above 13.5 h (Keating *et al.*, 1982).

The main carbohydrate storage organ of cassava is the root and each plant may have multiple

storage roots (Figure 1b). This pattern of rooting is characteristic of crops that are propagated by stem cuttings. Adventitious roots arise from the base of the cut surface or from subterranean buds. Some roots develop into storage roots while others will remain fibrous and serve to supply water and nutrients to the plant. The cassava storage root is not anatomically a tuber or stem due to the organization of the phloem and xylem (Wheatley and Chuzel, 1993). The fully developed cassava storage root can be subdivided into three distinct sections: the periderm (bark), peel, and

the parenchyma. The parenchyma is the edible part, which consists mainly of starch and makes up about 85% of the total root mass. The parenchyma is interspersed with xylary tissues, however, most of these are in the central core (Wheatley and Chuzel, 1993). The peel is made up of sclerenchyma, cortical parenchyma, and phloem and accounts for approximately 11–20% of the root weight (Barrios and Bressani, 1967). The periderm comprises about 3% of the total root mass and is a thin layer that can be readily removed from the exterior of the root. The size and shape of roots is contingent on the cultivar and environmental factors (Wheatley and Chuzel, 1993).

1.1.2 Physiology and agronomy

Cassava has many agronomic traits that are desirable for high-yielding crops. For a C3 plant it has an unusually high photosynthetic rate ($43 \mu\text{M m}^{-2} \text{s}^{-1}$) as well as a broad temperature range (20–45 °C) for carbon assimilation (Edwards *et al.*, 1990; Angelov *et al.*, 1993). In addition, it does not light saturate at high irradiance levels, the leaves maintain good stomatal control even when drought stressed, and it has a chlorenchymatous bundle sheath cell layer; traits similar to those of high-photosynthetic C4 plants (Edwards *et al.*, 1990; Angelov *et al.*, 1993). Significantly, cassava has the highest rate of sucrose production of any plant measured (Angelov *et al.*, 1993). Forty-five percent of the carbon fixed is converted into sucrose in less than 1 min. This rate is sixfold greater than for C4 species such as *Flaveria*. In addition, the high rate of sucrose synthesis does not vary with respect to leaf age, light intensity, or temperature (between 21 °C and 32 °C).

The distribution of dry matter in cassava is dependent on the age of the plant. During the first 60–75 days following planting dry matter accumulation is mainly in the leaves. However, after the fourth month, more dry matter is accumulated in the storage roots than the rest of the plant (Howeler and Cadavid, 1983; Tavora *et al.*, 1995). At 12 months of growth, the dry matter is present mainly in the storage roots followed by stem and leaves. The time for the maximal accumulation of dry matter depends on the cultivar and the environment. The growth

phase in high latitudes is 4–6 months (Lorenzi, 1978) and 7 months for high altitudes (Oelsligle, 1975). In the tropics, where growth is faster, the maximum rate of dry matter accumulation is between 3- and 5-month age (Howeler and Cadavid, 1983).

All cassava organs apart from seeds contain cyanogenic glycosides. Cultivars with less than 100 mg CN equivalents per kilogram fresh weight (kgfw) in their roots are called “sweet” while cultivars with 100–500 mg CN equivalents per kilogram are classified as “bitter” cassava (Wheatley *et al.*, 1993). The most prevalent cyanogenic glycoside is linamarin (95%) with lesser amounts of lotaustralin. The total amount of cyanogenic glycoside is dependent on cultivar, cultural practice, environmental conditions, and plant age (McMahon *et al.*, 1995). Linamarin is synthesized in the leaf and transported to the roots (Wheatley and Chuzel, 1993; Siritunga and Sayre, 2003). The hydrolysis of linamarin and breakdown of acetone cyanohydrin by hydroxynitrile lyase (HNL) or elevated pH (>5.0) leads to the release of HCN, which is poisonous (Cooke and Coursey, 1981). Compartmentalization of linamarase in cell walls and laticifers and linamarin in the vacuole guards against the release of HCN in intact plant tissues (McMahon *et al.*, 1995). Processing of cassava roots brings enzyme and substrate together leading to the hydrolysis of linamarin and breakdown of acetone cyanohydrin to produce cyanide (Mkpong *et al.*, 1990; Wheatley and Chuzel, 1993; McMahon *et al.*, 1995; White *et al.*, 1998).

Postharvest deterioration is a major constraint for marketing fresh cassava roots. Roots are very perishable and deteriorate within 24–72 h after harvest. This deterioration is manifested by the production of phenolic compounds whose polymerization leads to the discoloration of the roots (Wheatley and Chuzel, 1993). This process is oxygen dependent and can be inhibited by placing the roots in an anaerobic environment (Wheatley and Chuzel, 1993; Reilly *et al.*, 2007). The earliest events are mediated by the production of reactive oxygen species (ROS), which is followed by expression of genes involved in ROS metabolism (Reilly *et al.*, 2007). Secondary deterioration is associated with microbial infection of damaged tissues (Wheatley and Chuzel, 1993).

1.2 How Cassava Came to Africa

Botanical, genetic, and archeological evidence supports the South American origin of cassava, pointing to the Amazon region as the center of cassava domestication (Olsen and Schaal, 1999; Hillocks, 2002). Olsen and Schaal (1999) took advantage of the high variations in the noncoding sequence of the glyceraldehydes 3-phosphate dehydrogenase (*G3pdh*) gene to determine the center of origin and genetic diversity of cassava. They determined that cassava was domesticated from wild *M. esculenta* populations along the southern border of Brazil near Paraguay. Portuguese navigators took cassava with them from Brazil to West Africa in the 16th century (Jones, 1959) and then later to East Africa (Jennings, 1976). Even though cassava was grown in Fernando Po in the Gulf of Benin and around the Congo River in the 16th century, cassava's dispersal into West Africa did not take place until the 20th century (Hillocks, 2002). Cassava was initially grown mainly in the coastal areas. The inland spread of cassava cultivation was by African traders who were drawn to cassava for its fabled characteristic of providing security against famine. Cassava is now grown in most African countries especially south of the Sahara desert. Realizing the importance of cassava, the International Institute of Tropical Agriculture (IITA) was established with its headquarters in Ibadan, Nigeria in 1972 under the guidance of the Consultative Group on International Agricultural Research (CGIAR) to oversee the development of the crop in Africa. Together with Centro Internacional de Agricultura Tropical (CIAT) in Colombia, IITA has the global mandate for cassava improvement (Hillocks, 2002).

1.3 Cassava Production

Although cassava is native to the Amazon region, Africa now produces more cassava than the rest of the world combined. Africa's largest producers of cassava are Nigeria (35%), Democratic Republic of Congo (19%), Ghana (8%), Tanzania (7%), and Mozambique (6%). The top producers in Africa have increased their production greatly in the past two decades especially Nigeria (22–35%) and

Ghana (4–8%) (IITA, 1997). Other countries are on the decline.

The increases in production of cassava have come as a result of increase in the area of land cultivated as opposed to increase in yield per hectare. Average yields have increased by 33% in the past two decades while the cultivated land for cassava increased by 70% during the same time period (IITA, 1997). In Africa, cassava yield declined 1.2% to 0.6% in the past decade. Only Ghana has increased yield from 1990 to 1995. According to the survey conducted by the Collaborative Study of Cassava in Africa (COSCA), funded by Rockefeller Foundation, the main reason for the increase in cultivation is response to famine, hunger, and drought. This confirms the value of cassava as a security crop (Hillocks, 2002).

1.4 Economic Importance

1.4.1 Cassava starch

Starch is the most important form of carbon reserve in plants with respect to the amount produced, its distribution among different plant species, and its commercial importance (Martin and Smith, 1995). Starch comprises different polymers of glucose arranged in a three-dimensional, semicrystalline structure called the starch granule. Starch is composed of two types of glucan polymers: amylose and amylopectin (Martin and Smith, 1995). Amylose is made up of linear chains of α (1,4)-linked glucose residues, which are typically about 1000 residues long. It may have one α (1,6)-linkage per 1000 residue and makes up about 30% of total starch (Okita, 1992; Martin and Smith, 1995; Smith *et al.*, 1995). On the other hand, amylopectin consists of highly branched glucan chains and accounts for about 70% of total starch in most plants. Chains of about 20 α (1,4)-linked glucose moieties are joined by α (1,6)-linkages to other branches. Some branches of amylopectin are not substituted on the number 6 position and are called A chains. These are α (1,6)-linked to inner branches called B chains, which may be branched at one or more points. A single chain per amylopectin molecule has a free reducing end, the C chain (Kainuma, 1988; Smith and Martin, 1993; Martin and Smith, 1995).

The phosphorylation of starch is almost universal in plants (Blennow *et al.*, 2002). Glycogen, which is the analog of starch in animals, also contains phosphate groups, an indication that phosphorylation is a requirement for storage α -glucan metabolism (Vikso-Nielsen *et al.*, 2002). However, the level of starch phosphorylation varies among plants. Phosphorylation in cereal starch is low (less than 1 nM Glc6P/mg starch). Cassava starch has 2.5 nM Glc6P/mg starch, a low level of phosphorylation. In contrast, potato starch is highly phosphorylated (8–33 nM Glc6P/mg starch) (Blennow *et al.*, 2000, 2002). The phosphate groups are bound mainly to the amylopectin moiety of starch as monoesters at the C-6 and C-3 positions of glucose units (Bay-Smidt *et al.*, 1994; Blennow *et al.*, 2002). The phosphorylation of starch serves to increase the hydration capacity following gelatinization. Phosphorylation of starch prevents its crystallization and affects the viscosity of the final product. Engineering crop starch for higher phosphate content is important because it reduces expensive and environmentally hazardous chemical processing by the industries (Blennow *et al.*, 2002).

Cassava starch is stored in the amyloplasts of thickened roots. The starch content of cassava roots ranges from 74% to 85% of the dry weight (Rickard *et al.*, 1991; Munyikwa *et al.*, 1997). Cassava starch granules are round, flat on one side, and contain a conical pit, which extends to well-defined, eccentric helium, ranging in size from 5 to 40 μ m (Moorthy, 1994; Munyikwa *et al.*, 1997). An 18-month study of the growth of cassava granules indicated that growth continues up to the 6th month. No further growth was observed after 6 months (Moorthy and Ramanujan, 1986).

The amylose content of cassava starch is 15–26% (Ketiku and Oyenuga, 1972; Kawabata *et al.*, 1984). Starch is classified into three types based on different x-ray diffraction patterns, namely, A, B, and C. Cassava starch comprises mainly the A-type pattern that is characteristic of cereal starches (Guiltbot and Mercier, 1985). This A-type pattern is characterized by a closely packed double helices compared to the more open B-type arrangement. Raw cassava starch contains 0.08–1.54% crude fat, 0.03–0.6% crude protein, and 0.75–4% phosphorus (Soni *et al.*, 1985; Munyikwa *et al.*, 1997).

1.4.2 Cassava foods

Cassava serves as the main staple food for more than 500 million people in the tropical and subtropical regions of the world (Balagopalan, 2002). It also contributes significantly to the livelihood of these people. Cassava is eaten raw in Africa and other parts of the world where it is grown, after removing the skin. However, the cultivars that are eaten raw are the “sweet cultivars”, which have low cyanogenic glycoside content. The cultivars that have high cyanogenic glycoside content are processed and cooked before consumption (Balagopalan, 2002).

Cassava is used as food in various ways across the regions of the world. In South America, cassava tubers are grated and pounded into pulp. The dewatered pulp is often shaped into pies and cakes, wrapped in leaves, and baked on fire. In West Africa, cassava roots are fermented in a pot for 4–7 days, boiled, and pounded into *fufu* and eaten with vegetable soup supplemented with fish and meat (Lancaster *et al.*, 1982).

In Liberia, cassava is made into *dumby*, which is prepared by placing boiled cassava roots in mortar followed by pounding. Following pounding, the *dumby* is cut into pieces and put in soup supplemented with vegetables or meat. It is then swallowed whole. Cassava is also made into *farina* in South America and West Indies. *Farina* is prepared by removing the skin of the cassava root and grating. The mash is then depulped, sieved, and roasted in a slow fire. It could then be stored for several months and can be eaten as a cereal and in combination with other foods (Omole, 1977).

Cassava macaroni is prepared by blending cassava flour, groundnut flour, and wheat semolina in the ratio 60:12:15. It is enriched with 12% protein. The food is used to feed children because of its high protein content (Balagopalan, 2002).

In Ghana, Nigeria, Guinea, Togo, and Benin, *gari* is one of the most important cassava foods. Cassava roots are skinned, grated, and dewatered in sacks made from jute fibers and allowed to ferment for 2–4 days. This is followed by sieving to remove fibers from the roots. It is then fried in shallow iron pans and stirred continuously until it becomes dry and crisp. Palm oil is added sometimes during frying to prevent burning and also as a source of carotene in the food. *Gari* is prepared with boiling water to make a thick paste.

Cassava is also used in preparing fast food. It is used in preparing wafers, and made into fried chips, cakes, and doughnuts (Balagopalan, 2002).

Cassava is also important in animal feeds. Various parts of the plant such as leaves, stem, and roots are used to feed animals. The high energy value of cassava makes it a good source of carbohydrate in animal diets (Omole, 1977). However, roots have low protein content. This shortcoming is often overcome by supplementing it with soya, which is rich in protein.

Fresh roots are used in feeding farm animals such as cattle, goats, and sheep in the developing countries. Sometimes, the roots are boiled before feeding animals. Feeding livestock with fresh roots might cause cyanide toxicity depending on the level of cyanogenic glucosides in the roots. It has been reported that replacement of cereals with cassava up to 50–100% did not affect the milk quantity and quality in dairy animals (Mathur *et al.*, 1969). In some cases, higher milk yield has been reported up to 19.5% as a result of increased energy from cassava (Balagopalan, 2002).

Cassava leaves are used as forage in many developing countries especially in the dry season when other feeds are scarce. There is some opposition to the use of cassava leaves as forage, however, for fear of cyanogenic poisoning of the livestock (Balagopalan, 2002).

1.4.3 Cassava and industry

Cassava is an important commodity in industry mainly because of its starch, which is used in the production of various items. Cassava starch is used in the production of adhesives and a variety of other compounds (Balagopalan, 2002). Gums are made from cassava starch by heat treatment. Other varieties of adhesives are made from cassava starch by the addition of chemicals (Cock, 1985; Balagopalan, 2002).

Cassava gum, made without additives, is produced by cooking cassava starch with water. Preservatives may be added later if needed. This type of gum is used in bill pasting, making bags, and in the tobacco industry. Copper sulfate may be added to the paste to prevent microbial damage. Cassava starch is used for paste production because of its cohesiveness and clarity. Cassava starch is preferred for food packaging over other

types of starch because of its bland taste (Cock, 1985; Balagopalan, 2002).

The cassava gums containing chemicals are made by the addition of calcium chloride, magnesium chloride, borax, urea, and carboxymethyl cellulose during the process of gelatinization. The chemicals increase viscosity, flow ability and are used for controlling hydration. Such gums are used in the lamination of papers, production of wallpapers, and pasting labels (Abraham, 1996; Balagopalan, 2002).

Cassava starch is also used in production of dextrans. An aqueous solution of dextrin is used for bonding similar and dissimilar surfaces. Dextrans are not as strong adhesives as starch films but are preferred to starch because they can be used at higher concentrations than starch, which accelerates drying. Dextrans are used as envelope gums, bottle-labeling adhesives, postage stamps adhesives, in making cardboard boxes, and photographic mounting materials (Cock, 1985; Trim *et al.*, 1996; Balagopalan, 2002).

Glucose and dextrose, products of starch hydrolysis, are liquefied into sugar syrups. The syrup can be used in making confection and in the pharmaceutical industry. Cassava starch is also used in making fructose syrup and fructose crystals, which are used in the substitution of sucrose, glucose, and synthetic sweeteners. Fructose is 1.7 times sweeter than sucrose and 4 times sweeter than glucose (Abraham, 1996). Cassava starch is also used in the manufacture of maltodextrin, which substitutes for glucose as a sweetener. It is also used as a thickening agent (Balagopalan *et al.*, 1988; Balagopalan, 2002). Cassava starch oxidized with hypochlorite or chlorine is useful in the paper industry because of its low viscosity, film strength, and clarity in making glossy papers. These starches are also used in the textile industry for sizing warps of cotton and spun rayons and for laundry finishes. Cationic starches made by treating starch with amino, imino, ammonium, sulfonium, or phosphonium groups are used in the paper industry to provide glaze and strength to paper (Cock, 1985; Abraham, 1996; Trim *et al.*, 1996).

Cassava starch is used in blending synthetic polymers to give it biodegradable characteristics. Cassava starch, because of its low swelling and gelatinization temperature, is easily saccharified

to simple sugars. These simple sugars are used in the production of sugar alcohols such as sorbitol, mannitol, and maltol. Sorbitol can be used to replace glycerin in the production of toothpaste, cosmetics, and oil-based paints. It is also used as a raw material in the production of ascorbic acid (Ren, 1996). Mannitol has a wide range of applications in medicine as a dehydrating agent in blood vessel diastolic preparations. Mannitol is also used in the treatment of cerebral thrombosis and other circulating disorders and in the production of polyester, polyethylene, and solid foam plastics. Maltol, a food flavoring agent, is used in the confectionery industry (Ren, 1996).

2. CASSAVA BIOTECHNOLOGY

2.1 Plant Tissue Culture and Regeneration

Cassava, a very important crop in the tropics, is very difficult to breed by classical genetics. It takes 10 years to generate a new cultivar by conventional breeding (Alves, 2002). This is in large part due to inconsistent flowering coupled by poor seed set. Cassava is also heterozygous and clonally propagated (Jennings and Iglesias, 2002). In this circumstance, genetic engineering becomes attractive for substituting plant breeding for cassava improvement. One of the requirements for the generation of transgenic cassava is an efficient and reproducible plant regeneration system. Nearly all parts of cassava plant have been used to establish *in vitro* cultures (Roca, 1984).

Somatic embryogenesis is the method of choice for cassava regeneration. Explants have been mostly restricted to young leaves and shoot meristems. Somatic embryos have been induced from cassava young leaf lobes and cotyledons. Plants have also been regenerated from these tissues (Stamp and Henshaw, 1982, 1986, 1987a, b; Stamp, 1987; Szabados *et al.*, 1987; Mathews *et al.*, 1993; Raemakers *et al.*, 1993; Taylor and Henshaw, 1993; Konan *et al.*, 1994). Even though it is now possible, in principle, to regenerate cassava plants from a number of cultivars, the efficiency of regeneration achieved so far is low. In their best experience, Szabados *et al.* (1987) obtained an average of 1.15 plantlets

per primary explant. Stamp and Henshaw (1987a) studied the regeneration of somatic embryos derived from cotyledon explants and found that only 14% of the embryos that they started with eventually regenerated into plants.

Another strategy for plant regeneration is organogenesis. Cassava organogenesis is independent of callus formation and auxin treatment. Unlike most plants, there is no intermediary stage of callus production during organogenesis. Instead, shoot primordia are induced directly from cotyledons of somatic embryos and young leaf lobes on Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 6-benzyladenine (BA) (Li *et al.*, 1996; Mussio *et al.*, 1998).

Regeneration of cassava has also been reported using meristem culture techniques (Kartha *et al.*, 1974; Bajaj, 1983). Meristem culture could provide an alternative means to generate explants for transformation studies, but it is difficult to obtain adequate numbers of meristems for transformation experiments. Apart from serving as explants for cassava transformation, meristem culture serves a more important role in providing disease-free plants, especially for plants infected with viruses as meristems are generally devoid of viruses. Meristems, therefore, serve as a means for viral decontamination as well as in multiplication of cassava.

The isolation and culture of cassava protoplasts have been performed (Mabanza and Jonard, 1983; Mabanza, 1984; Szabados *et al.*, 1987; Villegas *et al.*, 1988; Nzoghe, 1989). Prior to the report of Sofiari (1996), only one successful regeneration from protoplasts has been reported (Shahin and Shepard, 1980) but this has not been reproduced.

Currently, the most reliable method for cassava regeneration is through the young leaf lobes (and perhaps, through suspension cultures, which is more time consuming and requires more dexterity). Somatic embryos have been obtained from a number of cultivars, but there is still the need for optimization of the regeneration procedures. Results from the field tests of cassava plants derived from embryogenesis showed that somaclonal variation is not a problem in cassava. This makes plant regeneration via somatic embryogenesis a reliable tool for genetic improvement of cassava.

2.2 Transformation

Cassava transformation has been reported via *Agrobacterium*-mediated transformation (Li *et al.*, 1996; Arias-Garzon, 1997; Sarria *et al.*, 2000; Zhang *et al.*, 2000b; Siritunga and Sayre, 2003; Siritunga *et al.*, 2004) and by particle bombardment (Raemakers *et al.*, 1996; Schopke *et al.*, 1996; Zhang *et al.*, 2000a). *Agrobacterium*-mediated transformation is favored over particle bombardment because of the lower number of integrated sequences and greater stability of transformants. *Agrobacterium*-mediated transformation usually results in the transfer of 1–3 copy(ies) of the transgene to plants compared to the particle gun, which has been known to deliver up to 12 copies of a transgene. Importantly, the introduction of multiple transgene copies can lead to gene silencing (Alien *et al.*, 1993; Matzke and Matzke, 1995; Meyer and Saedler, 1996).

The first published attempt to transform cassava cells was that of Calderon (1988). He inoculated leaf pieces, stem pieces, and embryogenic callus with *Agrobacterium*-containing plasmids with the coding sequences for neomycin phosphotransferase II (*nptII*), phosphinotricin acetyltransferase (*bar*), or β -glucuronidase (*uidA*). He isolated callus lines expressing the phenotype expected from the transformation tissue.

Subsequent reports of transient gene expression followed (Franché *et al.*, 1991; Arias-Garzon and Sayre, 1993; Luong *et al.*, 1995). Arias-Garzon and Sayre (1993) observed a tissue-specific inhibition of transient gene expression in cassava associated with elevated DNase activity. Using a cauliflower mosaic virus (CaMV) 35S promoter, they observed several hundred localized regions that expressed β -glucuronidase activity in cassava leaves but virtually none in the roots. This shows that different parts of cassava plant respond differentially to transient transformation treatments. Luong *et al.* (1995) reported transient expression of the *uidA* gene in cassava tissues by electroporation. However, in 1996, two reports of stable cassava transformation came out simultaneously; one using *Agrobacterium*-mediated transformation (Li *et al.*, 1996) and the other microparticle bombardment (Schopke *et al.*, 1996).

The earliest reports of cassava transformation were only with marker genes of little agronomic importance (Li *et al.*, 1996; Schopke *et al.*, 1996). Additional reports of genetic transformation of cassava followed (Zhang *et al.*, 2000a, b). Sarria *et al.* (2000) reported the transformation of cassava with *bar* gene which confers resistance to the herbicide Basta. Transformed plants tolerated 200 mg l⁻¹ Basta, which proved toxic to the untransformed cassava plants.

In 2003, Siritunga and coworkers reported the transformation of cassava with the gene encoding HNL driven by the 35S promoter. HNL catalyzes the breakdown of acetone cyanohydrin, a product of linamarin hydrolysis, to acetone and cyanide (Siritunga *et al.*, 2004). This was an important development in cassava processing, because cassava roots contain little HNL (0–6%) relative to that in leaves. The rate of acetone cyanohydrin breakdown in the transformed plants was 41–75% faster than wild-type cassava (Siritunga *et al.*, 2004). These plants are advantageous for detoxification and processing of cassava food products especially for the poor people of the African continent who are perennially dependent on cassava for food.

2.2.1 Transformed cassava plants

There are more than 1500 cultivars of cassava worldwide (Alves, 2002). The high level of heterozygosity could be a function of cross-pollination in cassava resulting from the differences in timing of male and female flowers. The heterozygosity in cultivars is also reflected in its genetic transformability. Not all cassava cultivars are transformable to date. Only a few cultivars have been successfully transformed as furnished below:

- TMS 60444 (Schopke *et al.*, 1996; Gonzalez *et al.*, 1998; Zhang *et al.*, 2000a, b).
- TMS 71173 (Ihemere *et al.*, 2006).
- MCol 2215 (Arias-Garzon, 1997; White *et al.*, 1998; Siritunga and Sayre, 2003).
- MCol 122 (Zhang *et al.*, 2000a, b).
- MPer183 (Sarria *et al.*, 2000).

Regardless of the paucity of transformation success among cultivars, not all transformable

cassava cultivars respond to the available methods of transformation. Only TMS 60444 has been successfully transformed using particle bombardment of friable embryogenic callus (FEC) (Raemakers *et al.*, 1996; Gonzalez *et al.*, 1998; Zhang *et al.*, 2000a). Mcol 2215 (White *et al.*, 1998; Siritunga and Sayre, 2003; Siritunga *et al.*, 2004) and TMS 71173 (Ihemere *et al.*, 2006) are the most responsive to *Agrobacterium*-mediated transformation of cassava somatic embryo explants. Success with other cultivars is limited (Fregene and Puonti-Kaerlas, 2002).

2.2.2 Methods of cassava transformation

Cassava has been transformed using various types of tissues and explants as starting material including somatic embryo cotyledon (White *et al.*, 1998; Zhang *et al.*, 2000b; Siritunga and Sayre, 2003), young leaf lobes (White *et al.*, 1998), and FEC (Raemakers *et al.*, 1996; Gonzalez *et al.*, 1998; Zhang *et al.*, 2000a) (Table 1). Embryogenesis has been reported from FECs, somatic embryo cotyledons, and young leaf lobes. Direct organogenesis has been reported

Table 1 Methods used in cassava transformation^(a)

Target tissue	Regeneration mode	Gene transfer system	Selection	Transgenic tissue	Analysis	References
Somatic embryos	Somatic embryogenesis	Electroporation	–	Chimeric embryos	Transient GUS expression	Luong <i>et al.</i> , 1995
Somatic cotyledons	Shoot organogenesis	<i>Agrobacterium</i>	Hygromycin, geneticin	Transgenic plants	Southern, northern	Li <i>et al.</i> , 1996
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Paromomycin	Transgenic plants	Southern	Schopke <i>et al.</i> , 1996
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Luciferase	Transgenic plants	Southern	Raemakers <i>et al.</i> , 1996; Schopke <i>et al.</i> , 1997
Somatic cotyledon	Somatic embryogenesis	<i>Agrobacterium</i>	Paromomycin	Transgenic plants	RT-PCR, western	Arias-Garzon, 1997
Young leaf lobes	Somatic embryogenesis	<i>Agrobacterium</i>	Paromomycin	Transgenic plants	RT-PCR, western	Arias-Garzon, 1997
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	–	Chimeric suspension	Transient gene expression	Munyikwa <i>et al.</i> , 1998
Embryogenic suspension	Somatic embryogenesis	Particle bombardment	Luciferase, phosphotricin	Transgenic plants	Southern, northern	Gonzalez <i>et al.</i> , 1998
Embryogenic suspension	Somatic embryogenesis	<i>Agrobacterium</i>	Paromomycin	Transgenic plants	Southern	Sarria <i>et al.</i> , 2000
Somatic cotyledon	Somatic embryogenesis	<i>Agrobacterium</i>	Basta	Transgenic plants	Southern	Zhang <i>et al.</i> , 2000a
Somatic cotyledon	Shoot organogenesis	Particle bombardment	Hygromycin	Transgenic plants	Southern, RT-PCR	Zhang and Puonti-Kaerlas, 2002
Somatic cotyledon	Shoot organogenesis	Particle bombardment	Mannose, hygromycin	Transgenic plants	Southern, northern	Zhang and Puonti-Kaerlas, 2002
Embryogenic suspension	Somatic embryogenesis	<i>Agrobacterium</i>	Mannose, hygromycin	Transgenic plants	RT-PCR	Zhang <i>et al.</i> , 2000b
Embryogenic suspension	Shoot organogenesis, somatic embryogenesis	<i>Agrobacterium</i>	Mannose, hygromycin	Transgenic plants	Southern, northern, RT-PCR	Zhang and Puonti-Kaerlas, 2002
Somatic cotyledon	Somatic embryogenesis	<i>Agrobacterium</i>	Paromomycin	Transgenic plants	Southern, RT-PCR	Siritunga and Sayre, 2003; Siritunga <i>et al.</i> , 2004; Siritunga and Sayre, 2004

^(a)Modified from Fregene and Puonti-Kaerlas (2002). © CABI Publishing

Table 2 Comparison of the three transformation systems; the different methods were scored on an 1–5 scale, with 5 being the best score

	FEC	Oganogenesis	GSE
Genotype capability ^(a)	4	1	2
Chimerism	3	2	3
Somaclonal variation	2	4	4
Transferability to other labs	2	2	4
Efficiency of transformation	3	3	3
Labor input	1 (High)	1 (High)	1 (High)
Time for product	2 (5–7 months)	4 (2 months)	3 (3–6 months)
Potential for scale-up	4	3	4
Scope for improvement	Yes	Yes	Yes
Marker use flexibility	3	3	3
Regeneration potential	2	4	5

^(a)Genotypes used: FEC (friable embryogenic calli)—TMS60444, Adera4, Mcol 1505, B. Rouge, Mtai5, R60 (Mtai 8), CM3306-4; Organo (organogenesis)—Mcol22, TMS60444, GSE—Mcol2215, MS71173, Mper183, Mcol22; plant regeneration: FEC—50% of the genotypes tested can be transformed; Organo—lower success on transformation of new varieties, GSE (germinating somatic embryo)—50% of the genotypes tested can be transformed

with somatic embryo cotyledons and young leaf lobes (Zhang *et al.*, 2000b). The efficiency of transformation of the different explants and methods was scored by scientists in the Advanced Cassava Transformation Group who met at CIAT in Palmira, Colombia (June 9–10, 2003). The somatic embryogenesis method using embryo cotyledons as explants was chosen to be the best method (Table 2) based on efficiency of regeneration, incidence of chimeras, speed of regeneration of transformed plants, and simplicity of transformation technique. The FEC system produced the largest numbers of transformants.

2.2.3 Gene promoters used in cassava transformation

Gene promoters are very important in the transformation process because they drive the tissue- and temporal-specific expression of genes. Without different promoters, controlled-gene expression is not possible. Some of the promoters used to date are listed in Table 3. Only a few of the promoters are root/tuber-specific, namely, Patatin and Potato GBSS.

The need for selectable markers in transformation arises from the need to distinguish between transformed and nontransformed tissues and plants. In cassava transformation, the selection of transformed tissues has been done using kanamycin and paromomycin (enabled by the *nptII* gene), hygromycin (enabled by *hpt* gene),

basta (enabled by *bar* gene), mannose (enabled by phosphomannose isomerase), phosphinotricin, and geneticin (see Table 2).

2.2.4 Problems with cassava transformation

Notwithstanding the successes recorded in cassava transformation, there are still many problems associated with cassava transformation. First and foremost, cassava has a low efficiency of transformation compared to other crops. The best recorded transformation efficiency for cassava is in the range of 3–5%. Most other crops have transformation efficiencies greater than 10% (Raemakers *et al.*, 1997). This makes it difficult to work with cassava. Added to the low efficiency of transformation is the difficulty to characterize transformed cassava plants molecularly. Southern blot analysis of cassava is very difficult. Part of the problem is that cassava genome is large. It has been reported that gene insertions from independently transformed cassava plants may have similar restriction patterns making it difficult to identify independent transformed lines (Siritunga, 2002).

Due to the low efficiency of cassava transformation, scientists working on cassava have come up with different tissues for cassava transformation. The most prolific regeneration system is the FEC, which has been reported to generate more transformed cassava (Raemakers *et al.*, 1996; Gonzalez *et al.*, 1998). However, the production

Table 3 Gene promoters that have been used in cassava transformation

Promoter	Source plant/organism	Size (kb)	Tissue specificity	References
CaMV 35S	CaMV	0.8	Constitutive	White <i>et al.</i> , 1998; Arias-Garzon and Sayre, 1993; Arias-Garzon, 1997; Siritunga <i>et al.</i> , 2004
NOS	Agrobacteria	0.26	Constitutive	Arias-Garzon and Sayre, 1993; Arias-Garzon, 1997
PTR-1, PTR-2	<i>Methanococcus jannaschii</i>	2.5	Constitutive	Ouhammouch and Geiduschek, 2001
P15-1.5	Cassava	1.46	Strongest in root	Zhang <i>et al.</i> , 2003
P54-1.0	Cassava	1.08	Strongest in root	Zhang <i>et al.</i> , 2003
SAG-12	Tobacco	1.5	Leaf	Chang <i>et al.</i> , 2003
CAB1	<i>Arabidopsis</i>	0.4	Leaf	Siritunga and Sayre, 2003
PATATIN	Potato (<i>Solanum</i>)	1.3 kb	Root	Siritunga and Sayre, 2004; Ihemere <i>et al.</i> , 2006
GBSSI	Potato (<i>Solanum</i>)	1 kb	Root	Salehuzzaman <i>et al.</i> , 1994
ACMV-DNA1	ACMV	2.7 kb	Constitutive	Frey <i>et al.</i> , 2001

of FECs requires that the cassava embryogenic tissues stay on high-auxin ($50 \mu\text{g l}^{-1}$) media for up to 6 months compared to the 1-month requirement for somatic embryogenesis. This long exposure to high auxin concentrations can lead to somaclonal variation in cassava.

2.2.5 Genetic manipulation of cyanogenesis in transgenic cassava

Daily consumption of cassava roots as a staple diet has been associated with the presence of chronic cyanide toxicity among the poor population in the tropics. The causative agents of cyanide toxicity are two cyanogenic glucosides, linamarin (95%), and lotaustralin (5%), which are present in all parts of the plant with the exception of seeds (Conn, 1979, 1994; Balagopalan *et al.*, 1988). Although various cultivars and hybrids of cassava are available, none has been found to be acyanogenic. Attempts to reduce cyanogens through conventional breeding have also not been successful. During the past 4 years transgenic strategies have been used to engineer acyanogenic cassava plants as well as plants in which the breakdown of cyanogenic glucosides and volatilization of cyanide is accelerated in the roots (Siritunga and Sayre, 2003, 2004; Siritunga *et al.*, 2004).

The starchy cortex of fresh cassava roots may contain between 15 and 1500 mg CN equivalents/kgfw depending on the cultivar and growth

conditions (O'Brien *et al.*, 1991). Cultivars having <100 mg CN equivalents/kgfw are designated as low-cyanide cultivars while cultivars having >500 mg CN equivalents/kgfw are known as high-cyanide cultivars. Interestingly, the outer peel (2–5 mm) of cassava roots has a high cyanogen content (average = 577 mg/kgfw) regardless of the cultivar and may serve to protect the root from herbivory (Bellotti and Riss, 1994). Leaf cyanogen content (average = 446 mg CN equivalents/kgfw) is also typically high regardless of the cultivar.

During the last 10 years the physiology and biochemistry of cyanogenesis in cassava has been elucidated (reviewed in McMahon *et al.*, 1995; Andersen *et al.*, 2000; Siritunga and Sayre, 2004). The major cyanogen in cassava is linamarin (95%), which is synthesized from valine (Figure 2). Two cytochrome P450s (CYP79D1 and CYP79D2) catalyze the first-dedicated step in linamarin synthesis. The end product of the cytochrome P450-catalyzed reactions is the hydroxynitrile intermediate, acetone cyanohydrin (Andersen *et al.*, 2000). Acetone cyanohydrin is subsequently glycosylated by a UDP-glucosyl transferase to yield the stable cyanogen, linamarin, which is stored in the vacuoles (see for review McMahon *et al.*, 1995).

Cyanogenesis in cassava is initiated by tissue disruption. Rupture of the plant cell vacuole releases linamarin, which is subsequently hydrolyzed by a cell wall or laticifer-localized β -glycosidase known as linamarase (Mkpong *et al.*,

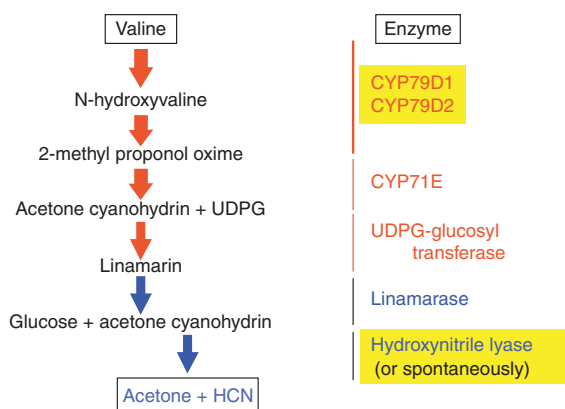


Figure 2 Linamarin synthesis and turnover pathway (high-lighted in yellow are the enzymes in the pathway that have been genetically manipulated to produce transgenic cassava with altered rates of cyanogen synthesis and breakdown)

1990; Hughes and Hughes, 1994). The products of linamarin hydrolysis are acetone cyanohydrin and glucose. Significantly, acetone cyanohydrin will spontaneously decompose to cyanide and acetone at pH >5.0 or temperature >35°C. Acetone cyanohydrin can also be broken down enzymatically by HNL. Cassava roots have much lower linamarase activities than leaves and very little or no HNL activity, presumably accounting for the accumulation of potentially toxic levels of acetone cyanohydrin in poorly processed cassava roots (White *et al.*, 1994, 1998).

Various human health disorders have been associated with the consumption of residual cyanogens from poorly processed cassava. Chronic exposure to low levels of cyanogens from poorly processed cassava has been associated with hyperthyroidism and neurological disorders including tropical ataxic neuropath (Mlingi *et al.*, 1992; Tylleskar *et al.*, 1992). In addition, the consumption of unprocessed, highly cyanogenic varieties of cassava may result in a permanent paralysis of the legs (*konzo*). The incidence of *konzo* is highest during crop failures or when less time is taken to process or remove cyanogens from cassava. Significantly, cyanide poisoning from poorly processed cassava is exacerbated by insufficient amounts of sulfur-containing amino acids (cysteine) in the diet (Tor-Agbidye *et al.*, 1999). Reduced sulfur or sulfur-containing amino acids are required for the detoxification of cyanide by the enzymes rhodanese and β -cyanoalanine

synthase. In the absence of sufficient cysteine, cyanide is converted to cyanate in the body. The brain is particularly sensitive to cyanate poisoning. The environmental conditions (e.g., drought and famine) that promote shortcut cassava-processing practices often go hand-in-hand with reduced availability of protein and/or cysteine in the diet.

In 2003 and 2004, Siritunga and Sayre reported the generation of transgenic cassava plants with reduced cyanogen potential in foods using two different strategies: (1) enhancement of HNL activity in roots to accelerate the turnover of cyanogens and cyanide volatilization during processing (Arias-Garzon and Sayre, 2000; Siritunga *et al.*, 2004) and (2) the inhibition of cyanogen production through suppression of *CYP79D1* and *CYP79D2* expression, the enzymes that catalyze the first-dedicated step in cyanogen synthesis (Figure 2) (Siritunga and Sayre, 2003, 2004).

Until recently, it had been assumed that the only cyanogen present in poorly processed cassava foods was linamarin. In 1992, however, Dr Hans Rossling and colleagues demonstrated that the major cyanogen present in poorly processed cassava roots was not linamarin but acetone cyanohydrin. In 1995, the Sayre lab characterized the distribution HNL in cassava roots, stems, and leaves (White and Sayre, 1995; White *et al.*, 1998). Their objective was to determine the biochemical basis for the unexpectedly high levels of acetone cyanohydrin in processed cassava food products. Significantly, they discovered that root HNL levels were <5% (protein basis) of those present in leaves. Thus, it was apparent that the low HNL activity in roots could contribute to the high acetone cyanohydrin levels present in poorly processed cassava roots. This observation led to the development of transgenic strategies to facilitate cyanogen elimination from processed cassava foods by overexpression of HNL in roots (Siritunga *et al.*, 2004).

Using an *Agrobacterium*-mediated transformation system, the Sayre lab introduced a cassava complementary DNA (cDNA)-encoding HNL driven by constitutive double CaMV 35S promoter into cassava. Transgenic cassava plants were verified by polymerase chain reaction (PCR) and Southern blot analysis for the integrated HNL cDNA (Siritunga *et al.*, 2004). The HNL activity of root extracts obtained from

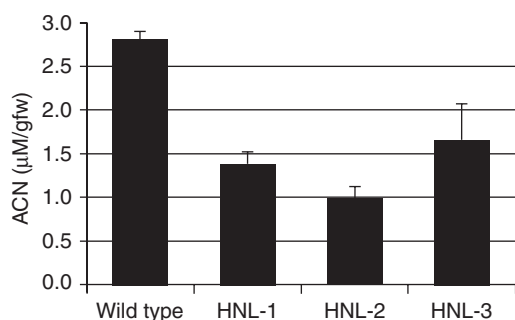


Figure 3 Acetone cyanohydrin content of 8-month-old (greenhouse-grown) wild type and HNL overexpressing transgenic roots 2-h posthomogenization

in vitro transgenic plants ranged from 0 to 0.56 mM CN/mg protein/h. These HNL activities were as high as 13-fold greater than that for wild-type plants.

Analyses of acetone cyanohydrin levels in processed roots of HNL-overexpressing plants indicated that the rate of acetone cyanohydrin turnover was more than three times faster than for wild-type plants (Figure 3). It was also demonstrated that the turnover of acetone cyanohydrin was stoichiometrically equivalent to the loss of cyanide from the processed root tissues. The presence of elevated amounts of HNL enzyme in the transgenic roots resulted in accelerated turnover of acetone cyanohydrins compared to untransformed wild-type plants. Significantly, plants overexpressing HNL had normal root linamarin levels and linamarase activity indicating that the protective benefits of cyanogenesis or herbivore deterrence should not be compromised in the transgenic plants (Siritunga *et al.*, 2004).

In 2002, Dr. Birger Möller's lab identified the genes encoding the cytochrome P450s (*CYP79D1* and *CYP79D2*) that catalyze the first-dedicated step in linamarin synthesis (Andersen *et al.*, 2000). This discovery made it feasible to block linamarin synthesis by suppression of *CYP79D1* and *CYP79D2* expression in transgenic plants (Siritunga and Sayre, 2003, 2004). To determine the most effective strategy for reducing root linamarin content and to evaluate the role of linamarin transport in determining root linamarin levels, Siritunga and Sayre generated transgenic cassava in which the expression of the *CYP79D1*

and *CYP79D2* genes was selectively inhibited in leaves or roots only (Siritunga and Sayre, 2003, 2004). This selective down-regulation was achieved through the use of two different organ-specific gene promoters, *cab1* (leaf) and *patatin* (root), to drive the antisense expression of the *CYP79D1* and *CYP79D2* genes.

Reverse transcriptase-PCR analyses of five independent *cab1*-*CYP79D1/D2* antisense transformants constructs demonstrated a range of suppression of *CYP79D1* and *CYP79D2* transcript levels in leaves. Concomitant with this reduction was a reduction in leaf linamarin content (Figure 4a). *Cab1*-*CYP79D1/D2* transgenic plants had leaf linamarin levels ranging from 6% to 40% of wild-type levels. Surprisingly, Siritunga and Sayre (2003) observed a 99% reduction in root linamarin content in all *cab1*-*CYP79D1/D2* antisense transformants even though the root *CYP79D1* and *CYP79D2* transcript levels were unaffected (Figure 4a). They also demonstrated that *patatin*-*CYP79* antisense plants, having no detectable *CYP79D1* or *CYP79D2* message in roots, had wild-type root linamarin levels (Figure 4b) (Siritunga and Sayre, 2004). These results indicated that in 3–4-month-old cassava plants nearly all the linamarin present in roots was synthesized in the leaves and transported to the roots. Linamarin has previously been shown to be transported from seeds to growing shoots in germinating rubber tree seedlings as well (Selmar, 1993).

Surprisingly, when *cab1*-*CYP79D1/D2* antisense plants were transferred from *in vitro* conditions, where the media contained nitrate (40 mM) and reduced nitrogen (20 mM NH_4), to potting soil, they initially all died. When the *cab1*-*CYP79D1/D2* antisense plants were grown on MS media in which the ammonia was replaced with nitrate (60 mM) they failed to produce strong roots (Siritunga, 2002; Siritunga and Sayre, 2004). In contrast, *patatin*-*CYP79D1/D2* antisense plants grew normally when grown in modified MS media lacking ammonia or in potting soil. These results lead to a model for linamarin-derived nitrogen (CN) assimilation into amino acids by cassava roots (Figure 5). Siritunga and Sayre (2004) proposed that linamarin transported to the roots has two possible fates, (a) storage in the vacuole or (b) assimilation into amino acids. In the latter case, linamarin is deglycosylated, presumably by

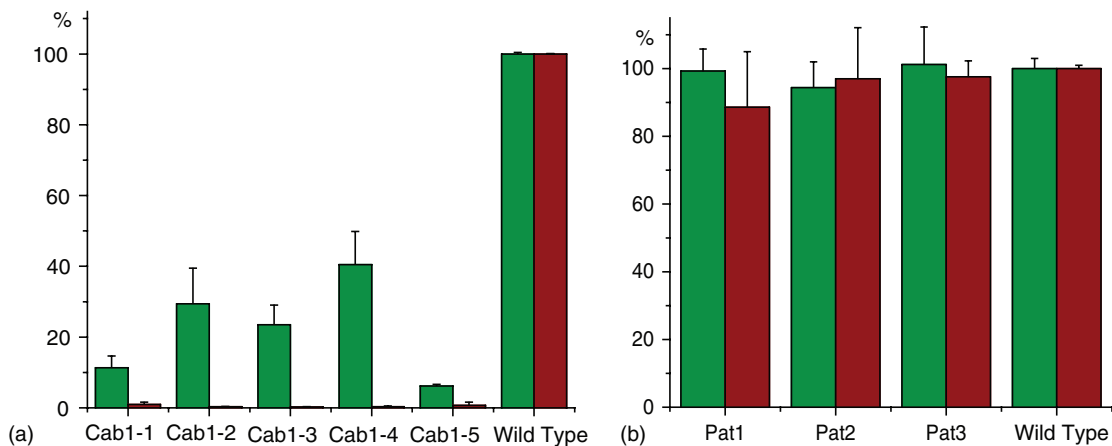


Figure 4 Linamarin content of leaves and roots from control: (a) cab1-CYP79 and (b) patatin-CYP79 cassava transformants; the maximum linamarin content (100%) in leaves (■) and roots (■) of wild-type plants was 80 and 3.4 μ g/gdw, respectively (Based on Siritunga and Sayre, 2003, 2004)

a generalized β -glucosidase, generating cyanide. The cyanide is then assimilated along with cysteine via β -cyanoalanine synthase to produce β -cyanoalanine and sulfide. The β -cyanoalanine is then hydrated to form the amino acid asparagine. Deamination of asparagine generates aspartate and free ammonia, which can be reassimilated by the glutamine synthetase/glutamate synthase cycle (Lea *et al.*, 1990, 1992). Consistent with this model, Nartey (1969) has reported that germinating cassava seedlings exposed to 14 CN incorporated 49% of the radioactive label into the amide carbon of asparagine and 6% into aspartate. Furthermore, it has been reported that the activities of β -cyanoalanine synthase and β -cyanoalanine hydrazase are threefold greater in roots than in leaves (Elias *et al.*, 1997a, b). Quantitative real-time PCR has also demonstrated that transcripts of β -cyanoalanine synthase are equally abundant in the leaf and roots of cassava (Siritunga *et al.*, personal communication). At present, the relative linamarin flux between storage and amino acid metabolism remains unknown and is the focal point in future research. But there is compelling evidence in support of linamarin being transported from leaves to root and playing a substantive role in root nitrogen metabolism.

More recently, cab1-CYP79D1/D2 antisense plants having root cyanogen levels greater than 0.3% of the wild-type level been recovered after transplantation to soil. These results

suggest that a threshold root linamarin level is required for adequate root formation. Putative transgenic plants that have been engineered to express CYP79D1/D2 RNA-interference (RNAi) constructs driven by the CaMV 35S promoter have been shown to root successfully; however, there was no analysis of the level of CYP79D1/D2 expression in these putative transgenic plants (Jørgensen *et al.*, 2005). Overall, these results suggest that linamarin is synthesized in roots and serves in part as a transportable form of reduced nitrogen in addition to providing protection from herbivory.

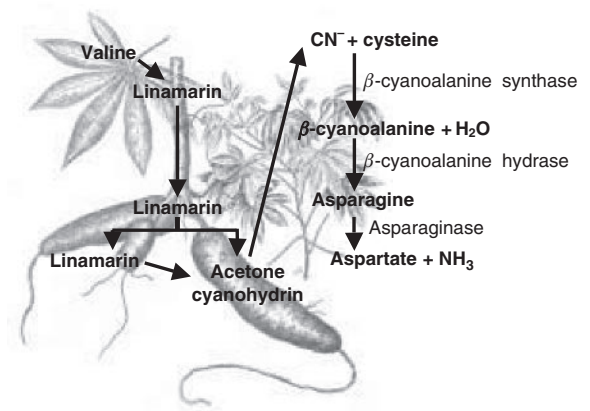


Figure 5 Model for linamarin-derived nitrogen (CN) assimilation into amino acids by cassava roots

2.2.6 Transgenic approaches to controlling viral disease in cassava

Cassava mosaic disease (CMD) causes substantial losses in productivity potentially impacting food security in Africa. In 2002, yield losses attributed to CMD were estimated to be 20–28% of the total cassava production in Africa. CMD is caused by a bi-partite gemini virus (African cassava mosaic virus, ACMV) that is transmitted by white flies in Africa. Various strains of ACMV have been identified that vary in the severity of disease symptoms. Complicating matters is the observation that recombination between virulent strains can occur in coinfecting plants accelerating the generation of new and potentially more virulent strains. ACMV-tolerant cultivars have been identified and incorporated into breeding programs in Africa. Marker-assisted selection strategies are being used to map the resistance gene(s). The use of conventional breeding strategies to develop ACMV-resistant lines have been impaired, however, due to the high heterozygosity and inbreeding depression in many farmer-preferred cultivars. As a result, a number of transgenic strategies have been employed to suppress viral replication and transmission.

The first transgenic plants resistant to ACMV were reported in 2004 (Chellappan *et al.*, 2004a, b). Introduction of the *AC1* gene, encoding the Rep protein, which is required for viral replication, induced post-transcriptional silencing of the *AC1* gene via the generation of multiple small interfering RNAs targeted against the *AC1* transcript. In addition, full length AC1 protein was generated in the transgenic plants. Transgenic plants had substantially reduced symptoms compared to wild-type plants even after 2-month postinfection. No apparent role for AC1 protein expression in viral resistance was established. The AC1 transgenic plants were generated, however, by particle gun-mediated transformation, which resulted in the integration of multiple copies of transforming DNA. Subsequently, after several subclonings of the plants, it was observed that the viral resistance was lost. This was presumably due to gene silencing.

In 2005, Zhang and coworkers reported the development of transgenic cassava with enhanced resistance to ACMV using a targeted antisense approach. Small sense- and antisense-interfering

RNAs were generated against several viral RNA targets including the Rep (*AC1*), TrAP (*AC2*), and REn (*AC3*) transcripts, which are involved in viral replication and expression. The antisense constructs were fused to the 3' UTR (3' untranslated region) (prior to the terminator sequence) of the selectable marker gene used for selection of transgenic plants. Viral replication and disease symptoms were substantially reduced in transgenic leaves using moderate and high infection pressures.

2.2.7 Genetic manipulation of starch in transgenic cassava

One of the most important steps in starch biosynthesis is the conversion of triose phosphates into hexose phosphates, which are required for starch biosynthesis. Starch biosynthesis in roots takes place in heterotrophic plastids that lack fructose-1,6-bisphosphatase, an important enzyme for the interconversion of triose phosphates to hexose phosphates (Entwistle and ap Rees, 1990) (Figure 6). The precursor for starch biosynthesis, adenosine diphosphate (ADP)-glucose is not always made in the organelle of starch synthesis. The lack of fructose-1, 6-bisphosphatase in heterotrophic plastids necessitates the supply of substrates (ADP-glucose) for starch synthesis (Entwistle and ap Rees, 1990) from the cytoplasm. The enzyme catalyzing the first-dedicated and rate-limiting step in starch synthesis is ADP glucopyrophosphorylase (AGPase). In dicots AGPase is localized typically in the plastids but may also be present in the cytoplasm in some plant species (Denyer *et al.*, 1996). In cereals and tomato fruits, AGPase is cytoplasmic and ADP-glucose is transported into the plastid in exchange for adenosine monophosphate (AMP) by the adenylate translocator (Mohlmann *et al.*, 1997).

ADP-glucose pyrophosphorylase is a heterotrimeric enzyme that consists of a large subunit (shrunken2 locus, 54–60 kDa) and a small subunit (brittle2 locus, 51–55 kDa.). These subunits are also known as S and B subunits named after the loci from which the first AGPase cDNAs were cloned from maize (Bae *et al.*, 1990; Preiss *et al.*, 1990). The individual subunits each are catalytically active, however, optimal activity is observed in the heteroenzyme complex. In potato,

In 2006, Ihemere and coworkers reported the transformation of cassava using *Agrobacterium* harboring a *patatin-chloroplast transit sequence-glgC* transfer-DNA (T-DNA) cassette (Ihemere *et al.*, 2006). Transformed plants were confirmed by RT-PCR and Southern blot analysis. Tuber-specific expression of the *glgC* gene as confirmed by RT-PCR analysis (Ihemere *et al.*, 2006). AGPase assays demonstrated that the transgenic had 70% higher total AGPase activity than the wild type plants. The phosphate insensitive (bacterial) AGPase activity was 13-fold greater in transgenic than wild type plants reflecting expression of the phosphate-insensitive *glgC16* gene (Ihemere *et al.*, 2006).

Greenhouse-grown transgenic plants (3D-1 and 3D-3) expressing the highest AGPase activity had greater root fresh weight, greater root leaf numbers and greater top fresh weight per plant than wild type plants (Ihemere *et al.*, 2006). The roots' fresh weight yields of transgenic cassava plants were 2.7-fold greater than those of wild type roots. The transformed plants also had more roots per plant (8–12 roots/plant) than the wild type plants (average = 7 roots/plant). Root and shoot dry weight were also greater in transgenic versus wild-type plants, possibly reflecting a reduction in feed back inhibition on carbohydrate accumulation (Ihemere *et al.*, 2006). Interestingly, the observed increases in dry weight were not associated with an increase in starch density but with an increase in root size and number. These results suggest that the density of amyloplasts/cell in roots is not subject to substantial variation.

2.2.8 Starch modification

In 2005, Raemakers and colleagues reported the generation of amylose-free transgenic cassava by antisense inhibition of granule-bound starch synthase in cultivar TMS 60444. Amylose biosynthesis in cassava roots is catalyzed mainly by granule-bound starch synthase 1 (*gbss1*; Raemakers *et al.*, 2005). A reduction in the amylose synthesis could be achieved by down-regulating *gbss1*. The suppression of *gbss1* expression in other crops (potato, maize, wheat, and rice) resulted in the production of amylose-free starch (Visser and Jacobsen, 1993; Chakraborty *et al.*, 2004). Amylose-free

starch has desirable physico-chemical properties compared to amylose-containing starch. For example, amylose-free starch has a higher granule-melting temperature, less retrogradation, and better adhesive characteristics than amylose-containing starch (Visser *et al.*, 1997). The cassava amylose-free starch generated by Raemakers and coworkers has similar properties as the preceding amylose-free starch in potato, maize, wheat, and rice (Visser and Jacobsen, 1993; Chakraborty *et al.*, 2004) and is characterized by increased stability of the gel (Raemakers *et al.*, 2005). The starch particle size distribution for amylose-free and amylose-containing plants was similar. The authors also observed that the amylose-free starch had a higher melting temperature than the amylose-containing plants. The chain length distribution of the amylose-free and amylose-containing starches were the same, hence, the down-regulation of the *gbss* activity did not affect starch chain length. The other known modification of the cassava starch biosynthetic pathway was the antisense inhibition of the AGPase gene, which resulted in drastic reduction of starch and an increase in the amount of sugars in cassava roots (Munyikwa *et al.*, 1998). Starch biosynthesis in the AGPase antisense plants was limited to the root epidermal layer. This has no known industrial or agricultural applications save for the validation of the role of AGPase in starch biosynthesis.

3. FUTURE DIRECTIONS FOR PRECISION-ENGINEERED CASSAVA

In 2005, the Bill and Melinda Gates Foundation announced support for a major initiative to metabolically engineer cassava to provide complete nutrition for persons subsisting on a cassava-based diet. The BioCassava Plus Program (<http://biocassavaplus.org>) has eight objectives. These objectives include, elevating protein, iron, zinc, vitamin A and E levels to meet the minimum daily requirement in a 500 g cassava meal. In addition, cyanogenic glycoside levels will be reduced in cassava foods to safe or nontoxic levels, the shelf life of harvested roots will be extended from 2 days to several weeks, and CMV resistance will be engineered into cassava. Once each of these individual objectives has been achieved the traits will be stacked into local cultivars, field

tested, using approved biosafety systems, and used in human feeding trials to determine the bioavailability of the introduced nutrients. Field trials of transgenic cassava will be conducted in the targeted African countries of Nigeria and Kenya as the products develop. The long-term objectives are to provide precision engineered products that provide complete nutrition as well as value-added traits that facilitate income generation.

Recognizing the global importance of cassava, the DOE-Joint Genome Sequencing Program was announced in 2006, a cassava genome sequencing project. Complete coverage of the cassava genome is anticipated by 2008. In addition, the availability of full-length EST libraries and a cassava DNA chip are anticipated in the near future. These tools will facilitate discovery of potentially novel genes or gene regulatory networks that impart enhanced drought and pathogen tolerance, high photosynthetic capacity, and polyterpenoid production to cassava. These tools also will facilitate precision engineering strategies to alter the metabolism of cassava roots and shoots to facilitate crop biofortification and the development of cassava as a biofuel crop for the tropics.

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Cotton

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Cotton is the world's most important natural fiber. Cotton plant has played a significant role in the economy, social structure, and history of many countries around the globe. Despite the availability of synthetic alternatives, it continues to be an important source of fiber for the textile industry. The word "cotton" is derived from the Arabic word "*al qatan*" (Chaudhry and Guitchounts, 2003). Cotton has been cultivated and used to make fabrics for over 7000 years. It is speculated that cotton may have existed in Egypt as early as 12 000 BC and fragments of cotton fabrics have been found by archeologists in Pakistan (3000 BC), Mexico (from 3500 BC), India (3000 BC), Peru (2500 BC), and the southwestern United States (500 BC) (Stewart, 2001).

Cotton is adapted to warm climates and is cultivated from 45° North latitude to 32° South latitude. It is grown in approximately 80 countries worldwide by over 20 million farmers. Over 90% of the cotton is grown in the Northern hemisphere. Amongst the commercially cultivated species, over 90% of worldwide acreage is devoted to the tetraploid, *Gossypium hirsutum* ($2n = 4x = 52$) or upland cotton (Brubaker *et al.*, 1999). Another tetraploid, *Gossypium barbadense* or Egyptian

cotton is also grown in some parts of the world for its prized extra-long staple. The two Old World diploid species, *Gossypium arboreum* and *Gossypium herbaceum*, are still cultivated in some parts of Africa and Asia. However, these occupy a very small percentage of the total worldwide cotton acreage because of their low productivity and poor quality fiber. The global area of cotton cultivation in 2006 is estimated to be approximately 34.4 million hectares leading to total fiber production of 25 million metric tons. Interestingly, the world average cotton yield was 564 kg ha⁻¹ in 1995 compared to 726 kg ha⁻¹ in 2006. Much of the yield gains in the last decade are attributed to adoption of biotech cotton varieties (Cantrell, 2006).

1.2 Taxonomy and Botanical Distribution

Gossypium is a complex taxon comprised of over 50 perennial species that span the globe (Fryxell, 1992). Thus far, on the basis of cytogenetics and interspecific hybrid viability/fertility, eight diploid genomes designated A through G plus K have been recognized (Percival *et al.*, 1999). Some of the diploid cottons are poorly described and new *Gossypium* species continue to be discovered in some parts of the world. The original lineage of *Gossypium* may have arisen 10–20 million years

ago judging from the diverse spp. of *Gossypium* now growing wild in Australia, Africa, Arabia, and Mesoamerica (Brubaker *et al.*, 1999). At one point, in ancient Africa a line of the cotton genus denoted *Gossypium* evolved to produce trichomes (plant hairs) on the seed. These migrated to different parts of the world. One species found wild in Southern Africa is *G. herbaceum*. A species established and cultivated in Southern Asia is *G. arboreum*. The third diploid species (putatively identified as *Gossypium raimondii*) is no longer found in Asia or Africa, but is believed to have migrated to the Western Hemisphere (New World) approximately 1–2 million years ago (Brubaker *et al.*, 1999). All three of these species were diploid and had a basic chromosome number of $2n = 2x = 26$. About the time of migration of one of the diploid species to the New World, a critical hybrid event occurred between two of the species (similar to *G. herbaceum* and *G. raimondii*) (Wendel and Cronn, 2002). This polyploid hybrid retained the ability to produce fibers and was more vigorous than the diploid parental species. It became established and radiated out to different regions of the New World. Migration of the polyploid to diverse regions and subsequent isolation contributed to the origin of *G. hirsutum* in the Yucatan and *G. barbadense* in Peru. Other tetraploids diverged in Brazil (*Gossypium mustelinum*), Galapagos (*Gossypium darwinii*), and Hawaii (*Gossypium tomentosum*). All of these tetraploid species are perennial and adapted to short-day tropical environments. This means they flower when daylengths are less than 12 h. This prevents migration and adaptation to more temperate regions. The discovery of genetic variants in *G. hirsutum* and *G. barbadense* allowed these perennial species native to the tropics to be grown as an annual crop with good yields in temperate long-day environments. This is very similar to domestication events for day neutrality in tropical maize (*Zea mays* L.) and grain sorghum (*Sorghum bicolor* L. Moench). Hendrix and Stewart (2005) estimated the 2C nuclear DNA content of the AD tetraploid cotton ($2n = 4x = 52$) as being about 4.91 pg (picogram) or 2401 Mb (1C content). This is approximately additive for the putative A genome donor, *G. herbaceum* ssp. *africanum* and the D genome donor *G. raimondii*.

1.3 Economic Importance

Cotton is the single most important textile fiber in the world, accounting for nearly 40% of total world fiber production. The top three cotton producers, China, the United States, and India, together provide over half the world's cotton. The United States, while typically ranking second to China in production, is the leading exporter, accounting for over one-third of global trade in raw cotton. The US cotton industry accounts for more than \$25 billion in products and services annually, generating over 400 000 jobs in the industry sectors from farm to textile mill.

The cotton fiber is remarkable and unique in the plant kingdom. It is a modified plant hair or trichome that differentiates from epidermal cells of the seed coat. Typically, there are about 25–30 ovules that develop into cottonseed within the cotton boll (Kim and Triplett, 2001). Fiber is a single living cell that can grow extremely fast to achieve an average length of 25–35 mm or a 3000-fold increase during development (Stewart, 1975; Applequist *et al.*, 2001; Taliercio *et al.*, 2005). Approximately 25% of the epidermal cells elongate and differentiate into fibers giving rise to 14 000 to 18 000 fibers per seed (Bowman *et al.*, 2001); however, as many as 30 000 fiber cells per seed have been reported (Basra and Saha, 1999). The process of fiber development has been categorized into following four overlapping stages: initiation, elongation, secondary cell wall synthesis, and maturation (Basra and Malik, 1984). The cotton fiber takes from 40 to 60 days to develop completely. The mature cotton fiber has two clearly defined cell walls, a primary and secondary cell wall. The degree of secondary wall thickness determines the fineness of the fiber. Micronaire (fiber's resistance to airflow) is the measure of fiber's fineness. Cotton fiber represents one of the purest forms of crystalline cellulose. It is a polysaccharide polymer composed of 100–15 000 glucose molecules. When the cotton boll opens, the mature fibers dry into flat, twisted, ribbonlike structures that become interlocked. This interlocking permits spinning into fine, strong thread. Its crystalline and porous structure makes a fiber that is versatile, capable of binding dyes, very absorbent, and withstands repeated washing.

The properties of the cotton fiber determine its utility to the textile industry. Fortunately, the length, strength, and fineness of the cotton fiber are heritable traits amenable to careful breeding and selection (Benedict *et al.*, 1999). All of these traits can be measured analytically and are displayed prominently on all bales of cotton grown in the United States. The value of the bale to producer is determined by these fiber properties.

There are diverse and important uses of cotton products beyond textiles (Cantrell, 2005). In addition to the fiber, cotton plant also produces large quantities of seeds. In fact, the plants produce 1.65 kg of seeds for every kilogram of fiber. In the year 2005, approximately 40 million metric tons of cottonseed was produced worldwide (FAO). This makes cotton the third largest field crop in terms of edible oilseed tonnage in the world. Cottonseed is also a balanced source of fiber and protein. The whole cottonseed or the meal following oil extraction is important animal feed, primarily for dairy cattle. Oil is extracted from cottonseed kernels following separation from the hulls. The kernels are flaked and crushed for oil extraction and cottonseeds typically yield 16% crude oil. Cottonseed oil is a major edible oil found in many food products. It is viewed as “naturally hydrogenated” with a 2:1 ratio of polyunsaturated to saturated fatty acids. It is valued as stable frying oil, not requiring additional processing thus avoiding the formation of trans fatty acids. It is rich in tocopherols, which act as natural antioxidants and prolong shelf life and stability. Although a substantial portion of global output is used for human nutrition in the form of edible oil, as mentioned earlier, the meal and a significant amount of cottonseeds are simply used as feed for cattle. A better utilization of this abundant but underutilized resource can help in meeting the nutritional requirements of the growing global population in future.

Very short fibers, referred to as linters, comprise about 8% of the weight of fuzzy cottonseed. The linters provide an inexpensive source of cellulose that requires less processing than that derived from wood pulp. These are used in many nonwoven household and medical products. In addition, linters find important usage in food products, photographic film, and molded plastic products. These fibers are used in the manufacture of high-

grade bond paper and are a major component of many currencies. Cellulose from cotton linters is used widely as carriers in cosmetics and many foods. Even the cottonseed hulls that are removed from kernels prior to oil extraction serve an important use as roughage in the feed for livestock.

Thus, cotton plant is not just a source of fiber, it provides many byproducts with diverse applications. Although fiber will continue to be the primary target for biotechnology-mediated improvement, various cottonseed components can also be modified and improved through genetic engineering to better serve the needs of mankind.

1.4 Traditional Breeding

Increasing profit per cotton field has been the ultimate objective for cotton plant breeders. Several strategies have been employed to meet this goal including improving host plant resistance, abiotic stress tolerance, fiber and seed qualities, and agronomic adaptability.

1.4.1 Breeding objectives

Breeding objectives are usually defined by the economic importance of the trait, availability of sources for improvement, and viability of incorporating targeted improvement into a high-yielding cotton variety.

1.4.1.1 Host plant resistance

Several insect, nematode, and disease maladies afflict cotton. Perhaps none are as notorious as the boll weevil (*Anthonomus grandis*) invasion of the US cotton belt, which financially ruined much of the cotton industry, created a societal upheaval and prompted scientists to begin searching for host plant resistance (HPR) to the pest (Jones, 2006). While no germplasm provided substantial resistance, early maturing varieties were developed that would provide temporal resistance or escape from the boll weevil (Walker, 1979). By developing cotton varieties that could produce a greater portion of their fruit early in the growing season before the second and third generations

of postdiapause boll weevils could reproduce, an economically acceptable cotton crop could be produced in many areas of the US cotton belt.

In the 1960s efforts to identify other mechanisms of resistance to different insect pests began to be sought. Several biochemical and morphological traits associated with cotton HPR have been identified with varying degrees of usefulness (El-Zik and Thaxton, 1989). Butler and Henneberry (1984) determined that fewer leaf trichomes resulted in greater cotton HPR to the whitefly, *Bemisia tabaci*. Increased tannins (Lege *et al.*, 1992) and gossypol (Bottger and Patana, 1966) as well as glabrousness (Lukefahr *et al.*, 1971) and nectariless (Butler *et al.*, 1972) traits have all been found to reduce *Heliothis* damage. Schuster *et al.* (1976) reported that plants lacking floral nectaries also were found to be less attractive to plant bugs (*Lygus hesperus*). The okra leaf trait confers resistance to pink bollworms (*Pectinophora gossypiella*) (Wilson *et al.*, 1986), and it proved to be a valuable means of improving pesticide penetration into the lower cotton canopy, which improves chemical pesticide efficacy.

Considerable breeding efforts have been directed toward improving nematode and disease resistance of cotton germplasm. Shepherd (1979) outlined methodology for screening germplasm for resistance to root-knot nematodes (RKNs) (*Meloidogyne incognita*) as well as developing several sources of resistant lines. Improving resistance to *Verticillium* wilt has been a major goal of several breeding programs throughout the world (Bell, 1992). To screen early-generation breeding lines for *Verticillium* disease resistance, breeders often establish fields with high fungal density that ensure plants are challenged by the pathogen. Occasionally breeders are capable of developing HPR to poorly defined diseases. In 1994, a new and devastating disease, bronze wilt, afflicted hundreds of thousands of US acres. Although Koch's postulate was never successfully completed with the disease, most breeders realized a strong relationship between cotton varieties, their pedigrees, and disease incidence. Consequently, breeders purged much of their material that was susceptible to bronze wilt. Since then, no major outbreak of the disease has been reported. In other situations, the disease has been very well defined but the economic effect is minimal. Such is the case with bacterial blight

(*Xanthomonas campestris*) (Chakrabarty *et al.*, 1997). Only minor commercial breeding efforts are made toward improving resistance because of limited and sporadic economic consequences of the disease (Blasingame, 2005).

1.4.1.2 Abiotic stress

Soil-moisture deficits generally are the most yield-limiting stress factor for cotton and can dramatically impact stability of performance. In certain growing areas, other abiotic stresses are of major concern such as excessive salt, heat and cold temperatures. Traditionally, abiotic stress screening entailed subjecting early-generation families and populations to extreme drought, heat, or cold conditions; then selecting the most productive plants to advance in breeding programs. Recently, more sophisticated methods of screening seedlings for drought tolerance were developed by Longenberger *et al.* (2006).

Heat tolerance usually is identified in plants by fertility ratings or pollen production in flowers. Programs approach screening for heat tolerance with different strategies. Some breeders emphasize early-generation screening to identify tolerance or sensitivity to extreme heat either under artificial heat or nurseries in specific areas where high temperatures are expected such as Arizona. Other breeders will wait and characterize heat tolerance of only advanced generation material that has undergone multiyear testing. Cool temperature stress typically receives very little attention during seedling stages of development and high levels of tolerance are often associated with larger seed size, and higher tolerance to seedling disease complexes.

For salt tolerance, usually minor efforts are made for selection and improvement. Gossett *et al.* (1994) indicated differences between New Mexico State University's Acala varieties and varieties developed in the mid-south, which suggest serendipitous enhancements due to the breeding program location.

1.4.1.3 Fiber quality

Because cotton's ultimate value is as a fiber crop, much attention has been given to improving fiber traits. By the early 1960s efforts were underway to

change the way cotton fiber quality was measured with high-volume instrument (HVI) testing. With this advent, breeders could then affordably evaluate fiber from individual plants and breeding lines and assign absolute and objective numbers to fiber properties. This technological innovation helped make rapid improvements in fiber traits.

Concomitant with HVI development, new techniques were being developed to break negative linkages among fiber properties and yield (Miller and Rawlings, 1967; Meredith and Bridge, 1971; Culp and Harrell, 1973). Fiber length and strength typically were characteristics most often targeted for improvement because of their importance to spinning quality, quantification by HVI, and existence of genetic improvement sources.

Today, many breeders employ very sophisticated crossing schemes designed to break linkages between yield and fiber qualities. Other fiber properties such as micronaire, elongation, length uniformity, fineness, and maturity also garner attention by breeders. Moreover, fiber properties in most lines are evaluated in several generations during the variety development process.

1.4.1.4 Seed quality

Because cottonseed represents only about 10% of the crop's economic value, minor breeding attention has been given to improving seed qualities. Seed size has typically been a trait receiving the most attention by breeders because small seed size can be associated with poor seedling vigor (Quisenberry and Gipson, 1974), but more importantly can cause problems at the gin by increasing seed coat fragments and neps (Barger and Garner, 1991). Cotton breeders monitor seed size through a seed index, which is the weight of 100-fuzzy seed. Unfortunately, smaller seed size is often associated with a greater lint-to-seed ratio, which is an important yield component.

A major devaluating characteristic of cottonseed is the presence of gossypol, a toxic secondary metabolite. Gossypol prevents nonruminants from consuming cottonseed and limits mature ruminant animals to safely consume only about 3 kg/day. In addition, gossypol makes oil extraction and utilization more expensive and less competitive against other oil crops such as soybeans (*Glycine max*) and canola (*Brassica napus*). The first

cotton variety with glandless seed was described by McMichael (1959). Less than two decades later several commercial glandless varieties were available (Hallowin *et al.*, 1978), but none of these lines were very successful because of the susceptibility of the plants to pests and lack of commercial value to growers.

1.4.1.5 Agronomic adaptation

Earliness of crop maturity can enhance yield and also reduce risk by avoiding late-season pests and aberrant weather. Early-maturing varieties are necessary for the northern areas of the cotton belt where heat units limit production. Early-maturing varieties also enable growers to more easily manage counter-season crop rotations such as winter wheat. Conversely, late-maturing varieties can improve drought tolerance (Dumka *et al.*, 2004). In latitudes closer to the equator, late-maturing varieties can exploit more heat units from the growing season, recover from fruit shedding, and translate that opportunity into greater yield potential than more determinant fruiting varieties.

The ideal boll type depends on location. On the high plains of Texas and Oklahoma where strong wind storms frequently occur, a tight storm-proof boll is preferred. However, the need for such a boll has decreased with the adoption of chemical harvest aids, which shorten the exposure time from defoliation until harvest. In the mid-south and southeast US, a looser boll, which is less prone to hard locking, is preferred. Bolls in this area need to be easily pulled out of the burr by mechanical pickers. Moreover, bolls need to open easily in the presence of low sunlight and high humidity, which are common in these regions during the harvest season.

1.4.2 Tools and strategies

A yield plateau was thought to have been reached in recent years due to several factors. Chief among these factors was a lack of genetic diversity caused in large part to, as Meredith (2002) suggested, breeding efforts directed toward added value traits. Genetic diversity is an important component for advancing breeding populations of cotton germplasm. Commercial level plant breeders

are pressed to quickly release high-performance varieties. Consequently, private breeders tend to use more in-house elite germplasm with a narrow genetic base in comparison to public cotton breeders (Bowman, 2000).

Most cotton breeders use a pedigree method of breeding, first described by Newman (1912), or slight derivation thereof. After initial hybridization, counter-season nurseries, especially for first filial plant generations, are frequently used to hasten development. Individual plants are selected in second filial or later generations after additive effects can be expressed. Many breeders use shuttle-breeding strategies to select in multiple environments such as a dryland nursery, a *Verticillium* wilt nursery, and an early-planted nursery. Initial testing of selected lines is done in high-yielding, well-controlled environments where the maximum amount of genetic yield and fiber potential can be expressed. As testing continues on advanced generations, trials tend to include more diverse growing environments to determine performance stability. The entire process generally takes at least 10 years from the initial cross-pollination to a finished cotton variety.

1.4.3 Achievements

In the year 1900, average cotton fiber yield per hectare was 218 kg in the United States (Ware, 1951); by 2005, it was 931 kg ha⁻¹ (Meyer *et al.*, 2006). While not all the gain can be attributed to genetic improvement, certainly cotton breeding programs deserve much credit. Meredith and Wells (1989) reported that in comparing modern cultivars versus antiquated cultivars, newer germplasm is capable of higher yields because of greater partitioning of dry matter to reproductive structures away from vegetation. Fiber quality is more heritable than yield potential and can affect crop quality even in the most environmentally diverse and challenging conditions (Krieg, 2002). Fiber quality has improved dramatically in almost all production regions. The most significant improvements have come in longer and stronger fibers.

Individual cotton breeding programs have made substantial contributions and developed reputations as centers of expertise in many areas of research and development. Public and private

breeding efforts in California made great strides in improving fiber quality and *Verticillium* and *Fusarium* wilt tolerance. Programs in Arizona are noted for improving fiber characteristics as well as heat and drought tolerance. The breeding program at New Mexico State University has developed Acala varieties that are well regarded for fiber quality and *Verticillium* wilt tolerance (Zhang *et al.*, 2005). The Texas A&M University breeding program in Lubbock released several lines with enhanced fiber qualities and *Verticillium* wilt tolerance. In College Station, TX, Texas A&M University breeding programs developed several germplasm lines and varieties with high levels of HPR to a wide array of maladies. Mid-south breeders have developed numerous lines that are highly adapted to a wide range of environments around the world. They also are noted for releasing germplasm and varieties with RKN resistance (Jenkins *et al.*, 1993). In the southeast, varieties, particularly from the USDA-Pee Dee program are well regarded for fiber properties. Internationally, breeders from Australia's Commonwealth of Scientific and Industrial Research Organization (CSIRO) program are responsible for releasing high-yielding, high-fiber quality varieties that are well adapted and widely grown throughout the world.

1.5 Limitations of Conventional Breeding, Rationale for Transgenic Breeding, and the First Generation of Engineered Cotton

Conventional cotton breeding can be a slow and methodical process with typical incremental improvements. Nontransgenic varieties have marginal retail value in comparison to other conventional cotton crop inputs. In contrast, transgenic cotton breeding increases the speed of developing high-valued cotton varieties with highly efficacious traits. Moreover, transgenic breeding not only offers better genes for specific traits, it also allows a faster, more convenient method of identifying these genes during the variety development process without the cumbersome negative linkages encountered during conventional breeding procedures. Use of polymerase chain reaction (PCR) technology and lateral flow strips allows breeders to identify target plants at the seedling stage or in environments where

screening is not possible. This greatly reduces the cost of nurseries and fallible phenotypic screening procedures.

Finding naturally altered and beneficial metabolic pathways that enhance abiotic stress tolerance is also virtually impossible due to screening inadequacies of large numbers of cotton genomes. With transgenic technology, pathways that can enhance stress tolerance can be altered, verified, and reliably reproduced in breeding programs.

The cotton plant is particularly susceptible to a wide variety of insect pests and nematodes. This vulnerability and the fact that cotton is generally not considered a food crop, production of cotton has traditionally relied on the use of large amounts of toxic pesticides. Prior to the introduction of Bt cottons, 5–10 insecticide applications were needed per season to control damage from insects (Benedict and Altman, 2001). Some estimates suggest that prior to the widespread adoption of GM (genetically modified)-cotton, nearly 25% of all insecticides used around the world were for production of cotton (Pannetier *et al.*, 1997; Gomez-Barbero and Rodriguez-Cerezo, 2006). Three classes of synthetic organic compounds including organophosphates, carbamates, and pyrethroids are used to control insect pests of cotton. These chemicals are expensive and harmful to people, wildlife, and the environment (Benedict and Altman, 2001).

With the exception of a few developed countries, cotton is mostly grown in developing countries as a cash crop by the resource-poor farmers. In some cases, their crops are destroyed by insects as some farmers cannot afford to buy the pesticides. In most cases, they resort to the use of some highly toxic chemicals leading to pesticide poisonings and deaths as they can rarely afford the proper equipment for safe chemical application (Mancini *et al.*, 2005).

The issues discussed above made cotton an ideal crop that could greatly benefit from genetic engineering. It was therefore, no surprise that upon receiving approval for commercial planting, of all the GM crops, cotton garnered the largest acreage for the first year in the United States (James, 1997). The GM cotton introduced in the United States was in the form of insect-resistant cotton (Bollgard® cotton; Jones *et al.*, 1996; Hardee and Herzog, 1997; Benedict and Altman, 2001;

Perlak *et al.*, 2001), herbicide (Buctril®)-resistant cotton (BXN57; Panter *et al.*, 1996), and herbicide (glyphosate)-resistant cotton (Roundup Ready® cotton; James and Krattiger, 1996). The gene-for-gene relationship demonstrated by Flor (1946) is an important concept in defending HPR traits against resistant pest populations. Pyramiding or stacking of genes, which offer different modes of resistance to the same pest, is much more feasible with transgenic technology. For instance, Monsanto now offers Bollgard II® technology and Dow offers WideStrike™ technology where each contains two independent genes responsible for cotton plants producing *Bacillus thuringiensis* (Bt) toxins to resist worms. This strategy decreases the likelihood of Lepidopteran insects developing resistance to both host plant gene products. The herbicide resistance traits mentioned earlier were the result of screening bacterial genomes for resistance. Resistant genotypes were then used as sources of tolerance in cotton plants. Thus, a vital component to ecologically and economically superior weed control was developed through transgenic breeding. Screening billions of cotton genomes for natural herbicide resistance would have been logistically impossible.

Cotton growers have adopted biotechnology at a faster pace than growers of any other crop. Ten countries, representing over 60% of the global area of cotton production, currently allow biotech cotton to be grown: Argentina, Australia, Brazil, China (Mainland), Colombia, India, Indonesia, Mexico, South Africa, and United States. In 2005/2006 crop season, it is estimated that 28% of the global area of cotton production was planted to varieties containing biotech traits, such as insect and/or herbicide resistance. This constitutes about 37% of total bales produced globally and about 38% of all exported bales. In the year 2004, GM cotton garnered 80% of US-, 66% of Chinese-, 80% of Australian-, and 85% of South African-cotton acreage (James, 2004). Often, cotton is the first crop to receive approval for commercial planting in many countries from their respective regulatory agencies. Biotech varieties are expected to account for 36% of 34.4 million hectares planted for cotton in 2006/2007 (Cantrell, 2006). GM varieties are projected to continue gaining their share of global cotton acreage. This bodes well for future products that are likely to be generated by further genetic modification of cotton.

2. DEVELOPMENT OF TRANSGENIC COTTONS

Several excellent reviews on transgenic cotton have been published in the last 15 years (Murray *et al.*, 1993; John, 1997; Paterson and Smith, 1999; Chlan *et al.*, 2000; Wilkins *et al.*, 2000; Rajasekaran *et al.*, 2001; Kumria *et al.*, 2003). In this chapter, we provide a comprehensive list of papers reporting genetic modification of cotton (Table 1). It includes reports published in international journals on the transformation of cotton using *Agrobacterium*, gene gun, or other alternative methods. Publications related to the intellectual property are sometimes overlooked in reviews, however, these provide additional and important new information not easily gleaned from traditional literature. Such information is essential in this age of rapidly advancing and increasingly complex biotechnology field. Therefore, a detailed list and discussion related to the relevant patents on cotton transformation methods, genes, promoters, and plants with engineered traits have been presented in this section. In addition, important issues concerning genetic engineering of cotton have also been highlighted. Production of transgenic cotton, especially via *Agrobacterium*-mediated transfer-DNA (T-DNA) delivery and somatic embryogenesis-mediated recovery of transformants, has become routine in some laboratories around the world. However, the fact remains that many laboratories, especially in the developing countries, continue to struggle with cotton transformation. This is because somatic embryogenesis in cotton is highly genotype-dependent and even with regenerable genotypes, the process is long, tedious, and requires a high level of tissue culture skills. Therefore, the laboratories that lack the necessary skills and resources are tempted to try alternative methods when embarking on projects involving cotton transformation. The results from many of these attempts have been questionable at best. We have addressed the issue of “cotton transformation methods” squarely, provided the necessary information to be able to identify the “real” transgenic events, and our hope is that investigators attempting cotton transformation for the first time will be able to make an informed choice.

2.1 Transformation Methods

2.1.1 *Agrobacterium*- or gene gun-mediated transformation of cotton cells followed by recovery of transgenic plants via somatic embryogenesis

The first two reports on successful transformation of cotton by *Agrobacterium*-method appeared as early as 1987 (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987). This feat was achieved only four years following the transformation of the model plant species—tobacco. However, the progress in producing transgenic cotton, especially in the academic laboratories, had been rather slow until the turn of this century. Although surprising considering the economic importance of cotton, the reasons for this dearth of reports on cotton transformation were the difficulties involved in producing transgenic cotton plants. A thorough investigation into various steps involved in generating transgenic cotton was conducted in the laboratory of K. Rathore at Texas A&M University and a comprehensive report unraveling the mystic surrounding cotton transformation was published in 2001 (Sunilkumar and Rathore, 2001). By using green fluorescent protein (*GFP*) as a reporter gene, this study revealed that the *Agrobacterium*-mediated transfer of T-DNA to the cotyledonary cells of cotton was a highly efficient process. In addition, the conversion of transient transformation events to stable events in cotyledon, hypocotyls, and petiole segments is quite efficient in cotton (Sunilkumar and Rathore, 2001; Rathore *et al.*, 2006). The difficulties in generating transgenic cotton are largely due to poor regeneration from cultured tissues. Recovery of transformed plants from cultured cotyledon-, hypocotyl- and petiole-derived callus tissues requires a careful selection of the friable, embryogenic callus, and several subcultures on various media under appropriate environmental conditions. Given the complexities of creating transgenic cotton, a simple yet robust method for cotton transformation was developed and has been described in detail (Rathore *et al.*, 2006). By following this protocol, it is possible to generate transformed cotton plants (cv. Coker 312) in 8–10 months. It should be noted,

Table 1 Summary of studies on transgenic cotton

Transformation method	Cultivar	Target tissue/mode of transformant recovery	Transgenes	Comments	Reference
<i>Agrobacterium</i>	Coker 310, 312, and 5110	H/somatic embryogenesis ^(c)	<i>cat</i> and <i>npII</i>	Enzyme assays and Southern for confirmation	Umbeck <i>et al.</i> , 1987
<i>Agrobacterium</i>	Coker 201	C/somatic embryogenesis ^(a)	<i>npII</i> and OCS	Immunoblot and Southern for confirmation	Firoozabady <i>et al.</i> , 1987
Gene gun	Coker 310	ESC/somatic embryogenesis ^(b)	<i>hpt</i>	Southern for confirmation	Finer and Mc-Mullen, 1990
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	<i>CryIAC</i> , <i>CryIAb</i> , and <i>npIII</i>	Western and bioassay for confirmation	Perlak <i>et al.</i> , 1990
<i>Agrobacterium</i>	Siokra 1-3	H/somatic embryogenesis ^(c)	<i>npII</i> and <i>gusA</i>	NPTII and GUS enzyme assays for confirmation	Cousins <i>et al.</i> , 1991
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	<i>npII</i> and <i>tfidA</i>	2,4-D monooxygenase activity, PCR, and 2,4-D resistance for confirmation	Bayley <i>et al.</i> , 1992
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	<i>npII</i> , <i>gusA</i> , and <i>tfidA</i>	Southern, GUS enzyme assay and 2,4-D resistance for confirmation	Lyon <i>et al.</i> , 1993
Gene gun	Delta Pine 50, Delta Pine 90, Sea Island, Pima S-6	SAM from mature seed/shoot regeneration in culture ^(c)	<i>gusA</i>	GUS histochemical analysis and Southern for confirmation	McCabe and Martinell, 1993
<i>Agrobacterium</i>	Coker 312	C/somatic embryogenesis ^(a)	<i>npIII</i> , protease inhibitors	Western for confirmation	Thomas <i>et al.</i> , 1995
Gene gun	Not stated	SAM from mature seed/shoot regeneration in culture ^(c)	<i>npIII</i>	Western for confirmation against insects	Chlan <i>et al.</i> , 1995
Gene gun	Delta Pine 50	SAM from mature seed/shoot regeneration in culture ^(c)	Fiber-specific E6 antisense and <i>gusA</i>	GUS assay, Northern, Western, and fiber quality analysis for confirmation	John, 1996
Gene gun	Coker 312, Delta Pine 50, Sea Island	SAM from mature seed/shoot regeneration in culture ^(c)	Fiber-specific, FbL2A promoter driving <i>phaB</i> and <i>phaC</i> , and <i>gusA</i>	GUS assay, Southern, Western, and biochemical analyses for confirmation	Rinehart <i>et al.</i> , 1996
Gene gun	Delta Pine 50	SAM from mature seed/shoot regeneration in culture ^(c)	Fiber-specific, E6 or FbL2A promoter driving <i>phaB</i> and <i>phaC</i> , and <i>gusA</i>	GUS assay, Southern, Northern, microscopic, and biochemical analyses for confirmation; fiber's thermal properties were altered	John and Keller, 1996; Chowdhury and John, 1998
<i>Agrobacterium</i> and gene gun	Coker 315 and Acala varieties	C, H, ESC/somatic embryogenesis ^{(a)(b)(c)}	<i>npIII</i> , mutant native AHAS genes	Southern and resistance to herbicides, imidazolinone and sulfonylurea, for confirmation	Rajasekaran <i>et al.</i> , 1996

(Continued)

Table 1 Summary of studies on transgenic cotton (*Continued*)

Transformation method	Cultivar	Target tissue/mode of transformant recovery	Transgenes	Comments	Reference
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	<i>npII</i> , FMV 35S promoter driving <i>CP4-EPSPS</i>	Southern, ELISA, and resistance to herbicide, glyphosate for confirmation	Nida <i>et al.</i> , 1996; Chen <i>et al.</i> , 2006
Gene gun	Coker 312, Delta Pine 50, El Dorado, and Pima S6	SAM from mature seed/shoot regeneration in culture ^(c)	<i>bar</i> and <i>gusA</i>	Southern, Northern and biochemical analyses, and resistance to herbicide, Basta, for confirmation	Keller <i>et al.</i> , 1997
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	<i>npII</i> , Mn-SOD	Biochemical analyses for confirmation; no significant improvements in chilling sensitivity	Payton <i>et al.</i> , 1997
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	<i>npII</i> , glucose oxidase	Biochemical analyses for confirmation; some protection against a root pathogen but phytotoxicity observed	Murray <i>et al.</i> , 1999
<i>Agrobacterium</i>	CUBQHRPIS	SAM from seedling/shoot regeneration in culture ^(e)	<i>npII</i> , <i>gusA</i>	Resistance to kanamycin and Southern for confirmation	Zapata <i>et al.</i> , 1999
Gene gun	Acala B1654 and Coker 315	ESC/somatic embryogenesis ^(b)	<i>npII</i> , <i>gusA</i>	GUS histochemical analysis and Southern for confirmation	Rajasekaran <i>et al.</i> , 2000
<i>Agrobacterium</i>	Coker 315	H/somatic embryogenesis ^(c)	Tobacco basic chitinase, glucose oxidase, <i>npII</i>	Some protection against Verticillium wilt with each gene	McFadden <i>et al.</i> , 2000
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	Cotton <i>Adh2</i> , rice <i>Pdcl</i> , <i>npII</i>	Biochemical and Western analyses; no increase in waterlogging tolerance	Ellis <i>et al.</i> , 2000
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Cotton seed-protein promoter or rbcS promoter driving <i>gusA</i>	PCR, histochemical, and biochemical analyses for confirmation; promoter tissue-specificity confirmed	Song <i>et al.</i> , 2000
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	<i>npII</i> , <i>gusA</i>	PCR and Southern for confirmation; a step-wise and comprehensive account of transgenic cotton production	Sunilkumar and Rathore, 2001; Rathore <i>et al.</i> , 2006
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Mn-SOD, APX, GR, <i>npIII</i>	Enzymatic assays confirmed overexpression; chilling-induced photoinhibition of photosystem II reduced	Korneyev <i>et al.</i> , 2001, 2003a, b
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Mn-SOD, APX, GR, <i>npIII</i>	Enzymatic assays confirmed overexpression; some protection of photosynthetic capacity during chilling	Payton <i>et al.</i> , 2001; Logan <i>et al.</i> , 2003
<i>Agrobacterium</i>	Coker 312	C/somatic embryogenesis ^(a)	Phaseolin promoter driving rapeseed mutant fad2	PCR and Southern for confirmation; seed oil with increased oleic acid and reduced linoleic acid levels	Chapman <i>et al.</i> , 2001
Polybrene/Spermidine-mediated DNA uptake	<i>G. barbadense</i> Giza 88	Cotyledon-derived culture/shoot regeneration	<i>hpt</i> , <i>gusA</i>	Histochemical and Southern analyses for confirmation	Sawahel, 2001

<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Cotton α -globulin promoter driving <i>gusA</i> , <i>nptII</i>	Histochemical and biochemical analyses; seed-specificity of promoter demonstrated	Sunilkumar <i>et al.</i> , 2002a
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	CaMV 35S promoter driving GFP gene, <i>nptII</i>	Fluorescence microscopy analysis; developmental- and tissue-specific activity of promoter demonstrated	Sunilkumar <i>et al.</i> , 2002b
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	Seed-specific RNAs of Cotton SAD-1 and Cotton FAD2-1, <i>nptII</i>	Southern, Northern, and biochemical analyses; seed oil with substantially higher stearic acid or oleic acid levels	Liu <i>et al.</i> , 2002b
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	Soybean lectin promoter driving <i>gusA</i>	Histochemical, biochemical, and Northern analyses; seed-specificity of promoter demonstrated	Townsend and Llewellyn, 2002
<i>Agrobacterium</i>	MCU5, DCH32, and Coker 310FR	Shoot tip from seedling/shoot regeneration in culture	<i>gusA</i> , <i>nptII</i>	Histochemical, PCR and Southern analyses and resistance of progeny to kanamycin for confirmation	Satyavathi <i>et al.</i> , 2002
<i>Agrobacterium</i>	Coker 312	C, H/somatic embryogenesis ^{(b)(c)}	Cotton β -tubulin promoter driving <i>gusA</i> , <i>nptII</i>	Histochemical analyses; preferential activity in fiber and root tip observed	Li <i>et al.</i> , 2002b
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Endochitinase gene from <i>Trichoderma virens</i> , <i>nptII</i>	Southern, Northern, and biochemical analyses; protection against <i>Rhizoctonia solani</i> and <i>Alternaria alternata</i> observed	Emami <i>et al.</i> , 2003
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	Sense and antisense suppression of sucrose synthase, <i>nptII</i>	Southern, immunolocalization, electron microscopy, and biochemical analyses; fiber-development inhibited	Ruan <i>et al.</i> , 2003
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	CaMV 35S promoter driving antisense <i>cdn1-C1</i>	Southern, Northern, and biochemical analyses; some reduction of gossypol and related terpenoids in the seed and leaves. Second report shows no correlation between the trait and presence of the transgene	Martin <i>et al.</i> , 2003; Benedict <i>et al.</i> , 2004
<i>Agrobacterium</i>	Coker 310FR	H and C/somatic embryogenesis ^{(a)(c)}	<i>nptII</i> , <i>gusA</i>	Southern for confirmation	Chaudhary <i>et al.</i> , 2003
<i>Agrobacterium</i>	Not stated	H/somatic embryogenesis ^(c)	<i>nptII</i>	Transformation and regeneration protocol provided	Wilkins <i>et al.</i> , 2004
Gene gun	Coker 310FR	H-derived friable callus/somatic embryogenesis ^(c)	Chloroplast-specific expression of <i>aphA-6</i> and <i>nptII</i>	PCR and Southern to confirm plastid genome transformation; strict maternal inheritance of Kanamycin resistance	Kumar <i>et al.</i> , 2004

(Continued)

Table 1 Summary of studies on transgenic cotton (*Continued*)

Transformation method	Cultivar	Target tissue/mode of transformant recovery	Transgenes	Comments	Reference
<i>Agrobacterium</i>	Coker 310	C- or H-derived embryogenic callus/somatic embryogenesis ^{(a)(c)}	<i>CryIa5</i> , <i>nptII</i>	Southern for confirmation; transgenic plants obtained in shorter time	Leelavathi <i>et al.</i> , 2004
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	<i>GF14λ</i> , <i>nptII</i>	Northern and Western analyses; obtained moderate drought tolerance	Yan <i>et al.</i> , 2004
<i>Agrobacterium</i>	Coker 312	H-derived embryogenic callus/somatic embryogenesis ^(c)	<i>gusA</i> , <i>nptII</i>	PCR and Southern for confirmation	Haq, 2004
<i>Agrobacterium</i>	G007	Pollen grain/fertilized zygotic embryo	<i>acsA</i> , <i>acsB</i> , <i>hpt</i> , <i>gusA</i>	PCR, Southern, and Northern for confirmation; improvements in fiber quality reported	Li <i>et al.</i> , 2004
Pollen-tube pathway	Xin-Cai, Lv-9902, Lv-9903	Pollen tube/fertilized zygotic embryo	GAFP, bar	Southern and RT-PCR for confirmation; resistance to Verticillium wilt reported	Wang <i>et al.</i> , 2004b
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Cotton <i>ghCTL2</i> promoter driving <i>gusA</i> , <i>nptII</i>	Preferential activity in different cell types during secondary wall deposition including lint fibers	Zhang <i>et al.</i> , 2004
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Seed-specific antisense of cotton FAD-2, <i>nptII</i>	Biochemical analysis; seed oil with higher oleic acid level	Sunilkumar <i>et al.</i> , 2005
<i>Agrobacterium</i>	Coker 315	C/somatic embryogenesis ^(a)	Soybean lectin promoter or CaMV 35S promoter driving antisense <i>cdh1-C4</i> , <i>nptII</i>	Southern, Northern, and Western analyses; no reduction in gossypol levels; induction of the target gene by bacterial blight was blocked	Townsend <i>et al.</i> , 2005
<i>Agrobacterium</i>	Coker 312	C, H/somatic embryogenesis ^{(b)(c)}	Synthetic antimicrobial peptide <i>D4E1</i> , <i>nptII</i>	Southern, PCR, and RT-PCR analyses; transgenic plants resistant to several fungal pathogens	Rajasekaran <i>et al.</i> , 2005
<i>Agrobacterium</i>	F846	Shoot tip from seedling/shoot regeneration in culture	Antisense of <i>AV2</i> , <i>nptII</i>	PCR and Southern for confirmation; some resistance to leaf curl virus reported	Sanjaya <i>et al.</i> , 2005
<i>Agrobacterium</i>	YZ-1	H-derived friable callus/somatic embryogenesis ^(c)	<i>gusA</i> , <i>nptII</i>	PCR and Southern for confirmation	Jin <i>et al.</i> , 2005
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Arabidopsis <i>NHX1</i> , <i>nptII</i>	PCR, Northern, and Western analyses; more biomass and more fiber produced under salt stress conditions	He <i>et al.</i> , 2005

Gene gun	7MH, CD-401, Antares and ITA94	SAM from mature seed/shoot regeneration in culture ^(c)	<i>Arabidopsis-ahas</i> gene, <i>gusA</i>	Selection on imazapyr. PCR, Southern and histochemical analyses for confirmation	Aragao <i>et al.</i> , 2005
<i>Agrobacterium</i>	Ekang 9 and Jihe 321	H-derived friable callus/somatic embryogenesis ^(c)	<i>CryIAC</i> , <i>API-B</i> , <i>nptII</i>	Southern for confirmation; transgenic plants showed resistance to cotton bollworm	Wu <i>et al.</i> , 2005a
Gene gun	Christina	Pollen grains/fertilized zygotic embryo	<i>hmgR</i> and <i>nptII</i>	PCR for confirmation.	Gounaris <i>et al.</i> , 2005
<i>Agrobacterium</i>	Coker 312	C, H/somatic embryogenesis ^{(a)(c)}	Cotton ACTIN1 promoter driving <i>gusA</i> , RNAi of <i>ghACT1</i> , <i>nptII</i>	Histochemical analyses; preferential activity observed in the fiber; RNAi-inhibition of fiber elongation	Li <i>et al.</i> , 2005
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Bean chitinase and <i>nptII</i>	PCR, Southern, Western, and biochemical analyses; <i>in vitro</i> inhibition of <i>V. dahliae</i>	Tohidfar <i>et al.</i> , 2005
<i>Agrobacterium</i>	Coker 312	H/somatic embryogenesis ^(c)	Tobacco glutathione S-transferase and <i>nptII</i>	PCR and Biochemical analyses; no protection to stress tolerance observed	Light <i>et al.</i> , 2005
<i>Agrobacterium</i>	YZ-1, Coker 312, and Coker 201	H-derived friable callus/somatic embryogenesis ^(c)	<i>nptII</i>	PCR and Southern for confirmation	Jin <i>et al.</i> , 2006
<i>Agrobacterium</i>	Zhongmiansuo 35	H/somatic embryogenesis ^(c)	Phloem-specific promoter driving <i>ACA</i> gene, <i>nptII</i>	Southern and Western for confirmation; resistance to cotton aphid observed	Wu <i>et al.</i> , 2006a
<i>Agrobacterium</i>	Cukurova 1518	SAM from seedling/shoot regeneration in culture ^(c)	<i>gusA</i> , <i>nptII</i>	Histochemical and PCR analyses for confirmation	Yuceer and Koc, 2006
<i>Agrobacterium</i>	Zhongmian 35	H/somatic embryogenesis ^(c)	<i>aroA-M1</i>	PCR, Southern, and Western for confirmation; transformants selected on glyphosate and transgenics resistant to this herbicide	Zhao <i>et al.</i> , 2006
<i>Agrobacterium</i>	G9803	H/somatic embryogenesis ^(c)	<i>GhExp1</i> , <i>nptII</i>	Southern and RT-PCR for confirmation	Zhu <i>et al.</i> , 2006
<i>Agrobacterium</i>	Coker 312	H, P/somatic embryogenesis ^{(c)(d)}	Seed-specific RNAi of cotton δ -cadinene synthase, <i>nptII</i>	Southern, RT-PCR, Northern, and biochemical analyses for confirmation; over 98% reduction in the seed gossypol level obtained	Sunilkumar <i>et al.</i> , 2006

^(a)C, cotyledon^(b)ESC, embryogenic cell suspension^(c)H, hypocotyls^(d)P, cotyledonary petiole^(e)SAM, shoot apical meristem

however, that regeneration of cotton plants from cultured tissue via somatic embryogenesis still remains highly genotype dependent (Trolinder and Xhixian, 1989). Information presented in Table 1 suggests that transformation of cells within hypocotyl or cotyledon segments via *Agrobacterium* method, selection and proliferation of transformed cells in culture, followed by somatic embryogenesis appears to be the most popular means of creating transgenic cotton. This seems to be the method of choice even in industrial laboratories. Some investigators have successfully utilized microprojectile bombardment (gene gun or biolistics) or *Agrobacterium* to transform embryogenic suspension cells and then recovered the transformed cotton plants via somatic embryogenesis (Finer and McMullen, 1990; Rajasekaran *et al.*, 1996).

2.1.2 Transformation of cells within the shoot apices to generate transgenic cotton plants

As described earlier, plant recovery systems based on somatic embryogenesis from cultured tissues are time and resource intensive and highly genotype dependent. It is precisely these difficulties with regeneration that have led many researchers to seek alternative procedures for the recovery of transgenic cotton plants following transformation. Some studies have reported direct transformation of cells in the shoot apical meristem, either via the gene gun or *Agrobacterium* method. Since plants can be recovered from these tissues fairly easily (Gould *et al.*, 1991), it was hoped that this system would make transformation of cotton more genotype independent. Particle bombardment studies have provided concrete evidence showing that this method can transform either epidermal cells of the L1 layer or the germline progenitor cells in the L2 or L3 layer (McCabe and Martinell, 1993; McCabe *et al.*, 1998). The progeny from L1 transformants did not inherit the transgene, while the germline transformants (recovered from transformation of L2/L3 cells) were able to pass on the transgenic trait to subsequent generations. These studies also suggest that the primary transformants recovered from these shoot apices are chimeric and the efficiencies of germline transformations are extremely low.

Three different laboratories have reported production of transgenic cotton plants via *Agrobacterium*-mediated transformation of the shoot apex (Zapata *et al.*, 1999; Satyavathi *et al.*, 2002; Yuceer and Koc, 2006). These reports have provided results from PCR and Southern analyses that suggest integration of transgenes. Transformation efficiencies as high as 60–70% were reported for three different cotton cultivars in the study conducted by Satyavathi *et al.* (2002). These rates would be considered high for any species, especially cotton. It should be noted that transformation efficiencies were based on the resistance of shoot tip explants to kanamycin, a highly unreliable measure of transformation in cotton. When shoot tip explants are used as the target tissues, the recovery of heritable transgenic events is expected to be due to the transformation of cells in the L2 or L3 layer of the shoot apical meristem. However, the ability of the shoot tip to survive kanamycin medium, as the sole criterion for transformation, can be misleading. This is because *Agrobacterium* can easily transform the cells within the wounded base of the shoot apex. In this situation, the shoot tip explant will be able to survive when cultured on kanamycin medium because of the protection conferred by the transformed cells within the wounded site at the base (unpublished results from K. Rathore's Laboratory). In addition, contaminating *Agrobacterium* cells can contribute to incorrect interpretation of the results from Southern analysis unless it is performed with proper controls and the use of an appropriate restriction enzyme. *Agrobacteria* are not always eliminated with antibiotic treatment, can persist in tissues following transformation, and are sometimes found even in the regenerated plants (Matzk *et al.*, 1996; Barrett *et al.*, 1997). None of the studies, involving *Agrobacterium*-mediated transformation of shoot apices, investigated the type of cells that were being transformed in the apical meristem. This is an important issue because the transformation of meristematic cells in the shoot apex remains controversial (Potrykus, 1991). As mentioned previously, gene gun studies conducted by scientists at Agracetus have shown that when the cells within the shoot apex are transformed, the resultant plant following regeneration is chimeric (McCabe and Martinell, 1993; McCabe *et al.*, 1998). By careful tracking of

the transgenic leaves and by selective pruning of nontransgenic branches, they were able to obtain a uniformly transformed mericlinal plant. The same principles should apply when *Agrobacterium* is used for transformation of cells in the shoot apex. Even if *Agrobacterium* is somehow able to transform deeper cells within the L2 or L3 layers of the shoot meristem, a chimeric plant will be produced. Without precise pruning of such a plant to eliminate nontransgenic branches, it will not be possible to obtain T₁ seeds that segregate in a Mendelian manner. Thus, it is important to identify the cell types within the shoot apex that are transformed by the *Agrobacterium* (*GFP* will make an excellent reporter gene here) and provide a clear-cut proof of integrative transformation. Addressing these critical aspects of the transformation process will ensure widespread adoption of the method because recovery of a normal, fertile plant from a shoot apex is relatively easy and genotype independent.

2.1.3 Alternative methods used to transform cotton

Regeneration of transformants is often the limiting step in the creation of transformed plants in any species and it is especially true in the case of cotton. Therefore, attempts have been made to completely bypass the *in vitro* regeneration process to produce transgenic cotton by introducing the transgene(s) into pollen/pollen tube. Gounaris *et al.* (2005) reported production of transformed cotton by using pollen that had undergone microprojectile bombardment to introduce the transgene. Results from PCR analysis were presented as proof of transformation. Without additional molecular and genetic proofs and some data on the transformation efficiency, it is difficult to assess the effectiveness of this method for cotton transformation. Li *et al.* (2004) described a method that involved direct co-cultivation of pollen grains of brown cotton with *Agrobacterium* under vacuum and then pollination of emasculated flowers with the pollen/*Agrobacterium* mixture. Seeds obtained from plants pollinated in this manner were screened on antibiotic medium to select transformants. However, given the fact that pollen from most commercial varieties of

cotton is extremely susceptible to bursting in an aqueous environment, the general applicability of this method remains questionable. Wang *et al.* (2004b) described a rather simplistic approach that required direct application of naked plasmid bearing the transgene to the stigmatic surface. Seeds obtained from the treated flowers were germinated on a medium containing the herbicide, BastaTM for the identification of transformants. Although the technique described is extremely simple and does not require a biological vector such as the *Agrobacterium*, it is difficult to envision how the transgenes are delivered into the target cells without application of any physical or chemical treatments. The nature of the target cell(s) is also unclear in this system. Without a clear understanding of the transfer mechanism and solid proof of integrative transformation, it is difficult to assess the suitability of these alternative methods for performing cotton transformations in a routine manner.

2.2 Regeneration of Cotton Plants from Transformed Cells

An important aspect of genetic engineering is efficient recovery of whole plants from the cells that have undergone stable transformation following the introduction of a transgene by physical, chemical, or biological means. This section will briefly describe the somatic embryogenesis-based methods for the recovery of plants following transformation. The reader is referred to several excellent accounts of cotton regeneration from cultured tissues for a more comprehensive understanding of the process (Trolinder and Goodin, 1987; Trolinder and Goodin, 1988a, b; Firoozabady and DeBoer, 1993; Wu *et al.*, 1998; Mishra *et al.*, 2003). Some alternative methods related to the recovery of transgenic plants have been described earlier in the chapter and are not repeated in this section.

The reports included in Table 1 show that somatic embryogenesis from cultures derived from transformed cotyledonary cells or hypocotyls cells remains the most popular means to obtain transgenic cotton (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987; Cousins *et al.*, 1991; Bayley *et al.*, 1992; Lyon *et al.*, 1993; Sunilkumar and Rathore, 2001; Liu *et al.*, 2002b; Townsend and

Llewellyn, 2002; Townsend *et al.*, 2005; Rathore *et al.*, 2006; Sunilkumar *et al.*, 2006). Also, in some cases where the target tissue for transformation is either hypocotyl/cotyledon-derived embryogenic suspension culture or embryogenic callus culture, the recovery of the transformants is via somatic embryogenesis (Finer and McMullen, 1990; Rajasekaran *et al.*, 1996; Leelavathi *et al.*, 2004). In a majority of these studies, Murashige and Skoog (MS) medium with some variation was used for initiating and maintaining the cultures. A combination of an auxin (2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthaleneacetic acid, or indole-3-butyric acid) and a cytokinin (kinetin or N6-(2-isopentenyl)adenine) is used for callus induction, proliferation, and obtaining embryogenic cultures. Usually, a hormone-free medium in combination with additional KNO₃ is used for somatic embryo development. Some laboratories utilize an intermediate suspension culture phase (Cousins *et al.*, 1991; Bayley *et al.*, 1992; Payton *et al.*, 1997; Wilkins *et al.*, 2004) to hasten embryogenesis and to increase the number of somatic embryos. However, the maintenance of suspension culture necessitates the use of shakers and the method involves pipetting and fractionation steps. These procedures are not only more labor intensive, they add to the risk of contamination and, therefore, many laboratories maintain their cultures on semisolid medium throughout the regeneration process (Firoozabady *et al.*, 1987; Thomas *et al.*, 1995; Sunilkumar and Rathore, 2001; Townsend and Llewellyn, 2002; Rathore *et al.*, 2006). Although cells at the wound site in the cotyledon-, hypocotyl-, or cotyledonary petiole explants are highly amenable to *Agrobacterium*-mediated transformation, the recovery of plants from these cells in cotton depends on somatic embryogenesis. However, this type of regeneration process is genotype dependent, highly labor intensive, and necessitates lengthy culture periods. It is possible to shorten the period between transformation step and the recovery of transgenic cotton plant by using pre-established embryogenic calli or suspensions as the target explants for *Agrobacterium* or the gene gun (Rajasekaran *et al.*, 2000; Leelavathi *et al.*, 2004). However, this procedure necessitates periodic initiation of callus cultures and regular maintenance of embryogenic callus/suspension cultures. In order to bypass the problems encountered with somatic

embryogenesis, direct transformation of cells within the shoot apical meristem has been attempted. These methods with their associated strengths and weaknesses have been described in the previous section.

2.3 Selectable Marker Genes Used for Generating Transgenic Cotton

The list of papers presented in Table 1 suggests that neomycin phosphotransferase II (*nptII*) gene, the first selectable marker gene used to create transgenic cotton, remains a popular choice. Its widespread popularity in obtaining transgenic cotton is due to the fact that kanamycin-based selection is relatively inexpensive and does not have adverse effects on the regeneration of plants from cultured tissues. The hygromycin phosphotransferase (*hpt*) has been used as a selectable marker gene in a few studies suggesting its suitability in generating transgenic cotton (Finer and McMullen, 1990; Li *et al.*, 2004). There are two reports listed in the table involving incorporation of the *bar* (bialaphos resistance) gene into cotton; however, in both cases this gene was not used in the initial selection of transformants (Keller *et al.*, 1997; Wang *et al.*, 2004b). An interesting use of a different herbicide resistance gene, *Arabidopsis-ahas*, was described by Aragao *et al.* (2005), who used it in conjunction with gene gun-mediated transformation of shoot apical meristem in cotton. The *nptIII* gene, present in most of the GM cotton products, has been shown to be safe for both the environment and the consumer. Therefore, it will continue to be used as a selectable marker gene for routine cotton transformation. However, if needed, a variety of means that has been tested in other species is available to generate marker-free transgenic cotton plants (Yoder and Goldsbrough, 1994; Ebinuma *et al.*, 2001; Breitler *et al.*, 2004).

2.4 Reporter Genes Used in Cotton

Chloramphenicol acetyltransferase (*cat*) gene, widely used as a reporter gene in animal systems, was also used in the first published study on cotton transformation (Umbeck *et al.*, 1987). In the same year, Jefferson *et al.* (1987) published

a report describing the use of β -glucuronidase (*gusA*) as a reporter gene in plant systems. This gene allowed monitoring of transformation by either a simple histochemical method or a fluorescence-based, highly sensitive, quantitative assay. The information provided in Table 1 shows that this remains the gene of choice to evaluate various transformation methods as well as for the characterization of promoter activities in various tissues in cotton (Lyon *et al.*, 1993; McCabe and Martinell, 1993; Sunilkumar *et al.*, 2002a; Li *et al.*, 2005). In fact, in studies involving gene gun-mediated transformation of shoot apical meristems where the primary transformants recovered are transgenic chimeras, the selection of transformed tissues was based entirely on the monitoring of *gusA* activity and selective pruning of the nontransformed parts (McCabe and Martinell, 1993). Its use for transgenic research in cotton will continue because of the simple and relatively inexpensive assays to monitor GUS activity. The more recently developed marker GFP gene, which allows noninvasive monitoring of its expression (Chalfie *et al.*, 1994; Haseloff *et al.*, 1997), has not been widely utilized in cotton. Despite its superiority, the need for an expensive microscope with fluorescence capability discourages its widespread use. Nevertheless, the utility and versatility of this reporter gene was elegantly demonstrated by revealing the tissue- and development-specific activity of cauliflower mosaic virus (CaMV) 35S promoter in cotton (Sunilkumar and Rathore, 2001; Sunilkumar *et al.*, 2002b). The use of GFP and other related fluorescence reporter genes will increase in future as the fluorescence microscopy becomes more affordable.

2.5 Traits Introduced into Cotton Through Genetic Transformation

2.5.1 Insect resistance

Bollgard or Bt cotton, introduced by Monsanto in 1996, was one of the first commercially successful GM products (Perlak *et al.*, 1990; Jones *et al.*, 1996; Hardee and Herzog, 1997; Benedict and Altman, 2001). These plants expressed *CryIAc* gene from *Bt* whose protein product (a δ -endotoxin) is toxic mainly to tobacco budworm

and American bollworm. The success of this product provided a powerful demonstration of the power of biotechnology in addressing an important agronomical problem whilst helping the environment by reducing our dependence on harmful chemicals. In fact, the problem of Lepidopteran insect pests is so severe in all cotton-growing areas of the world that Bt cotton technology is usually the first GM crop to receive approval from the respective regulatory agencies. Bollgard II, which in addition to *CryIAc* also contains *Cry2Ab*, became available in 2003 (Micinski *et al.*, 2006; Robinson, 2006). This second *Bt* gene extends the resistance to fall armyworm, beet armyworm, cabbage looper, and soybean looper (Perlak *et al.*, 2001). Syngenta has recently developed a new generation of genes from *Bt* called the vegetative insecticidal protein (VIP; Estruch *et al.*, 1996). The *VIP* gene encodes an exotoxin that is structurally, biochemically, and functionally different from the Bt δ -endotoxins and exhibits insecticidal activity against a variety of Lepidopterans (McCaffery *et al.*, 2006). Another insect-resistant cotton has been developed by Dow AgroSciences by combining *CryIF* and *CryIAc* genes. This product, WideStrike cotton, also confers resistance to several Lepidopteran pests (Bacheler *et al.*, 2006; Micinski *et al.*, 2006). A wider choice of more than one insect resistance genes, especially if they are stacked, will help delay build up of resistance in the target insects.

2.5.2 Herbicide resistance

Roundup Ready cotton, introduced in 1997 by Monsanto, confers resistance to glyphosate-based herbicide (Nida *et al.*, 1996). This trait was engineered by expressing *Agrobacterium* sp. strain CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) under the control of FMV 35S promoter. Glyphosate-tolerant cotton helps in the effective management of weeds and was also quickly adopted by cotton growers in the United States. Insect resistance and the herbicide resistance traits are also available in combination. Since their introduction a decade ago, GM cottons with these two traits either combined or separate are grown on 80% of the acreage devoted to cotton in the United States, and their share is rapidly increasing in other cotton-growing

countries (James, 2004). Although Roundup Ready cotton provides sufficient tolerance to glyphosate applications during the vegetative state of plant development, late-stage application of the herbicide causes male sterility due to weak activity of the FMV 35S promoter in male reproductive tissues (Chen *et al.*, 2006). However, this weakness has been overcome by engineering an additional expression cassette that contains the CP4-EPSPS gene under the control of *Arabidopsis* elongation factor-1 α promoter (Chen *et al.*, 2006). This doubled-cassette cotton, that allows safe application of the herbicide well beyond the 5-leaf stage, was marketed as Roundup Ready Flex[®] cotton in 2006. In addition to these two commercially successful traits, there are a number of reports from both industrial and academic laboratories on successful engineering of cotton with useful genes (Table 1). These include genes that confer resistance to four different types of herbicides, namely 2,4-D, bromoxynil, phosphinothricin/bialaphos, and imidazolinone/sulfonylurea (Bayley *et al.*, 1992; Lyon *et al.*, 1993; Panter *et al.*, 1996; Rajasekaran *et al.*, 1996; Keller *et al.*, 1997). Of these, only phosphinothricin/bialaphos-tolerant cotton, developed by Bayer CropScience and marketed by FiberMax under the name LibertyLink[®], is available commercially (Perkins, 2004).

2.5.3 Resistance to pathogens and abiotic stresses

There are some reports on engineering of cotton to confer resistance to various fungal diseases (Murray *et al.*, 1999; McFadden *et al.*, 2000; Emani *et al.*, 2003; Wang *et al.*, 2004b; Rajasekaran *et al.*, 2005). Although these studies appear quite promising, in each case, the transgenic trait confers protection against only a limited spectrum of pathogens. A number of studies have been conducted that attempted to engineer cotton plant to confer resistance to abiotic stresses that include freezing, waterlogging, salt stress, and drought (Ellis *et al.*, 2000; Korniyev *et al.*, 2001, 2003a, b; Logan *et al.*, 2003; Payton *et al.*, 2001; Yan *et al.*, 2004; He *et al.*, 2005; Light *et al.*, 2005). However, these investigations have had varying degrees of success in making the cotton plants tolerant to the intended environmental stresses.

2.5.4 Fiber modification

Cotton is grown mainly for its fiber and, therefore, there is an obvious interest in applying biotechnological tools to alter fiber traits for superior product performance. The desired properties of cotton fiber include strength, fineness, length, and uniformity. Dye binding, wrinkle resistance, and shrinkage resistance are additional characteristics that will help cotton fiber compete effectively against synthetic fibers. The number of genes involved in controlling some of these traits is likely to be large and the mechanism controlling these characteristics is expected to be complex. As more genes involved in fiber initiation, elongation and development become available and are characterized, their coding sequences and promoters will be used in future for engineering cotton to address issues related to fiber quality improvement. Nevertheless, some interesting work to modify cotton fiber has been already conducted by scientists at Agracetus, Inc. and others. Three different strategies were used to alter fiber characteristics (John, 1998, 1999). The first approach involved reduction in the expression of certain fiber-specific genes; however, this manipulation did not result in any obvious changes in the fiber properties (John, 1999). Endogenous levels of hormones play important roles in the growth and development of various cell types including fiber cells. However, genetically engineered, enhanced levels of auxin and cytokinins did not influence fiber elongation, strength, or micronaire (John, 1999). The third approach included synthesis of novel biomaterials in the fiber. This research involved expression of genes from *Alcaligenes eutrophus* in developing cotton fibers that resulted in the deposition of poly-D-(–)-3-hydroxy-butyrate (PHB) in their lumens (John and Keller, 1996; Rinehart *et al.*, 1996). This modification resulted in a fiber with altered thermal properties such that its insulating characteristics were enhanced (Chowdhury and John, 1998). Although this modified fiber cotton has not been commercialized, this successful engineering feat demonstrates the feasibility of improving cotton fiber in ways that is not possible by traditional breeding methods. Recent studies by some other groups involving manipulation of fiber-specific gene expression in cotton also suggest that it will be possible to genetically engineer cotton

to modify its fiber characteristics (Ruan *et al.*, 2003; Arioli, 2005; Li *et al.*, 2005). A number of laboratories around the world are conducting gene isolation/identification/characterization, EST sequencing, and functional genomics studies related to the cotton fiber (John and Crow, 1992; Wilkins, 1993; Wan and Wilkins, 1994; Hasenfratz *et al.*, 1995; John, 1995; John and Keller, 1995; Ma *et al.*, 1995, 1997; Pear *et al.*, 1996; Song and Allen, 1997; Shimizu *et al.*, 1997; Orford and Timmis, 1997; Kawai *et al.*, 1998; Orford and Timmis, 1998; Smart *et al.*, 1998; Delmer, 1999; Loguercio *et al.*, 1999; Orford *et al.*, 1999; Whittaker and Triplett, 1999; Wilkins and Jernstedt, 1999; Liu *et al.*, 2000; Orford and Timmis, 2000; Cui *et al.*, 2001; Delmer and Haigler, 2002; Harmer *et al.*, 2002; Li *et al.*, 2002a; Zhao and Liu, 2002; Ji *et al.*, 2003; Suo *et al.*, 2003; Zhang *et al.*, 2003; Zhu *et al.*, 2003; Arpat *et al.*, 2004; Kim and Triplett, 2004; Ruan *et al.*, 2004; Sun *et al.*, 2004; Wang *et al.*, 2004a; Zhang *et al.*, 2004; Haigler *et al.*, 2005; Hsu *et al.*, 2005; Humphries *et al.*, 2005; Ruan, 2005; Wilkins and Arpat, 2005; Wu *et al.*, 2005b; Udall *et al.*, 2006; Wu *et al.*, 2006b; Yang *et al.*, 2006; Burr, 2000). Expression of only a handful of the genes identified from these investigations has been studied in detail thus far. Functional analysis involving overexpression and/or RNAi-mediated silencing of individual genes should be possible now that cotton transformation has become routine in several laboratories around the world. As additional genes (and their promoters) that are involved in fiber growth and development are characterized, these will be employed to genetically engineer the cotton plant to obtain desired fiber properties.

2.5.5 Cottonseed modification

An important and abundant byproduct of fiber production is the cottonseed. A substantial portion of the global cottonseed output is used for edible oil extraction. Gene silencing technologies have been used to alter cottonseed oil fatty acid composition in favor of higher oleic acid. Sunilkumar *et al.* (2005) used antisense technology to double the oleic acid from a wild-type level of ~15% to ~30% while reducing the linoleic acid level from ~55% to ~35%. A more powerful RNAi-mediated silencing of the same target gene

resulted in a fivefold increase in oleic acid level and a concomitant reduction in linoleic acid content (Liu *et al.*, 2002b). In the same study, RNAi-mediated down-regulation of the *ghSAD-1* gene resulted in >10-fold increase in stearic acid levels in cottonseed oil. These results show the possibility of improving the quality of cottonseed oil by modifying a biosynthetic pathway in a tissue-specific manner.

In addition to oil, cottonseed contains 23% protein that is of relatively high quality. Worldwide cottonseed output can potentially meet the protein requirements of 500 million people. However, the ability to utilize this protein-rich resource for food is hampered by the presence of toxic terpenoid, gossypol, which is unique to the tribe Gossypieae. This cardio- and hepatotoxic terpenoid, present in the cottonseed glands, renders the seed unsafe for human and monogastric animal consumption. Since traditional breeding methods may not be able to bring about a significant reduction in gossypol in a seed-specific manner, biotechnological approaches are being tested in many laboratories around the world to solve this long-standing problem of cottonseed toxicity. Most of these attempts over the last decade have been unsuccessful (see Townsend *et al.*, 2005 and references therein). However, in a recent breakthrough, the feat of selective and significant reduction of gossypol level in the cottonseed through metabolic engineering has been finally achieved (Sunilkumar *et al.*, 2006). This study utilized RNAi to disrupt gossypol biosynthesis in cottonseed tissue by interfering with the expression of the δ -cadinene synthase gene during seed development. Some of the RNAi lines obtained showed a 98% reduction in the levels of gossypol in the seed while maintaining this and other protective terpenoids in all other parts of the plant at the wild-type concentrations where they provide protection against insects and diseases. Several members of the δ -cadinene synthase gene family have been cloned from both, the diploid, *G. arboreum* and the tetraploid, *G. hirsutum* (see Townsend *et al.*, 2005 and references therein). In addition, genes encoding other enzymes in the terpenoid biosynthesis have been obtained from cotton (Liu *et al.*, 1999, 2002a; Luo *et al.*, 2001). Thus, it may also be possible to utilize one or more of these genes to modulate terpenoid biosynthesis in the seed or elsewhere in the cotton plant.

The results from oil and gossypol studies suggest that cotton plant, in addition to meeting the clothing requirements of humanity, can also play an important role in meeting its nutritional requirements in the future.

2.6 Intellectual Property Rights Related to Genetic Modification of Cotton

Plant biotechnology industry largely depends on the revenues generated by the sale of seeds from the crop plants that have been genetically modified. Currently, genetic modification involves introduction of a novel trait by overexpression of homologous or heterologous gene(s) and/or suppression of endogenous plant gene(s). Therefore, the basic research tools and techniques required for plant genome modification such as the methods for plant transformation and regeneration, genes that confer useful traits, and the DNA sequences needed to regulate the expression of the transgenes in either a constitutive-, tissue-specific, or temporal manner are the potential targets for obtaining patent rights. By creating biotechnology products and/or enabling technologies, companies as well as the academic laboratories have made contributions to the intellectual property resource for cotton improvement over the last two decades. This section will highlight some of the key patents that have been issued for cotton transformation techniques, the promoters and genes isolated from cotton for its improvement, and the genetically altered cotton plants with desired traits (Tables 2, 3, 4, and 5).

2.6.1 Cotton transformation techniques

The first paper on cotton transformation was published in 1987 by researchers from Agracetus (Umbeck *et al.*, 1987). They filed and obtained patents for this commercially valuable technique in several cotton growing countries including the United States, Brazil, China, and India. The company was granted a US patent on transgenic cotton plant produced by *Agrobacterium*-mediated transformation of hypocotyl segments followed by recovery of transformants via somatic

embryogenesis (US 5,004,863, April 1991; Table 2). Subsequently, upon filing a continuation application the company successfully obtained a broader patent (US 5,159,135). This patent covered all genetically engineered cotton plants and seeds regardless of the gene used for the transformation. Following the controversies over the broad nature of its coverage, the patents granted initially in the United States and India, were revoked in both countries in 1994 (NBIAP News Report, January 1995 (<http://www.isb.vt.edu/news/1995/news95.jan>); van Wijk, 1995). However, both the United States patents have been re-examined by the US Patent and Trademark Office (USPTO) and granted patentability of all of the claims listed in the original patents. Monsanto, in 1996, acquired Agracetus with its plant biotechnology assets including the key patent on cotton transformation. In 2001, Aventis CropScience was added as a joint owner of this cotton transformation patent as part of the settlement for the pending lawsuit filed against Monsanto (<http://www.seedquest.com/News/releases/usa/Monsanto/n3410.htm>). Bayer CropScience acquired Aventis CropScience in 2002, thereby becoming a co-owner of this patent.

After the first patent on cotton transformation was granted, various alternative methods were developed to create transgenic cotton plants. These involved the use of different gene transfer techniques or modifications in the original cotton transformation protocol such as use of a tissue explant other than hypocotyl for transformation, use of novel methods for regeneration of the transformed cells, transformation of cotton cultivars other than Coker, etc. Patents on the use of alternate explants for the *Agrobacterium*-mediated transformation protocol were obtained by several companies. A method developed by Calgene used *Agrobacterium* or the gene gun to transform hypocotyl segments excised from dark-grown cotton seedlings (US 5,846,797). Although transgenic plants were regenerated in this case via the usual somatic embryogenesis process, this method relied on media that was devoid of the phytohormones during co-cultivation and embryogenesis. Production of transgenic cotton plant from *Agrobacterium*-transformed cells in the “transition-region” of the cotton seedling has been patented by Southplains Biotechnologies

Table 2 Patents related to cotton transformation methods

Patent number	Issue date	Inventors	Assignee	Patent title
US 5,004,863	April 02, 1991	Umbeck	Agracetus Inc., USA	Genetic engineering of cotton plants and lines
US 5,159,135	October 27, 1992	Umbeck	Agracetus Inc., USA	Genetic engineering of cotton plants and lines
US 5,164,310	November 17, 1992	Smith <i>et al.</i>	Texas A&M University System	Method for transforming plants via the shoot apex
WO 97/43430	November 20, 1997	Chapman <i>et al.</i>	University of North Texas	A rapid in vitro regeneration scheme of cotton plants compatible with <i>Agrobacterium</i> -mediated transformation
US 5,846,797	December 8, 1998	Strickland	Calgene Inc, USA	Cotton transformation
EP 531,506	December 16, 1998	McCabe and Martinell	Monsanto Co., USA	Particle-mediated transformation of cotton
US 5,929,300	July 27, 1999	Burke <i>et al.</i>	Secretary of Agriculture, USA	Pollen-based transformation system using solid media
US 5,998,207	December 07, 1999	Reichert <i>et al.</i>	Mississippi State University	Method for transformation of cotton and kenaf and organogenic regeneration
US 5,986,181	August 24, 2000	Trolinder <i>et al.</i>	Southernplains Biotechnologies Inc., USA	Transformation and regeneration of fertile cotton plants
WO 00/53783	September 14, 2000	Jiao and Liu	Institute of Molecular Agrobiolgy, Singapore	<i>Agrobacterium</i> -mediated transformation of cotton with novel explants
WO 00/77230	December 21, 2000	Chen and Zhang	Institute of Molecular Agrobiolgy, Singapore	High efficiency <i>Agrobacterium</i> -mediated transformation of cotton using petiole explants
US 6,479,287	November 12, 2002	Reichert <i>et al.</i>	Mississippi State University	Method for transformation of cotton and organogenic regeneration
US 6,483,013	November 19, 2002	Reynaerts <i>et al.</i>	Bayer Bioscience, Belgium	Method for <i>Agrobacterium</i> -mediated transformation of cotton
US 6,573,437	June 03, 2003	Anderson <i>et al.</i>	Mycogen Corp., USA	Transformation of cotton plants
US 6,620,990	September 16, 2003	Rangan <i>et al.</i>	Mycogen Corp., USA	Transformation of cotton plants
US 6,624,344	September 23, 2003	Rangan <i>et al.</i>	Mycogen Corp., USA	Transformation of cotton plants
US 6,660,914	December 09, 2003	Rangan <i>et al.</i>	Mycogen Corp., USA	Transformed cotton plants
US 6,730,824	May 04, 2004	Petolino <i>et al.</i>	Dow Agrosciences LLC, USA	Whisker-mediated transformation of cotton embryogenic callus tissues and regeneration of plants thereof
US 6,858,777	February 22, 2005	Zhong <i>et al.</i>	Syngenta Participations AG, USA	Methods for stable transformation of plants
US 7,122,722	October 17, 2006	Trolinder <i>et al.</i>	Cotton Inc., USA	Methods for producing transgenic cotton plants using chilled apical shoot tips

Inc., USA (US 5,986,181). Bayer BioScience was granted a patent on *Agrobacterium*-mediated transformation of embryogenic callus itself that was derived from a hypocotyl tissue from the cotton seedlings (US 6,483,013). Furthermore, their method involved transformation in the presence of a plant phenolic compound (acetosyringone). Mycogen Corp., USA patented a protocol involving *Agrobacterium*-mediated transformation of hypocotyl, cotyledon, or immature zygotic embryo from the Acala cotton varieties (US 6,573,437). In subsequent patents, the company claimed the

use of the same method to transform additional cultivable cotton varieties (US 6,620,990, US 6,624,344, and US 6,660,914). The Institute of Molecular Agrobiolgy, Singapore was granted worldwide patents on cotton transformation that involved *Agrobacterium*-mediated transformation of callus tissues obtained from fibrous root explants or petiole explants (WO 00/53783 and WO 00/77230). In each case, the transgenic plants were regenerated via somatic embryogenesis.

As mentioned earlier, somatic embryogenesis is one of the limiting steps for the development of a

Table 3 Patents related to genes obtained from cotton

Patent number	Issue date	Inventors	Assignee	Patent title
WO 96/40924	December 19, 1996	McBride <i>et al.</i>	Calgene, Inc., USA	Cotton fiber transcriptional factors
US 5,792,933	August 11, 1998	Ma	Mississippi State University	Fiber-specific protein expression in the cotton plant
US 5,932,713	August 03, 1999	Kasukabe <i>et al.</i>	Toyo Boseki Kabushiki Kaisha, Japan and Texas Tech University	Cotton fiber tissue-specific genes
US 6,166,294	December 26, 2000	Kasukabe <i>et al.</i>	Toyobo Co. Japan and Texas Tech University	Cotton fiber tissue-specific genes
US 6,169,174	January 02, 2001	Hasegawa <i>et al.</i>	Nisshinbo Industries, Inc., Japan	Cotton plant gene
US 6,271,443	August 07, 2001	Stalker <i>et al.</i>	Calgene LLC., USA	Cotton and rice cellulose synthase DNA sequences
US 6,495,740	December 17, 2002	Arioli <i>et al.</i>	The Australian National University, Australia	Manipulation of cellulose and/or β -1,4-glucan
US 6,576,818	June 10, 2003	Stalker <i>et al.</i>	Calgene LLC, USA	Plant cellulose synthase and promoter sequences
US 6,828,476	December 07, 2004	Wilkins	University of California, USA	Cotton transcription factors and their uses
US 6,995,256	February 07, 2006	Li <i>et al.</i>	Temasek Life Sciences Laboratory Ltd., Singapore	Isolation and characterization of a fiber-specific actin promoter from cotton
US 6,998,478	February 14, 2006	Cai <i>et al.</i>	Temasek Life Sciences Laboratory Ltd., Singapore	Isolation and characterization of a fiber-specific β -tubulin promoter from cotton
US 7,060,874	June 13, 2006	Wilkins	University of California	Bioengineering cotton fiber properties
US 7,098,324	August 29, 2006	Haigler <i>et al.</i>	Texas Tech University	Chitinase encoding DNA molecules from cotton expressed preferentially in secondary walled cells during secondary wall deposition and a corresponding promoter

cotton transformation protocol that is universally applicable to a range of cotton cultivars. Therefore, several attempts were made to develop a cultivar-independent transformation method either by using shoot apex as the explant or by using direct organogenesis as a means to regenerate plants. McCabe and Martinel (EP 531,506) produced a transgenic cotton plant from the embryonic axis following transformation of the shoot apical meristem with a gene gun. The method was originally developed at Agracetus; however, the patent has been awarded to Monsanto following the acquisition. *Agrobacterium*-mediated transformation of shoot apices from monocots and dicots including the cotton plant was patented by Smith *et al.* (US 5,164,310). A variation on this method that involved treatment of apical shoot tips at 2–8 °C for 24 h prior to transformation with *Agrobacterium* or gene gun was patented by Cotton Incorporated (US

7,122,722). Syngenta Participations AG obtained a patent for a method of producing transgenic plants via multiple shoot formation from shoot meristem of several dicot plants including cotton (US 6,858,777). Reichert *et al.* of Mississippi State University describe a method for organogenic regeneration of cotton plants from leaf explants (US 5,998,207) or hypocotyl explants (US 6,479,287) that may be transformed with *Agrobacterium* or the gene gun. University of North Texas has been granted a patent on a regeneration method using explants of apical and/or nodal meristematic tissues that is compatible with *Agrobacterium*-mediated transformation (WO 97/43430). With the exception of the gene gun-based method patented by Agracetus/Monsanto (EP 531,506), the practical utility of the other alternative methods that are designed to bypass somatic embryogenesis remains uncertain in generating transgenic cotton plants in a routine manner (see

Table 4 Patents related to promoters isolated from cotton

Patent number	Issue date	Inventors	Assignee	Patent title
WO 96/40924 US 5,792,933	December 19, 1996 August 11, 1998	McBride <i>et al.</i> Ma	Calgene, Inc., USA Mississippi State University	Cotton fiber transcriptional factors Fiber-specific protein expression in the cotton plant
US 6,040,504	March 21, 2000	Rice <i>et al.</i>	Novartis Finance Corporation, USA	Cotton promoter
US 6,096,950 US 6,211,430 US 6,259,003	August 01, 2000 April 03, 2001 July 10, 2001	John John Fujisawa <i>et al.</i>	Monsanto Co., USA Monsanto Co., USA Toyo Boseki Kabushiki Kaisha, Japan and Texas Tech University	Cotton fiber-specific promoters FbLate promoter Cotton plant promoters
US 6,566,586 US 6,576,818	May 20, 2003 June 10, 2003	Stalker and Pear Stalker <i>et al.</i>	Calgene LLC, USA Calgene LLC, USA	Cotton expansin promoter sequence Plant cellulose synthase and promoter sequences
US 6,995,256	February 07, 2006	Li <i>et al.</i>	Temasek Life Sciences Laboratory Ltd., Singapore	Isolation and characterization of a fiber-specific actin promoter from cotton
US 6,998,478	February 14, 2006	Cai <i>et al.</i>	Temasek Life Sciences Laboratory Ltd., Singapore	Isolation and characterization of a fiber-specific β -tubulin promoter from cotton
US 7,098,324	August 29, 2006	Haigler <i>et al.</i>	Texas Tech University	Chitinase encoding DNA molecules from cotton expressed preferentially in secondary walled cells during secondary wall deposition and a corresponding promoter
US 7,132,526	November 07, 2006	Liu <i>et al.</i>	Temasek Life Sciences Laboratory Ltd., Singapore	Isolation and characterization of an anther-specific promoter (CoFS) in cotton

discussion related to the transformation of cells within the apical meristem in Section 2.1.2).

Dow Agrosociences LLC, USA used an alternate, nonconventional method to transform cotton. A US patent issued to the company describes the use of a whisker (silicon carbide) system for introducing transgenes into embryogenic callus cultures derived either from hypocotyl or cotyledon tissue from a cotton seedling (US 6,730,824). In a patent assigned to the Secretary of Agriculture, USA, a method to transform cotton pollen by *Agrobacterium* followed by pollination to obtain transgenic cottonseed is described. Using this method, they obtained one transgenic seed for every 3000 seeds tested (US 5,929,300).

2.6.2 Genes obtained from cotton

As a first step toward fiber quality improvement via genetic engineering, identification of genes involved in the development of fiber has been the primary focus of cotton molecular biologists. A

list of patents related to the genes obtained from cotton has been provided in Table 3.

Several useful genes that expressed specifically during various stages of fiber development have been identified in the last two decades. A joint patent was awarded to Texas Tech University and Toyo Boseki Kabushiki Kaisha, Japan for fiber-specific genes that expressed during fiber elongation stage (US 5,932,713 and US 6,166,294). Hasegawa *et al.*, isolated a transcription factor-like gene that was expressed during fiber formation in cotton (US 6,169,174). Wilkins (University of California) isolated six genes encoding MYB transcription factors from cotton (US 6,828,476). Expression of two of the MYB genes in transgenic tobacco increased the number, distribution, density, length, and morphology of trichomes. The patent covers the use of these genes to improve cotton fiber quality. Wilkins also isolated the following genes from cotton: phosphoenol pyruvate carboxylase, expansin, endoglucanase, xyloglucan endoglycosyltransferase, and pectin methyl esterase (US 7,060,874). Overexpression of

Table 5 Patents related to transgenic cotton

Patent number	Issue date	Inventors	Assignee	Patent title
US 5,188,960	February 23, 1993	Payne and Sick	Mycogen Corp., USA	<i>Bacillus thuringiensis</i> isolate active against lepidopteran pests, and genes encoding novel lepidopteran-active toxins
US 5,338,544	August 16, 1994	Donovan	Ecogen Inc., Germany	CryIIIB protein, insecticidal compositions, and methods of use thereof
US 5,474,925	December 12, 1995	John	Agracetus Inc., USA	Immobilized proteins in cotton fiber
US 5,495,070	February 27, 1996	John	Agracetus Inc., USA	Genetically engineering cotton plants for altered fiber
US 5,521,078	May 28, 1996	John	Agracetus Inc., USA	Genetically engineering cotton plants for altered fiber
US 5,602,321	February 11, 1997	John	Monsanto Co., USA	Transgenic cotton plants producing heterologous polyhydroxy(e) butyrate bioplastic
US 5,608,142	March 04, 1997	Barton and Umbeck	Agracetus Inc., USA	Insecticidal cotton plants
US 5,608,148	March 04, 1997	John	Agracetus Inc., USA	Transgenic cotton plants producing heterologous peroxidase
US 5,597,718	January 28, 1997	John <i>et al.</i>	Agracetus Inc., USA	Genetically engineering cotton plants for altered fiber
US 5,620,882	April 15, 1997	John	Agracetus Inc., USA	Genetically engineering cotton plants for altered fiber
US 5,633,435	May 27, 1997	Barry <i>et al.</i>	Monsanto Co., USA	Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases
US 5,827,514	October 27, 1998	Bradfish <i>et al.</i>	Mycogen Corp., USA	Pesticidal compositions
US 5,869,720	February 09, 1999	John	Monsanto Co., USA	Transgenic cotton plants producing heterologous peroxidase
US 5,880,275	March 09, 1999	Fischhoff <i>et al.</i>	Monsanto Co., USA	Synthetic plant genes from <i>Bt kurstaki</i> and method for preparation
US 5,981,834	November 09, 1999	John <i>et al.</i>	Monsanto Co., USA	Genetically engineering cotton plants for altered fiber
US 6,054,318	April 25, 2000	Murray <i>et al.</i>	CSIRO, Australia	Expression of the glucose oxidase gene in transgenic organisms
US 6,107,549	August 22, 2000	Feng <i>et al.</i>	Monsanto Co., USA	Genetically engineered plant resistance to thiazopyr and other pyridine herbicides
US 6,218,188	April 17, 2001	Cardineau <i>et al.</i>	Mycogen Corp., USA	Plant-optimized genes encoding pesticidal toxins
US 6,308,458	October 30, 2001	Volrath <i>et al.</i>	Novartis Finance Corporation, USA	Herbicide-tolerant plants and methods of controlling the growth of undesired vegetation
US 6,329,570	December 11, 2001	Martineau	Calgene, LLC, USA	Cotton modification using ovary-tissue transcriptional factors
US 6,448,476	September 10, 2002	Barry	Monsanto Co., USA	Plants and plant cells transformation to express an AMPA-N-acetyltransferase
US 6,472,588	October 29, 2002	Haigler and Holaday	Texas Tech University	Transgenic cotton plants with altered fiber characteristics transformed with a sucrose phosphate synthase nucleic acid
US 6,559,363	May 06, 2003	Allen <i>et al.</i>	Toyo Boseki Kabushiki Kaisha, Japan	Cotton plants with improved cotton fiber characteristics and method for producing cotton fibers from these cotton plants
US 6,563,022	May 13, 2003	Kasukabe <i>et al.</i>	Toyo Boseki Kabushiki Kaisha, Japan and Texas Tech University	Cotton plants with improved cotton fiber characteristics and method for producing cotton fibers from these cotton plants
US 6,703,540	March 09, 2004	Jacks <i>et al.</i>	Secretary of Agriculture, USA	Transformation of plants with a chloroperoxidase gene to enhance disease resistance
US 6,710,228	March 23, 2004	Yenofsky <i>et al.</i>	Mycogen Corp., USA	Cotton cells, plants, and seeds genetically engineered to express insecticidal and fungicidal chitin binding proteins (lectins)
US 6,753,463	June 22, 2004	Rangan <i>et al.</i>	Mycogen Corp., USA	Transformed cotton plants
US 6,818,807	November 16, 2004	Trolinder <i>et al.</i>	Bayer BioScience, Belgium	Herbicide tolerant cotton plants having event EE-GHI
US 6,943,282	September 13, 2005	Adang <i>et al.</i>	Mycogen Plant Science, Inc., USA	Insect resistant plants
US 6,974,898	December 13, 2005	Green <i>et al.</i>	CSIRO, Australia	Method of modifying the content of cottonseed oil
US 7,041,877	May 09, 2006	Anderson <i>et al.</i>	Hexima, Ltd., Australia	Defensin-encoding nucleic acid molecules derived from <i>Nicotiana glauca</i> , uses thereof and transgenic plants comprising same
US 7,053,270	May 30, 2006	Kasukabe <i>et al.</i>	Toyo Boseki Kabushiki Kaisha, Japan and Texas Tech University	Cotton plants with improved cotton fiber characteristics and method for producing cotton fibers from these cotton plants
US 7,091,400	August 15, 2006	Haigler and Holaday	Texas Tech University	Transgenic fiber producing plants with increased expression of sucrose phosphate synthase

expansin gene in a constitutive manner enhanced the fiber length and yield. The patent covers the coding sequences and the promoters from these genes as well as the use of these components to modify cotton fibers. Calgene LLC owns the intellectual property rights to cotton cellulose synthase gene, *CelA1* (US 6,271,443 and US 6,576,818). They isolated the catalytic subunit of cellulose synthase gene from cotton that expressed in developing fibers at the onset of secondary wall cellulose synthesis. Calgene obtained a worldwide patent on two cotton fiber-specific transcriptional factor genes *rac13* and *4-4* (WO 96/40924). Australian National University has been granted a patent on the catalytic subunit of cellulose synthase gene from various species including a complementary DNA (cDNA) sequence obtained from cotton (US 6,495,740). Mississippi State University patented cotton fiber-specific lipid transfer protein gene *GH3* (US 5,792,933). A fiber-specific actin gene, *CFACT1* and a β -tubulin gene, *CFTUB2* from cotton were patented by Temasek Life Sciences Laboratory, Singapore (US 6,995,256 and US 6,998,478). Haigler *et al.* (Texas Tech University) isolated genes encoding endogenous chitinase from cotton that expressed during the secondary wall deposition in cotton fibers (US 7,098,324).

2.6.3 Promoters isolated from cotton

The upstream regulatory sequences of the fiber specific and other genes from cotton constitute good source of promoters that can be used to control the expression of transgenes in desired cells/tissues or at certain stage of development. The promoters from the following fiber-specific genes isolated from cotton have been patented: lipid transfer protein genes *GH3*, *PLTP6*, and *PLTP12* (US 5,792,933); *KC03*, *KC18*, *KC22*, and *Gh3* genes (US 6,259,003); expansin gene that is expressed in developing fiber (US 6,566,586); cellulose synthase gene *CelA1* (US 6,576,818); endochitinase gene that specifically expressed during secondary wall deposition (US 7,098,324); actin gene (US 6,995,256); β -tubulin gene (US 6,998,478); *E6-3B* and *FbLate-2* genes (US 6,096,950); *FbLate* gene (US 6,211,430); and transcription factor genes *rac1* and *4-4* and a lipid transfer protein gene *ltp* (WO 96/40924).

In addition to the fiber specific promoters, a few other cotton promoters have been patented. The promoter region from the genes encoding the small subunit of ribulose biphosphate carboxylase *rbc-gY* and *rbc-gX* from cotton were patented by Novartis Finance Corporation (US 6,040,504). Temasek Life Sciences Laboratory, Singapore obtained a patent for an anther-specific promoter from the *CoFS* gene from cotton (US 7,132,526). A list of patents related to the promoters obtained from cotton has been provided in Table 4.

2.6.4 Genetically engineered cotton plants

A list of patents related to genetically engineered cotton plants has been provided in Table 5.

2.6.4.1 Fiber quality and productivity improvements

Fiber length, strength, and fineness are the three top qualities that determine value of the fiber in the textile industry. Several patents have been issued on the enhancement of some of these qualities by expressing certain genes in cotton fibers. Agracetus obtained patents on the introduction of fiber-specific promoter::reporter gene sequences as well as coding sequences of fiber-specific genes into the cotton genome (US 5,495,070 and US 5,521,078). Agracetus also expressed peroxidase gene in the lumen of seed floss fiber cell for the purpose of increasing the fiber strength (US 5,608,148 (Agracetus), US 5,869,720 (Monsanto)). They also obtained patents on the isolation of fiber-specific genes and their promoters as well as their overexpression/suppression in cotton fiber (US 5,597,718 (Agracetus), US 5,620,882 (Agracetus), and US 5,981,834 (Monsanto)). Calgene LLC patented a method to alter fiber dimension and strength by expressing isopentenyl transferase gene in cotton ovule tissues (US 6,329,570). Haigler and Holaday (Texas Tech University) patented a method for improving various fiber qualities by overexpressing or suppressing sucrose phosphate synthase gene in cotton fibers (US 6,472,588). Furthermore, these researchers were awarded a second patent that described a method to increase the ratio of cellulose to total dry weight components of cotton plants by expressing sucrose phosphate

synthase gene, thereby enhancing the yield and fiber quality (US 7,091,400). Joint patents awarded to scientists from Texas Tech University and Toyo Boseki Kabushiki Kaisha, Japan, described a method to enhance the fiber qualities such as length, fineness, or strength by expressing genes coding for peroxidase (US 6,559,363), catalase (US 6,563,022), or endoxyloglucan transferase gene from cotton (US 7,053,270) in cotton fibers.

2.6.4.2 Fatty acid manipulation to modify cottonseed oil

Cotton plant is also an excellent source of oil in addition to being a major source of fiber. However, cottonseed oil contains high levels of polyunsaturated fatty acids and high levels of palmitic acid. Green *et al.* (CSIRO) modified the fatty acid composition of cottonseed oil by reducing the expression of endogenous *GhFAD2-1* or *GhSAD-1* in cottonseed using various gene-silencing techniques (US 6,974,898).

2.6.4.3 Insect-resistant cotton plants

Monsanto produced transgenic cotton plants, commercially marketed as Bollgard cotton, that are resistant to Lepidopteran insect pests by expressing Bt crystal protein gene *CryIAC* (US 5,880,275). They also created and are marketing Bollgard II cotton with broader spectrum resistance against insects by adding a second Bt gene *Cry2Ab* (US 5,338,544; Perlak *et al.*, 2001) to the plants containing *CryIAC* gene. Agracetus had also produced transgenic cotton plants containing a Bt δ -endotoxin gene that provided resistance to *Heliothis zea* (US 5,608,142). Mycogen Corp. transformed cotton with pesticidal genes that encode barley, hevein, or nettle lectins (US 6,710,228). In 2005, Mycogen Plant Science, Inc. was awarded a patent that described creation of a transgenic cotton plant expressing crystal protein gene from *Bt* strain HD-1 or HD-73 (US 6,943,282). A different approach using a cDNA encoding floral defensin from *Nicotiana glauca* was used by Hexima, Ltd. to enhance the resistance of cotton plants to *Helicoverpa armigera* larvae (US 7,041,877). Recently, DOW

AgroSciences have begun to market WideStrike cotton that contains a combination of *CryIAC* and *CryIF* (US 5,188,960, US 5,827,514, US 6,218,188). As mentioned earlier, VIP is a new generation of insecticidal protein with a different mode of action. Syngenta Biotechnology Inc. and Delta & Pine Land Company are developing a transgenic cotton, VipCot™ (patent pending), by expressing *vip3A* gene from *Bt* in addition to *CryIAb*. This cotton provides a broader spectrum resistance against Lepidopteran insects.

2.6.4.4 Herbicide-resistant cotton plants

Monsanto patented a method for producing transgenic cotton that confers resistance to herbicide thiazopyr by expressing rabbit liver esterase gene (US 6,107,549). In 1996, Monsanto also produced and marketed transgenic cotton plants (Roundup Ready cotton) resistant to the herbicide glyphosate by expressing EPSPS gene from *Agrobacterium* sp. strain CP4 (US 5,633,435). Recently, Monsanto has released an improved glyphosate-resistant transgenic cotton variety called Roundup Ready Flex cotton. As mentioned earlier, the weakness related to male sterility in the Roundup Ready cotton has been overcome by engineering an additional expression cassette that contains the CP4-EPSPS gene under the control of *Arabidopsis* elongation factor-1 α promoter. This modification provides the male reproductive system an enhanced level of tolerance to glyphosate, thereby preventing the early boll loss that was observed in the Roundup Ready plants. Monsanto also obtained a patent on a cotton plant that was co-transformed with glyphosate oxidase (GOX) gene and *phnO* gene encoding AMPA-N-acetyltransferase. Expression of *phnO* gene conferred protection against bleaching that is induced by the degradation product, AMPA, that accumulates as a result of oxidation of glyphosate by GOX (US 6,448,476). Glyphosate-tolerant transgenic cotton plant was also produced by Mycogen Corporation by expressing an altered form of *AroA* gene from *Salmonella typhimurium* (US 6,753,463). Bayer BioScience obtained a patent in 2004 on the production of glufosinate herbicide-tolerant transgenic cotton plants by expressing *bar* gene from *Streptomyces*

hygrosopicus (US 6,818,807). This product is being marketed as LibertyLink cotton since 2004. An alternative strategy to control weeds through the use of a modified protoporphyrinogen oxidase gene has been patented by Novartis Finance Corporation (US 6,308,458).

2.6.4.5 Cotton fiber as a platform for the expression of novel proteins

In addition to its use in manufacturing textiles, cotton fiber can be utilized as a system for producing and storing useful proteins. Agracetus developed a system in which an industrial enzyme was expressed in the lumen of cotton fibers. The expectation is that the fibers containing the immobilized enzyme, packed into reaction columns can be used in industrial enzymatic processes. A US patent awarded to the company described the immobilization of recombinant β -glucuronidase and parathion hydrolase in transgenic cotton fiber (US 5,474,925). Furthermore, Monsanto created transgenic cotton that makes bioplastic molecule PHB in the fiber by expressing genes that encode ketothiolase, acetoacetyl CoA reductase and PHB synthase (US 5,602,321). The fibers from these plants had altered flexibility, stiffness, strength, absorbency, and thermal properties.

2.7 Benefits of Genetically Modified Cotton

Insect-resistant, Bollgard cotton (Jones *et al.*, 1996; Hardee and Herzog, 1997; Perlak *et al.*, 2001), herbicide (Buctril®)-resistant, BXN57 cotton (Panter *et al.*, 1996), and herbicide (glyphosate)-resistant, Roundup Ready cotton (James and Krattiger, 1996) were the first GM cottons marketed in the United States in 1996/1997. Cotton garnered the largest acreage for the first year of commercialization of GM crops in the United States (James, 1997). In the year 2004, GM cotton was planted on 80% of the US, 66% of the Chinese, 80% of the Australian, and 85% of the South African cotton acreage (James, 2004). Often, GM cotton is the first genetically engineered crop to receive approval for commercial planting in many other countries.

Although Bt-cotton varieties have been cultivated in the major cotton producing countries for

only 10 years or less, studies conducted thus far indicate that economic benefits to the farmers have been significant (Benedict and Altman, 2001; Pray *et al.*, 2002). There were substantial reductions in the number of pesticide applications in the United States following adoption of Bt-cotton. In six cotton-growing states in the United States following the introduction of Bt cotton in 1996, the number of insecticide treatments per season dropped from an average of 4.85 in 1995 to 1.88 in 1998 (Betz *et al.*, 2000). Similarly, following the introduction of Bt cotton, reductions of 3–5 sprays per ha per season have occurred in Australia (Benedict and Altman, 2001). In the developing countries, Bt cotton has increased the yields while decreasing the cost of production by reducing the pesticide use (Pray *et al.*, 2002; Brookes and Barfoot, 2005a; Gomez-Barbero and Rodriguez-Cerezo, 2006). Interestingly, smaller farms consistently obtained larger increases in net income compared to the larger farms in China by growing Bt cotton. Following its introduction in 2002 when Bt cotton was grown on 50 000 ha in India, the insect-resistant cotton plants were cultivated by 2.3 million small farmers on 3.8 million hectares in the year 2006 (James 2007). The average yield of cotton in India, one of the lowest yields in the world, increased from 308 kg ha⁻¹ in 2001–2002 to 450 kg ha⁻¹ in 2005–2006. Most of the improvement in yield has been attributed to the adoption of Bt cotton (James, 2007). The cost of pest control also fell in the developed countries due to adoption of insect-resistant cotton. However, the benefits of fewer pesticide applications, to some degree, were offset by the higher cost of GM cottonseeds resulting in smaller gains in the farm income for the growers, especially in the United States (Brookes and Barfoot, 2005a; Gomez-Barbero and Rodriguez-Cerezo, 2006). Nevertheless, the rapid adoption of GM varieties by cotton growers in both developed and developing countries clearly suggests significant benefits to the farmers. There have been many obvious and direct benefits of the Bollgard cotton including reduced use of broad-spectrum insecticides, lower farming risks and production costs, and better yields. An excellent analysis of these as well as indirect and less tangible advantages of this technology has been provided by Edge *et al.* (2001). Globally, the farm level impact of using Bt cotton was \$1.47

billion in the year 2004 (Brookes and Barfoot, 2005a, b).

Roundup Ready cotton, introduced in 1997, was also immediately accepted by cotton growers in the United States. As discussed earlier, the limitations of the original Roundup Ready cotton are expected to be overcome by Roundup Ready Flex cotton introduced in 2006. An alternative weed management system, LibertyLink cotton developed by Bayer CropScience, became commercially available in 2004. This cotton has been engineered to resist glufosinate ammonium-based herbicide, Ignite[®] (also called Liberty[®], Finale[®], Basta, and Rely[®]). Herbicide-tolerant cotton helps in the effective management of weeds and benefits the environment by encouraging the adoption of soil conservation practices. The enhancement in no-till acreage has been higher for cotton compared to any other crop (Sankula, 2006). This increase has largely been attributed to the availability of herbicide-resistant cotton. In addition, glyphosate, used on Roundup Ready cotton, is less toxic and more environmentally benign compared to the herbicides it replaces (Fernandez-Cornejo and Caswell, 2006). Herbicide-resistant cotton varieties are being grown in the United States, Australia, and South Africa and their share of the global cotton acreage is likely to increase in future (Brookes and Barfoot, 2005a).

Although real and significant, the benefits conferred by the two GM traits to the environment and in saving labor and fuel costs are not easily quantified (Gomez-Barbero and Rodriguez-Cerezo, 2006). However, analyses published by Edge *et al.* (2001) and Brookes and Barfoot (2005b) provide informative accounts of the impact of GM crops on the economics and the environment. GM varieties are expected to continue increasing their share of the global cotton acreage in the future as more Indian and Chinese farmers accept and adopt the GM technology and as more countries approve planting of GM crops.

2.8 New Technological Advances and Their Role in Cotton Improvement

Most transgenic crop plants including cotton have been generated by random integration of transgenes into their nuclear genomes. Although the commercial success of these first generation

GM crops has been astounding, new advances in biotechnology, molecular biology, and genomics promise even larger array of products in the future that are based on the alteration of organelle genome, more subtle changes in the nuclear genome in a very precise manner or gene knockout/silencing.

Transformation of the plastid genome has been reported for cotton recently (Kumar *et al.*, 2004). Compared to nuclear transformation, chloroplast transformation is more difficult and less efficient; however, it offers many advantages, including transgene containment because of maternal inheritance and a high level of consistent expression. By expressing two selectable marker genes to detoxify kanamycin in the green and nongreen tissues, Kumar *et al.* (2004) were able to regenerate cotton plants from cells containing plastids that were stably transformed. These transformants were fertile and more importantly, showed maternal inheritance of the transgene. Because of the lower efficiency and complexity of the plastid transformation system, widespread adoption of this technology is currently unlikely; however, in cases where a high level of transgene expression and/or its containment are desired, it will play an important role.

Modification of endogenous genomic sequences by homologous recombination or gene targeting has a lot of potential in plant improvement. Although efforts are being made to improve gene targeting in plants, it remains inefficient in comparison to what has been achieved in animal systems (Iida and Terada, 2005). However, in combination with a technology based on the use of designed zinc finger nucleases that can efficiently create double stranded breaks in a desired portion of the DNA, the efficiency of the targeting process can be substantially improved (Bibikova *et al.*, 2003). Recent successes with model plant species raise the possibility that the cotton genome can also be modified using this novel technology (Wright *et al.*, 2005). Another strategy for genome modification makes use of self-complementary, chimeric RNA/DNA oligonucleotides to create specific 1–2 bp alteration. This technology has been successfully used to mutate a gene encoding an enzyme of the branched-chain amino acid pathway in various plant systems to confer resistance to either imidazolinone or sulfonylurea herbicides (Beetham *et al.*, 1999; Zhu *et al.*, 1999;

Kochevenko and Willmitzer, 2003). Although promising, the utility of each of these technologies, especially to introduce recessive modifications, may be complicated by the fact that a majority of the commercial cotton varieties grown today are tetraploid.

The two examples of RNAi-mediated modification of seed-oil fatty acid composition and gossypol elimination show clearly that this newly discovered gene-suppression mechanism will play an important role in the future improvement of cottonseed. Once the genes involved in controlling various aspects of fiber growth and development are identified, RNAi will be an extremely valuable tool in engineering the desired characteristics in the fiber. In addition to the use of RNAi to improve the properties of the fiber and the seed, it may be possible to use this powerful gene silencing mechanism to address the problem of parasitic nematodes in cotton. Yadav *et al.* (2006) transformed tobacco plants to express dsRNA against important genes of an RKN that led to a virtual elimination of the target messenger-RNA (mRNA) in the parasite and significant resistance in the host plant. In another recent report, Huang *et al.* (2006) describe results of *in vivo* expression of dsRNA in *Arabidopsis* targeting an RKN gene that encodes *I6D10*, a secretory peptide essential for the nematode parasitism of the plant. These transgenic plants showed significant resistance to four major RKN species. It should be possible to engineer cotton plants in a similar way by targeting these particular genes or other gene(s) essential for parasitism to confer resistance to RKN.

2.9 Regulation of Agricultural Biotechnology

As research in plant biotechnology in the early 1980s geared toward developing commercial applications, the US government moved to provide guidance and a comprehensive regulatory oversight system. In 1984, the US government developed a coordinated framework to regulate agricultural biotechnology based on legislation that was already in place to protect the environment, agriculture, and food. This framework ensured that plants and plant products produced by biotechnology were as safe as their conventional counterparts for the environment, for agriculture,

and for use as food or feed. Since the authority to regulate agricultural biotechnology was based on existing laws, the involvement of each regulatory agency depends on the specific product and phase of product development.

2.9.1 US agencies involved in the regulation of agriculture biotechnology

United States Department of Agriculture (USDA), Food and Drug Administration (FDA), and Environmental Protection Agency (EPA) are the primary agencies that regulate plants and products derived from them that are generated using modern biotechnology. Details on the regulatory aspects related to agriculture biotechnology in the United States can also be obtained from the following links:

- <http://pewagbiotech.org/resources/issuebriefs/1-regguide.pdf>
- <http://www.agbioforum.org/v3n4/v3n4a15-belson.htm>

2.9.1.1 USDA

The Animal and Plant Health Inspection Service (APHIS) branch of USDA regulates agricultural biotech plants under the Plant Protection Act, a consolidation of 10 previous laws including the Plant Pest Act, the Plant Quarantine Act, and the Noxious Weed Act. The goal of the Plant Protection Act and its preceding acts is to protect US agriculture from the introduction of pests. Using the broad authority provided in the Plant Protection Act, plants that are genetically engineered are considered regulated articles under the APHIS regulations (7 CFR Part 304), if they meet the following definition:

Any organism which has been altered or produced through genetic engineering, if the donor organism, recipient organism, or vector or vector agent belongs to any genera or taxa designated in Section 340.2 and meets the definition of plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced

through genetic engineering which the (APHIS) Administrator determines is a plant pest or has reason to believe is a plant pest.

For any biotech plant meeting the definition of a regulated article, one must obtain approval from APHIS prior to importation, interstate movement, field release, or commercialization of the product. For details check the following websites: <http://usbiotechreg.nbi.gov/roles.asp> and <http://www.aphis.usda.gov>.

2.9.1.2 FDA

FDA has authority under the Federal Food, Drug, and Cosmetic Act to ensure the safety and wholesomeness of most foods, except meat, poultry, and eggs, including foods developed through modern biotechnology. Based on this authority, FDA issued a policy in 1992 on the assessment of the safety of "Foods Derived from New Plant Varieties" that was based on existing food law and required that genetically engineered foods meet the same rigorous safety standards required of all other foods. FDA's biotechnology policy treats genetically engineered substances that are intentionally added to food as food additives if they are significantly different in structure, function, or amount than substances currently found in food. For details check the following website: <http://www.cfsan.fda.gov>.

2.9.1.3 EPA

The BioPesticides and Pollution Prevention Division (BPPD) branch of the Office of Pesticide Programs (OPP) regulates the distribution, sale, use, and testing of plants and microbes producing pesticidal substances, including biotech plants, under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Federal Food, Drug and Cosmetic Act (FFDCA), and the Food Quality Protection Act (FQPA). The goal of these acts is to protect human health and to safeguard the natural environment. EPA ensures the safety of pesticides, both chemical and those that are produced biologically. This includes pesticidal proteins that are produced *in planta* such as Bt proteins. Under the FFDCA, EPA sets tolerance limits (safe upper bounds) for substances used as

pesticides on and in food and feed, or establishes an exemption from the requirement of a tolerance (any level is assessed to be safe). EPA also establishes tolerances for residues of herbicides used on herbicide-tolerant crops that create novel use patterns. For details check the following website: <http://www.epa.gov/pesticides>.

2.9.2 Regulatory oversight during product development in the US

Initial regulatory oversight for development and testing of biotech products in the laboratories and growth chambers are overseen by local peer-reviewed committees known as Institutional Biosafety Committees (IBCs). Such committees are mandatory for institutions that receive federal funding to support their biotech research and are used widely in the private sector on a voluntary basis. These committees are responsible for ensuring that the recombinant DNA research conducted or sponsored by the institute is compliant with the National Institutes of Health Guidelines for research involving recombinant DNA molecules (59 Federal Register 34496). For details see the article: http://www4.od.nih.gov/oba/rac/guidelines_02/NIH_Gdlnes.lnk_2002z.pdf.

For importation, interstate movement, or testing biotech plants in the field, approval must be obtained from USDA either in the form of a permit or notification, a streamlined version of the permit. The applicant must identify the plant species, genetic elements, the researchers, and locations of trials. Both the permit and notification process impose requirements to make certain that the movement of biotech material or fields trials are conducted in a manner that will ensure containment of the material, and that the material does not persist in the environment and thus does not negatively impact agriculture or the environment. For importation and interstate movement, precautions include clear markings for identification and double packaging for containment. For field trials, typical precautions include clearly marking the plot area, isolating the regulated plants from sexually compatible relatives, and monitoring for volunteer plants for an appropriate amount of time following the trial. If the size of the trials reaches 10 acres per

assessment of efficacy per pest, and the plant produces a pesticidal trait, an Experimental Use Permit may be required by EPA for such field testing and shipment of materials.

The field trial stage not only allows one to evaluate the performance of the biotech plant but also allows one to collect field data and samples for further study and analyses that will be required by the regulatory agencies if a request to commercialize the biotech plant is pursued.

2.9.3 Regulatory authorizations to allow for commercialization in the US

After collecting laboratory and field data on the biotech plant, documents may be submitted to the regulatory agencies requesting authorizations that allow the biotech plant to be commercialized. For example, cotton is grown as a row crop. So, USDA and EPA address the impacts on the environment and agriculture. While FDA addresses the use for food and feed of cottonseed and its products such as cottonseed oil and meal.

USDA. To allow for unconfined plantings, such as commercial use, biotechnology-derived plants must be deregulated by USDA-APHIS by petitioning the agency in a formal submission. The deregulation request (also referred to as a petition for a determination of nonregulated status) asks APHIS to no longer consider the biotech plant to be a regulated article and, thus, no longer subject to its regulations. The petition should contain information on the biology of the crop species, the transformation system, the introduced genes and regulatory sequences, the genetic analysis (molecular characterization), the agronomic performance, and the environmental consequences of the unconfined release of the regulated article. After review of the petition document, USDA prepares a draft decision document and an environmental assessment document and solicits input from the public. If deregulated, the product can be imported, shipped, and planted without permit or notification and allows for product commercialization pending input from FDA and EPA, if required based on the introduced trait.

FDA. As stated in their 1992 policy document, FDA has a voluntary assessment program that allows developers of biotechnology-derived plants

to consult with FDA to ensure that the agency has no questions regarding the food and feed safety of the biotech plant or its derived products. The consultation process may involve early discussions to determine the type of data needed for a particular product. In general, the agency requests information on the following: name and use of the crop, the inserted genetic material, the resulting phenotype, the expressed protein(s), the potential allergenicity and toxicity of the introduced protein(s), and the nutritional composition of the resulting product. Following a review of the data, FDA issues a memorandum stating that the consultation is complete and agency has no further questions. To date, all of the biotech plant products that have been commercialized have gone through the FDA consultation process.

EPA. A biotechnology-derived product with a pesticidal trait (e.g., the trait is intended to prevent, repel, or mitigate a pest) is referred to as a plant-incorporated protectant (PIP) by the EPA. For such products, EPA requires a registration for sale and distribution of the PIP and a tolerance or exemption from the requirement of a tolerance to allow the PIP to be used in food. EPA requires the registrant to submit data on the following: product characterization such as protein expression, mammalian toxicity, gene flow, nontarget effects, and environmental fate. For insecticidal traits, EPA requires the submission of a plan to delay development of resistance to the PIP. Following commercialization, EPA may require additional data and impose reporting obligations to ensure effective insect resistance management.

2.9.4 International regulations

Many countries have regulations for the importation, testing, and commercialization of biotechnology-derived plants. The specific requirements vary for each country depending on the specific regulatory action requested, such as approval for importation and/or production. In general, all regulating countries require specific data to be submitted to address the food, feed, and environmental safety of biotech products that would be coming into the country. An excellent account of legislation and regulations related to the release of GM crops in various countries has been provided by Nap *et al.* (2003).

3. FUTURE PERSPECTIVES

The first reports on cotton transformation were published in 1987, some 20 years ago. Since its introduction to the US farmers in 1996, cotton has been the most widely planted GM crop around the world. However, the current commercial GM varieties carry only the input traits benefiting largely the cotton growers. Of course, the associated environmental benefits have been significant. The research studies and patents described in this chapter show the efforts being made to engineer a number of useful output traits into cotton. As discussed earlier, strength, fineness, length, and uniformity are not the only desired fiber characteristics, it will also be necessary to incorporate properties such as wrinkle resistance, shrinkage resistance, color retention, flame resistance, etc. for this natural fiber to effectively compete against petroleum-based, man-made fibers. It may be possible to achieve these goals through "Rational Fiber Design" by integrating plant biotechnology with textile chemistry to create value-added fibers for existing and new applications (Cantrell, 2005). As new genes become available from cotton and other organisms and as the genetic modification technologies are refined and improved, it will become possible to incorporate additional input and output traits into this important crop to benefit growers, consumers, and the environment.

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Tobacco

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1. INTRODUCTION

In January of 1983, the Miami Winter Symposia series hosted the conference on “Advances in Gene Technology: Molecular Genetics of Plants and Animals.” This conference would serve as a forum for unveiling one of the milestones in plant biology research—the introduction of a bacterial gene into a plant species. Two groups, one led by Mary-Dell Chilton and another led by Jeff Schell and Marc Von Montagu presented evidence that a bacterial gene for antibiotic resistance had been successfully integrated into the genome of tobacco plants. Since this seminal breakthrough, tobacco has become a widely used system for studies of gene function. It is the established system for plastid transformation; however, *Arabidopsis* has become the system of choice for nuclear gene integration due to the ease of transformation and a short generation cycle. This review summarizes the use of tobacco in dissecting plant biology concepts pertaining to the three important compartments of the cell that harbor genetic material within them. The chapter

begins with familiarizing the reader with the sequence of events that led to the development of tobacco regeneration and transformation systems. Next, the utility of the transgenic tobacco system is discussed, detailing the seminal research that enlightened a new understanding of gene function based on important *in planta* observations.

2. TRANSGENIC RESEARCH IN TOBACCO

2.1 Development of a Regeneration System and the Discovery of Cytokinins

Tobacco and carrot are the original model systems for plant tissue culture. While regeneration by somatic embryogenesis was first demonstrated with carrot cultures, the principles underlying regeneration via organogenesis were developed with tobacco. Tobacco tissue culture also has a historic role in hormone biology as the bioassay system used in the discovery of cytokinins in Folke Skoog’s lab at the University of Wisconsin-Madison. Cell division factors could not have been

discovered without a system in which their effects would be easily and unambiguously ascertained. Moreover, efficient regeneration is a prerequisite for transformation in plants. By virtue of its effective regeneration protocol, tobacco was the first plant to be transformed and has for many years been an important model plant for testing of transgenes.

2.1.1 Historical perspective: regeneration and the discovery of cytokinins

Early work on plant tissue culture focused on enticing cells to divide in culture. Important results were published by three researchers in the 1930s. Nobécourt (1938, 1939) and Gautheret (1939) in France reported on the maintenance of carrot callus cultures while White (1939a) in the United States concentrated his research on tobacco. It should be noted that White's tobacco cultures were derived from the hybrid *Nicotiana glauca* × *Nicotiana langsdorfii*, which is prone to form tumors *in planta*. White (1939a) showed that when these tumor tissues are placed *in vitro*, cell division could be maintained. Although this study only demonstrated the ability of naturally tumorous tissues to continue to divide *in vitro*, it did set the stage for further experiments using cultured tobacco tissues, eventually leading to the discovery of cytokinins and their role in organogenesis.

White (1939b) observed the occasional formation of shoots on tumorous tissues submerged in liquid medium. Skoog, whose research had focused on auxins, showed that the tumorous tissues contained high levels of β -indoleacetic acid (IAA) and that addition of IAA to the liquid culture medium suppressed the sporadic formation of buds in these tissues (Skoog, 1944). Roots appeared in a few of the cultures, always attached to the basal end of shoots (Skoog, 1944). This was the first demonstration of complete plantlet formation from callus tissue.

Subsequent research was performed with *Nicotiana tabacum* W38, a genetically normal tobacco variety as opposed to the tumorous plants employed by White, which would infrequently form callus, roots, and buds on stem segments in culture. It was also observed that callus formation could be stimulated and bud formation suppressed by exogenous auxin (Skoog and Tsui,

1948). Interestingly, addition of adenine sulfate could overcome this inhibitory effect of IAA but to accomplish that, very high adenine levels were required (Skoog and Tsui, 1948). These observations led the authors to conclude that "formation of roots, buds, or undifferentiated growth of tissues can be obtained by the application of different proportions of auxin and adenine to the medium". This was the first clear support for the concept that relative levels of growth factors control plant differentiation.

Pith tissue, which is rich in parenchyma but lacks vascular bundles, responded poorly to IAA but cell division was highly stimulated when natural extracts, such as coconut or malt extract, were added (Jablonski and Skoog, 1954). The biologically active fraction in yeast extract was isolated by Carlos Miller and found to be rich in purines (Skoog, 1994), which was consistent with the effects of adenine observed earlier. Further efforts by Miller in Skoog's lab focused on isolation of purines from natural substances and testing for their activity in tobacco cultures. The most potent substance was contained in autoclaved herring sperm DNA. The active compound was identified as N-furfuryladenine and its structure was subsequently confirmed by its chemical synthesis (Miller *et al.*, 1955a, b, 1956). The compound was given the name "kinetin" and was the first of a new class of plant growth regulators, named cytokinins for their ability to promote cytokinesis (Skoog *et al.*, 1965).

The discovery of kinetin led to an upsurge in plant tissue culture research. Skoog and Miller (1957) showed in the classical experiment that kinetin and auxin together could give rise to shoots, roots, or callus, depending on the concentrations and ratios between the two types of compounds. High kinetin with low IAA resulted in shoot formation, low kinetin with high IAA in root formation, and high concentrations of both in callus formation. Since that time, this principle was found to apply generally to regeneration of many plant genera through organogenesis. Application of plant growth regulators to control cell division and differentiation is used as an essential tool for plant biotechnology, from micropropagation to transformation.

Related research resulted in further refinements in tissue culture methods. To optimize callus growth, a chemically defined formulation was

devised based on analysis of ash from tobacco tissues (Murashige and Skoog, 1962). Further tests of organic nutrient requirements indicated that besides auxin and cytokinin, the only compounds necessary for tobacco tissue cultures, are sucrose, *myo*-inositol, and thiamine (Linsmaier and Skoog, 1965). This Murashige and Skoog (MS) mineral nutrient medium is still the most versatile and widely used plant tissue culture medium. Although many modified mineral media were developed after 1962 and given different names, they were often based on the MS medium. For more details on the development of the tobacco tissue culture system and the discovery of cytokinins, the reader is referred to Skoog (1994) and Armstrong (2002).

2.1.2 Development of a transformation system

Tobacco transformation was made possible by availability of a regeneration system as well as years of research on *Agrobacterium*, starting with the demonstration by Braun (1958) that tobacco crown gall tissues could grow in the absence of cytokinin and auxin even when *Agrobacterium* was eliminated from the tissues. This clearly showed that the tumor-inducing factors were maintained in the tissues even after many cell divisions. It should be noted that tobacco is an excellent host for *Agrobacterium*, forming large galls upon infection of stems, and has been used widely for crown gall research. An extensive treatise of *Agrobacterium*-based vector development is beyond the intention of this chapter and only a few of the important advances leading to tobacco transformation will be highlighted.

Two milestones on the road to transformation were the isolation of the *Agrobacterium* Ti plasmid (Zaenen *et al.*, 1974) and the demonstration that bacterial DNA was inserted in the DNA of host cells (Chilton *et al.*, 1977). They formed the basis for the identification of the essential genes inside as well as outside the transferred region and for the design of modified vectors for plant transformation. Also crucial to development of suitable gene vectors was incorporation of selectable markers, such as resistance to kanamycin (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983), which

made it possible to delete the tumor-forming genes. These and many other important contributions culminated in production of transformed tobacco and petunia plants that developed normally and formed progeny segregating for the transgene in a Mendelian fashion (De Block *et al.*, 1984; Horsch *et al.*, 1984). The procedures used to generate the transformants involved co-cultivation with protoplasts.

The tobacco protoplast transformation system was soon replaced by a much simpler system involving incubation of leaf discs with *Agrobacterium* (Horsch *et al.*, 1985, 1988). Tobacco leaf discs respond very well to cytokinin and auxin, just like the pith or callus tissues used by Skoog and Miller (1957). Shoots, roots, and callus can be obtained on appropriate combinations of the two hormones (Figure 1). Shoot formation at optimal growth regulator concentrations is very prolific, which is essential to efficient transformation. Moreover, callus formation is limited at concentrations used for shoot formation and thus the occurrence of somaclonal variants is minimized.

At about the same time as *Agrobacterium*-mediated systems were established, success was also reported on tobacco transformation by direct gene transfer with small plasmids (Paszkowski *et al.*, 1984). Direct gene transfer methods have,

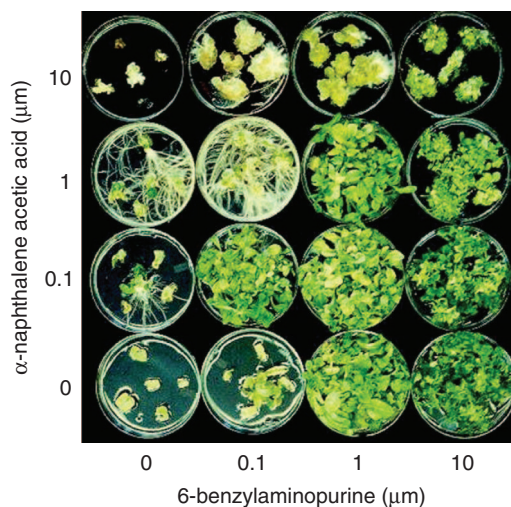


Figure 1 Interaction between α -naphthaleneacetic acid (NAA) and N^6 -benzyladenine (BAP) in formation of roots, shoots, and callus from *N. tabacum* leaf discs

however, lower success rates and insertion into plant DNA is less precise, accompanied with more rearrangements. For practical purposes *Agrobacterium*-mediated transformation of leaf discs has remained the method of choice. An exception is transformation of chloroplasts, for which direct transformation is applied (discussed in Section 2.5).

2.1.3 The use of tobacco haploids to obtain lines homozygous for multiple inserts

N. tabacum is a disomic tetraploid, meiotically and genetically behaving like a diploid. As with most other species, there is an inherent problem when the objective is to generate transformed lines with multiple inserts (Smith *et al.*, 1994; Lee *et al.*, 2003). Although lines with more than one insert are often found after tobacco transformation, as indicated by ratios of 15:1, 63:1, etc., in the T₁, only one locus needs to be homozygous to render a T₂ completely resistant to the selective agent. Such lines are generally still segregating for the other insert loci and several further generations need to be characterized by extensive Southern analyses to identify completely homozygous lines for three or four insert loci.

Transformation of haploid tobacco followed by doubling of the chromosome number is an alternative and much quicker method for obtaining homozygous lines with multiple inserts. Haploid plants can be obtained through several routes (Nitsch and Nitsch, 1969; Burk *et al.*, 1979) and haploid materials are usually maintained as micropropagated shoots. Leaf discs from these shoots can be used for *Agrobacterium*-mediated transformation as described before for tetraploid tobacco, resulting in formation of transformed haploid shoots. The plants obtained after rooting of these shoots are normal but sterile. Interestingly, cultured midrib segments from fully expanded leaves can give rise to doubled haploid shoots whereas those from younger leaves only yield haploid shoots (Kasperbauer and Collins, 1972). Most of the plants (about 70%) obtained from the older leaves have doubled chromosome numbers and can be distinguished from haploids by their fertility. Progeny from each fertile plant should be homozygous for all transgenes.

The only disadvantage of this method resides in the fact that the number of independent inserts is initially unknown; however, Southern analyses after digestion with a restriction enzyme that does not cut the insert will quickly determine the number of independent inserts in each line. Overall, this procedure results in significant savings in time and effort.

2.2 Transgenic Tobacco and Light-Regulated Gene Expression

Not long after the introduction of transgenes into tobacco was published, several groups aggressively began functional characterization of promoter sequences *in planta*. Of particular interest were regulated promoters, especially those where activity was modulated by light. Light is essential for plant metabolism; therefore, it is little wonder that light also has significant roles in regulating plant growth and development. In this way plant form and composition are best adjusted to capture and utilize ambient light energy. Because of the clear importance of light in affecting plant biology, scientists have carefully studied the effects of specific wavelengths, intensities, and photoperiods on plant processes of agricultural or research interest. Before the widespread availability of molecular tools and techniques, hundreds if not thousands of careers were well spent characterizing the physiology associated with light developmental processes, the biochemical and spectral properties of regulatory pigments and the components that comprises transduction schema. While these scientists postulated the properties of various photosensors and signaling networks, their efforts preceded the availability, utility, and agility of *Arabidopsis* as a transformable genetic system.

In these cases, transgenic tobacco served as an important transitional system, owing to its ease of transformability and ability to produce ample resources for meaningful study in a short time. Transgenic tobacco was the structure–function workhorse, the best system to test various hypotheses of gene form and function throughout the 1980s and into the 1990s. With the advent of simple “floral dipping” (Clough and Bent, 1998) transformation of *Arabidopsis*, it then became possible to test constructs and heterologous

gene expression in this useful genetic system, and the tests in tobacco fell from favor. Still, tobacco's utility is realized in a contemporary context (Pierik *et al.*, 2004). The following pages present a historical account of experiments where tobacco served as a surrogate for gene constructs devised to dissect the roles of photoreceptors and their signaling mechanisms in regulating photomorphogenic phenomena.

2.2.1 Photoreceptors and photophysiology

The transgenic tobacco system was valuable in early characterization of photoreceptor contributions to plant growth and development. With *Arabidopsis* photomorphogenic mutants just being characterized, overexpression of photosensor constructs in a tobacco background allowed gain-of-function analyses that would allow description of receptor function. These were valuable because they could complement the many careful studies of action spectra or absorption spectra that described physiological phenomena and the receptors that guided them.

Phytochrome is a pigment that activates developmental, molecular, and biochemical processes upon absorption of red light (Quail, 2002; Casal and Yanovsky, 2005). The red light activated receptor is toggled off by far-red light. Under natural conditions the ratio of red to far-red is dictated by the position of the sun and the amount of atmosphere it travels through, so the red/far-red ratio is an accurate indicator of time and perhaps season. Far-red light is readily transmitted through, and reflected from plant tissue, therefore the red/far-red ratio is an important barometer of plant neighbor density or plant position within a canopy (Kim *et al.*, 2005; Vandenbussche *et al.*, 2005).

While the physiology and biochemistry of phytochromes was advanced in the early 1980s, little was known about the molecular mechanisms of *phy* signaling. In the absence of a simple transgenic genetic system like *Arabidopsis*, it was necessary to exploit the most agile systems of the day, in this case the recently defined transgenic tobacco system. More importantly, such a system would allow study of plant gene expression and characterize the regulation of genes and light-sensing mechanisms from monocotyledonous

species. Although critical to agriculture, efficient transformation of monocots has always lagged behind that of eudicots, such as tobacco. Because of this barrier, several early studies validated tobacco as an effective system to study regulation of light-regulated gene expression using constructs obtained from monocots (Lamppa *et al.*, 1985). Later, a rice type-I phytochrome (most likely phytochrome A by recent comparisons) was introduced into tobacco for studies of *phy* effects on circadian rhythms and plant growth. These studies were important because type-I phytochromes are typically absent from mature tissues and transgenic studies provided a platform to test the effects of type-I on various processes of biological interest. Tobacco plants containing the rice type-I *phy* cassette expressed the protein highly in leaves. The protein was abundant and associated correctly with its chromophore. Since there was ample evidence of expression, assembly, and photoactivation, the effect of the overexpressor was assessed in a transgenic context. When the mRNA levels of the rhythmically-expressed chlorophyll a/b binding protein (*cab*) gene were assessed, it became clear that *phy* overexpressors had greater amplitude of *cab* expression under free-run conditions, indicating a role for the receptor itself in maintenance of circadian rhythms. In a subsequent study, the effects of rice type-I *phy* overexpression on plant stature were evident, as *phy* overexpressors were short as seedlings and mature plants (Nagatani *et al.*, 1991). The change was attributed to a difference in cell elongation and not cell number.

A series of reports from Harry Smith's laboratory utilized transgenic tobacco to analyze the role of specific phytochrome receptors in mediating various plant responses to light. Transgenic lines of *N. tabacum* and *Nicotiana glauca* were constructed, overexpressing the phytochrome A (*PhyA*) gene from oat (*Avena sativa*) (Keller *et al.*, 1989). The initial characterizations showed that overexpressors generated a spectrally active chromoprotein of the proper size. The transgenic overexpressors exhibited dwarfish growth, a loss of apical dominance, and dark green leaves. The same plants were used by McCormac *et al.* (1992) to describe the relationship between far-red fluence rate and various plant responses. The study demonstrated that oat *phyA* was able to function

in a tobacco background, as transgenic seedlings possessed hypocotyls that were shorter than wild type when grown under a given fluence rate of far-red light. More importantly, wild type seedlings exhibited a loss of sensitivity to far-red whereas transgenic overexpressors did not, reinforcing a well-described theme of transcriptional down-regulation of *PhyA* upon illumination. Additional characterization of *Avena PhyA*-overexpressing tobacco seedlings was performed, measuring cotyledon angle in response to extremely low fluence pulses of red light (Casal *et al.*, 1994). The study confirmed that *phyA* is the photoreceptor mediating response to the most minor red irradiances.

These studies were expanded by assessment of *phy* degradation patterns in transformed cells in culture. Here McCormac *et al.* (1993) established liquid suspension cultures of tobacco cells overexpressing the oat *phyA*. The overexpressed *phyA* was the predominant form detected and was analyzed for degradation patterns following red or far-red irradiation, as well as for degradation dependence on chromophore association. *In vitro* changes in gene expression mirrored those induced *in vivo*. This work established and validated a single-cell system where phytochrome activation, signaling, response, and degradation could be further studied. Tobacco *PhyA* overexpressors also were used to study the effect of *PhyA* expression on germination. Tobacco and *Arabidopsis* differ in their spectral response to germination. Whereas *Arabidopsis* germination is induced with all wavelengths (Shinomura *et al.*, 1996), tobacco exhibits far-red induced inhibition of germination, much like that observed in Borthwick's classical experiments with "Grand Rapids" lettuce (Borthwick *et al.*, 1952). However, when harboring a *PhyA* transgene, seeds were less sensitive to the far-red block of germination (McCormac *et al.*, 1993).

The set of *phyA* studies in transgenic tobacco culminates with studies showing the effect of *phyA* overexpression on traits relevant to agriculture. Robson and Smith (1996) showed that strong overexpression of *phyA* results in a complete suppression of shade avoidance phenotypes, changes in architecture that render the plant elongate and hyponastic. High overexpression of *phyA* induced a proximity-conditional dwarf phenotype whereas some lines with lower expression levels

exhibited normal phenotypes in white light but shade avoidance defects when the red/far-red ratio was lowered (Schmitt *et al.*, 1995). In *PhyA* overexpressors, photosynthate was allocated away from stems in these plants, increasing assimilates in leaf tissue. The report concludes that overexpression of *PhyA* may be useful in other crops to increase harvest index. Similar neighbor insensitivity was noted by Casal and Sanchez (1994) when they carefully assessed the roles of red/far-red ratio on plant growth, concluding that overexpression of *phyA* would cause a lag in response to detection of neighbors. A study was designed to test if these effects had ecological ramifications. The adaptive plasticity hypothesis, the notion that environmentally induced changes in plant form and composition would actually have an effect on fitness, was tested using these same lines (Schmitt *et al.*, 1995). Analysis of *PhyA* overexpression and wild type lines showed that the ability to take on shade avoidance characters allowed plants to better compete, as evidenced by a greater dry mass in wild-type plants upon harvest. These findings support the adaptive plasticity hypothesis.

The studies of *Avena phyA* indicate a role for phytochrome in mediating responses to a shade environment, directed as a decrease in red/far-red ratio. Blue light and the gaseous hormone ethylene also have a role in regulating shade avoidance response (Pierik *et al.*, 2004). A report showed shade avoidance responses in transgenic tobacco bearing an overexpressed mutated copy of the *Etr1* gene, the gene encoding the ethylene receptor. These plants exhibit a dominant insensitivity to ethylene, which accumulated to high levels in dense stands of plants. The ethylene response was shown to be independent of the red/far-red ratio and was related to blue light fluence rates. This study demonstrates that shade avoidance symptoms are not simply affected by the red/far-red ratio and phytochrome activity.

Later, oat *phyA*, and *Arabidopsis* phytochrome B (*phyB*) and phytochrome C (*phyC*) were introduced independently to a tobacco background to probe the roles of individual phytochromes in various light responses (Halliday *et al.*, 1997). This study reiterated the respective sensitivities of *phyA* and *phyB* to far-red and red light, as well as demonstrated a role for *phyC* for the first time. Furthermore, this study showed the long-term

effects of *phy* overexpression on photoperiodic flowering. A cultivar normally insensitive to a night break on short days could be made sensitive with *phy* overexpression, and a cultivar normally exhibiting a delay in flowering after a night break showed an increased delay. This study provided a mechanistic complement to the plethora of photophysiological studies that tied phytochrome to regulation of photoperiodic flowering.

Studies in blue light signaling utilized the tobacco system as well. The aforementioned *Avena PhyA* overexpressor showed enhanced hypocotyl growth inhibition phenotypes under blue light (Casal and Sanchez, 1994). Transgenic tobacco systems were also used to characterize newly discovered blue light sensors. The *hy4 Arabidopsis* mutant was identified as a seedling with defects in its response to blue and white light (Koornneef *et al.*, 1980). The mutants possessed long hypocotyls and poorly developed cotyledons, indicating a major role for the HY4 protein in regulation of photomorphogenic development. Later, the *hy4* mutant would be found to encode a protein with homology to microbial photolyases (Ahmad and Cashmore, 1993), the light-activated flavoproteins that catalyze repair of thymidine dimers in DNA caused by ultraviolet damage. The photolyases absorb optimally in the blue and UV-A portion of the spectrum, correlating well with the wavebands that produce conspicuous phenotypes in the *hy4* mutant. These findings led to the compelling conclusion that *Hy4* encoded a flavin-based photoreceptor that regulated stem elongation during early light development. Although identified genetically in *Arabidopsis*, the transgenic tobacco system was used because of its ability to rapidly produce plants that could be used in further description of this new light sensor's function, as well as uncover structure–function relationships.

An important study by Lin *et al.* (1995) provided the overexpression data that complemented the *Arabidopsis* loss-of-function genetic studies. Transgenic tobacco seedlings were hypersensitive to UV-A, blue and green light treatment, growing with shorter stems than wild-type seedlings. *Hy4* overexpressing seedlings were not distinguishable from wild-type seedlings in darkness, red light, or far-red light. Here tobacco served as a rapid means to test the effect of a photoreceptor in a

heterologous system, showing that the receptor, as well as the transduction mechanism, is conserved between the two different species representing two different plant families.

2.2.2 Elucidating light signal transduction mechanisms

The use of transgenic tobacco provided a foundation to study the mechanism of phytochrome signal transduction. Studies of *Sinapis cab* promoter activity in transgenic tobacco demonstrated that the promoter's activity is dictated by the host context, as tobacco has contrasting sensitivity to phytochrome induction compared to mustard (Kretsch *et al.*, 1995). Initial studies of cell fractionation and immunocytochemical analyses provided evidence that PHYA and PHYB were localized to the nucleus upon light activation (Mosinger *et al.*, 1988; Sakamoto and Nagatani, 1996). These observations led to the exciting hypotheses repartitioning of the photoreceptor itself may constitute an important step in light signaling. However, cell fractionation studies were inconclusive due to the fact that phytochrome is a notoriously “sticky” protein, and may be improperly ascribed to a given location or condition. To bypass this problem, Kircher *et al.* (1999) studied the kinetics of photoreceptor repartitioning in transgenic tobacco. The authors created a fusion protein between phytochrome B and green fluorescent protein (GFP), allowing them to nondestructively track the movement of the protein through time following light treatment. The results indicate that the photoreceptor::GFP fusion is detected in the cytosol in dark-grown plants and then reapportioned to the nucleus following illumination, although PHYA and PHYB translocate with different kinetics and spectral sensitivities. The movement of PHYB to the nucleus is driven by red light, is far-red reversible, and has a low-fluence illumination threshold. Import of PHYA is much more light sensitive and occurred in response to both red and far-red light. These studies in tobacco illustrated that photoreceptor localization to, and removal from, the nucleus plays a central role in the regulation of gene expression by light, a theme that would form the foundation of further investigations.

2.2.3 Analyses of light-regulatory sequences

As useful as tobacco was to understand the physiology of photosensor function and the mechanisms of transduction, tobacco first served to establish a basis for light-regulated promoter studies. The first efforts appear in the literature from N.H. Chua's laboratory in the mid-1980s, where efforts centered upon the promoters of two light-regulated nuclear genes—*cab* and the small subunit of ribulose 1-5 biphosphate carboxylase/oxygenase (*rbcS*). These studies allowed a primary glimpse into the structural elements that coupled ambient light conditions to regulation of gene expression in plants. A study by Lamppa *et al.* (1985) indicated that the wheat *cab* genes were properly regulated in the heterologous tobacco system. Next Nagy *et al.* (1986) demonstrated the phytochrome reversibility of wheat *cab-1* in wheat, and showed that a 1.8 kb promoter fragment could faithfully execute phytochrome-mediated induction in transgenic tobacco. A follow-up study pared down the wheat *cab* regulatory regions to a 286 bp promoter region as that conferring phytochrome responsiveness, and showed that phytochrome did not affect transcript stability (Nagy *et al.*, 1987). A related study of the light-regulated 2.4 kb pea *rbcS* promoter showed that it could be properly regulated in the heterologous context, and that a significant deletion construct leaving only 352 bp was still sufficient to confer a phytochrome response. Kuhlemeier *et al.* (1989) used transgenic tobacco to characterize both positive and negative regulators of *rbcS* activation. The use of protein synthesis inhibitors in transgenic tobacco provided evidence indicating that light control of *cab* transcripts was due to labile factors (Lam *et al.*, 1989).

The utility of tobacco as a transgenic system was realized in the definition of sequence motifs that narrowed the analyses of large expanses of light-responsive promoters. One element with demonstrated light regulatory capacity is the GT-1 motif. A study by Lam and Chua (1990) utilized tobacco to illustrate that the GT-1 motif of the pea *rbcS* promoter could confer light-regulated activity to a normally constitutive promoter. Proper induction was seen in cells that contained chloroplasts, suggesting a signal from the organelle was necessary. While necessary, the GT-1 motif was not sufficient to confer

responsiveness (Cuozzo-Davis *et al.*, 1990). The GT-1 motif was later shown to be specifically part of a phytochrome responsive element (Gilmartin and Chua, 1990) and that the precise spacing between GT-1 motifs was critical for full activity (Gilmartin and Chua, 1990). A DNA binding activity designated 3AF1 bound to the light responsive promoter, but the elements bound did not confer light regulation to a constitutive promoter (Lam and Chua, 1990). Later transgenic suspension cultures would be used to illustrate that phytochrome induction of light-regulated genes could be induced by addition of calmodulin and inhibited by appropriate calmodulin inhibitors (Zhou *et al.*, 2001).

Analysis of regulatory regions of other light-responsive genes raised exciting new questions about the role of post-transcriptional processes in the maintenance of transcript abundance. In etiolated seedlings, the pea ferredoxin 1 (*Fed1*) gene exhibited unusual induction kinetics relative to *cab*, *rbcS*, and other light-regulated transcripts (Kaufman *et al.*, 1985, 1986). These findings prompted Elliott *et al.* (1989) to express the modular components of the pea *Fed1* gene in transgenic tobacco. Long (~2 kb) and short versions of the *Fed1* promoter were still active when driving a reporter gene, but no light regulation was observed. Conversely, the cauliflower mosaic virus 35S (CaMV 35S) promoter::*Fed1* transcript was strongly regulated by light, indicating that the regulatory sequences were downstream of the transcriptional start. Attachment of the *Fed1* sequence to a CaMV 35S-driven β -glucuronidase (GUS) construct conferred light regulation to the normally constitutive GUS transcript. Later analyses in transgenic tobacco showed that the upstream regulatory regions from pea were most active during the dark-light transition and that transcript regulation was more evident in the green tissues (Gallo-Meagher *et al.*, 1992).

To further dissect the regulatory mechanisms Petracek *et al.* (1997) studied accumulation of CaMV 35S-driven *Fed1* transcripts in response to the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), as well as *Fed1* association with polysomes in light-grown and dark-adapted tissues. DCMU blocked accumulation of *Fed1* transcripts indicating an association with active photosynthesis. In light-grown tissue, *Fed1* transcripts were associated

with polyribosomes, whereas such relationships were not observed in darkness, in subthreshold illumination or after DCMU treatment. Later it would be shown that stability is associated with specific sequences in the 5'-UTR and coding region that would promote polysome association in light conditions, stabilizing the transcript (Dickey *et al.*, 1998). The stability could be broken with the introduction of termination codons into the coding region suggesting that active translation is required for optimal stability (Petracek *et al.*, 2000).

Regulation of another gene associated with photosynthesis was similarly studied using the transgenic tobacco system. A deletion series of the pea plastocyanin promoter (petE) was fused to GUS and introduced into transgenic tobacco (Pwee and Gray, 1993). The results described a host of positive and negative regulatory elements that occurred between -784 and +3 relative to the transcription start, and also required a plastidic signal. However, the light induction observed from truncated promoters never was as robust as that exhibited by the endogenes, leading the authors to conclude that other sequences located 3' to the transcription start may modulate gene expression. This hypothesis, initiated from a study in transgenic tobacco, revealed a post-transcriptional mechanism that would later be described in *Arabidopsis* with further resolution (Brown *et al.*, 2005).

Key enzymes of the phenylpropanoid pathway are regulated by light, and they too became targets for promoter analyses. A 1.1 kb fragment of the *Phaseolus vulgaris* phenylalanine lyase (PAL2) promoter was placed upstream of a GUS reporter and introduced into tobacco (Liang *et al.*, 1989). The resulting plants indicated that the promoter was active in specific tissues and could be regulated in a wound- and light-inducible manner. Similarly the promoter that drives the parsley *4CL-1* gene (encoding 4-coumarate:coenzyme A ligase) was placed upstream of the GUS reporter, and its activity was tested in a deletion series (Hauffe *et al.*, 1991). Transient assays in protoplasts isolated the regulatory regions to a 210 bp fragment, and activity was shown to be tissue specific in mature plants. The "G-box" motif is present in the promoters of many plant genes, and it was shown to be a functional part of the parsley *chalcone synthase* gene when activated by UV light (Kaulen *et al.*, 1986).

Although functional gene studies in tobacco have fallen from favor, it is important to remember that *Nicotiana* species still maintain many advantages over *Arabidopsis*. The large plants and organs are more amenable to biochemical techniques. Recently *Nicotiana benthamiana* and *N. tabacum* have been used for transient assays of transgenic protein function, as well as in vivo protein-protein interaction studies (Clemente, 2006). Plus, tobacco is a solanaceous crop, serving as a useful intermediate for translational genomics studies of light signaling in other solanaceous crop species, such as tomato. Although not a central system in the study of light-mediated processes, it is important for us to bear in mind the advantages of this system in elucidation of light-signaling mechanisms and their translation to relevant crop species.

2.3 Tobacco Transformation Studies for Biotic and Abiotic Stresses

Establishment of transgenic technology in tobacco ushered in an era of research to engineer resistance to various forms of stress. Crops are constantly being challenged by biotic and abiotic factors. Tobacco became a host to study and test the genes that could protect important crops in the future.

2.3.1 Biotic stress

2.3.1.1 Insect resistance

It has been well documented through pioneering research in tobacco that expression of *Bacillus thuringiensis* (*Bt*) endotoxin genes, as well as proteinase inhibitor (PI) genes, confer insect resistance. However, it must be noted that novel sources of resistance are continuing to be explored for future use in insect control.

Most Lepidoptera species are susceptible to the Cry 1 and Cry 2 insecticidal crystal proteins produced by *Bt*. The first studies to examine insect resistance following transfer of *Bt* genes were in tobacco in 1987 (Barton *et al.*, 1987; Vaeck *et al.*, 1987). These transgenic tobacco plants carried truncated versions of either *cry1Aa* or *cry1IAb*, respectively, and were resistant to the larvae of

Manduca sexta (L.). However, Cry protein levels were low (less than 0.001% of leaf soluble proteins) and this initially led to the use of synthetic *cry* genes whose expression levels were higher in plant cells. The codons of these bacterial *cry1Ab* and *cry1Ac* genes, as well as other features, were optimized for usage in plants and this raised the level of expression in transgenic tobacco (0.02–1% of leaf soluble proteins) (Perlak *et al.*, 1991). In these examples, the *cry* genes were driven by the constitutive CaMV 35S promoter. However, Williams *et al.* (1993) used the promoter from the pathogenesis-related *PR-1a* gene to control expression of *cry1Ab*. More recently, *cry2Aa2* was placed behind the promoter from the *Solanum tuberosum* leaf- and stem-specific (*ST-LS1*) gene to allow expression in green tissues of tobacco (Zaidi *et al.*, 2005). In this study, CRY2AS2 levels reached 0.21% of leaf soluble protein and plants were highly resistant to the larvae of *Heliothis virescens*. Another approach to increase *cry* gene expression (and improve transgene containment) has been to insert these genes into the tobacco chloroplast genome. Integration of a *cry1Ac* gene (McBride *et al.*, 1995), *cry2Aa2* (Kota *et al.*, 1999) and *cry11a5* (Reddy *et al.*, 2002) into the chloroplasts of tobacco led to high levels of expression (3–5% of leaf soluble proteins), and resistance to a number of economically important lepidopteran pests without deleterious effects on plant growth. De Cosa *et al.* (2001) expressed the *cry* operon in chloroplast transgenic tobacco resulting in accumulation of Cry protein to 46.1% of tsp with devastating effect on the lepidopteran pests. Recently, Chakrabarti *et al.* (2006) showed that expression of a truncated *cry9Aa2* in tobacco chloroplasts resulted in resistance to the potato tuber moth (PTM), *Phytorimacea operculella* as measured by leaf bioassays. Levels of CRY9AA2 were extremely high (10% of leaf soluble proteins); however, such high expression led to delayed development.

Other microbial genes also have proved useful. The *Agrobacterium tumefaciens* isopentenyl transferase (*ipt*) gene, which is important for cytokinin production, was introduced into tobacco under the control of the potato PI II gene promoter (Smigocki *et al.*, 1993). This wound-inducible system resulted in decreased feeding of *M. sexta* and increased mortality for the peach potato aphid, *Myzus persicae*. However, plant

development was negatively altered resulting in poor root systems and a reduction in chlorophyll levels.

Viral genes also have been introduced to curb insect feeding. Enhancin genes from *Trichoplusia ni* or *Helicoverpa armigera* baculoviruses encoding metalloproteases that breakdown mucin, which compromises the peritrophic membrane, were introduced into tobacco. *Trichoplusia ni* larvae allowed to feed directly on transgenic leaves showed reduced growth and development as well as increased mortality (Cao *et al.*, 2002). Long-term feeding studies using transgenic tobacco expressing the *Trichoplusia ni* baculovirus enhancin gene in artificial diets showed similar results with *Pseudaletia separata* and *Spodoptera exigua* larvae (Hayakawa *et al.*, 2004).

Plant PIs have been quite effective in reducing insect growth both *in vitro* and *in vivo*. The PIs are classified as inhibiting serine, cysteine, metallo or aspartyl proteases. The first PI gene shown to be effective against insects, cowpea trypsin inhibitor (CpTI), a serine PI, was introduced into tobacco in 1987 and displayed resistance to *M. sexta* and *H. virescens* (Hilder *et al.*, 1987). Since that time, numerous serine PI genes, including those expressing potato (*PPI-II*), tomato (*TI-II*), and sweet potato (*spTI-I*) PIs have been introduced into tobacco and shown to slow the growth of several species (Johnson *et al.*, 1989; McManus *et al.*, 1994; Yeh *et al.*, 1997). In 2000, three soybean genes (*KTi3*, *C-II*, and *PI-IV*) coding for serine PIs were introduced into tobacco (Marchetti *et al.*, 2000). In this study, 100% mortality was achieved for *S. littoralis* larvae fed on certain tobacco transgenic lines.

Lectins, a group of carbohydrate-binding proteins, have shown insecticidal properties. Tobacco expressing a pea lectin was toxic to *H. virescens* (Gatehouse *et al.*, 1992), while those expressing the snowdrop lectin (GNA) have displayed resistance to the peach potato aphid *M. persicae* (Hilder *et al.*, 1995). More recently, tobacco plants expressing the *Pinellia ternata* agglutinin transgene (*pta*) also reduced the growth of *M. persicae* (Yao *et al.*, 2003). Therefore, the *pta* gene could be used in conjunction with or pyramided with the snowdrop lectin gene (*gna*) to control aphids.

A CaMV 35S tobacco anionic peroxidase gene was able to provide broad-spectrum insect

resistance particularly to caterpillars, aphids, and whiteflies in both greenhouse (Dowd and Lagrimini, 1997) and field studies (Dowd and Lagrimini, 2006). However, the mode of action of the peroxidase is believed to be indirect and is not well understood.

Gamma-aminobutyrate (GABA), a nonprotein amino acid, accumulates in plants following various abiotic stresses. GABA is an inhibitory neurotransmitter that acts at insect neuromuscular junctions, and therefore may contribute to insect resistance. MacGregor *et al.* (2003) showed that tobacco transgenics expressing a glutamate decarboxylase complementary DNA (cDNA) and accumulating large amounts of GABA were not preferred by *H. virescens* compared to nontransgenics. Tryptamine is another neuroactive agent that results in insect antifeeding behavior. Transgenic tobacco plants expressing the tryptophan decarboxylase 1 gene leading to increased accumulation of tryptamine reduced the feeding and slowed the growth of *M. sexta* larvae (Gill *et al.*, 2003).

Caffeine was produced in tobacco via insertion of three coffee N-methyltransferase genes (Uefuji *et al.*, 2005). In preference studies, *S. litura* larvae fed on wild-type tobacco leaf discs rather than those of the transgenics. Therefore, caffeine may act as a feeding deterrent.

To date, genes derived from insects have conferred only low levels of insect resistance when expressed in tobacco. Chitin is a structural polysaccharide that is a component of the insect exoskeleton, as well as the peritrophic membrane, which separates food from midgut tissue. Genes coding for chitinases, normally produced by insects during moulting, have been cloned and introduced into plants for insect control. A *M. sexta* chitinase was expressed in transgenic tobacco, and when purified and fed to merchant grain beetle, *Oryzaephilus mercator*, resulted in toxicity (Wang *et al.*, 1996). A chitinase cDNA from *M. sexta*, introduced into tobacco, reduced feeding damage and growth of *H. virescens* larvae, but not of *M. sexta* larvae (Ding *et al.*, 1998). A synthetic gene containing multiple copies of the trypsin modulating and oostatic factor (TMOF) from *Aedes aegypti* (Aea-TMOF) was introduced into tobacco (Tortiglione *et al.*, 2002). *H. virescens* larvae fed with transgenic leaves expressing this gene showed a reduction in growth compared to

those fed with control plants. These genes may be more effective when used in conjunction with other control strategies.

Genes from animals also affect insectivory. Expression of a bovine spleen trypsin inhibitor (SI) gene in transgenic tobacco leaves was effective in decreasing both survival and growth of *H. armigera* larvae (Christeller *et al.*, 2002). When expressed at high levels, both biotin-binding proteins conferred a high level of insect resistance on transformed tobacco plants to larval PTM, *P. operculella* (Zeller) (fam. Gelechiidae), and on apple plants to larvae of the lightbrown apple moth (LBAM), *Epiphyas postvittana* (Walker) (fam. Tortricidae). More than 90% of PTM larvae died on tobacco plants expressing either avidin or streptavidin genes within nine days of inoculation (Marwick *et al.*, 2003).

The venom toxin, ω -ACTX-Hv1a (Hvt), from the Australian funnel web spider (*Hadronyche versuta*), acts as a calcium channel antagonist. Transgenic tobacco expressing Hvt were effectively protected with 100% mortality of *H. armigera* and *S. littoralis* larvae (Khan *et al.*, 2006).

2.3.1.2 Virus—pathogen- and nonpathogen-derived resistance

There are two main approaches to engineering plants for viral resistance. One approach is pathogen-derived resistance (PDR) where a portion or a complete viral gene is inserted into the plant conferring resistance. This was first demonstrated in transgenic tobacco plants containing the coat protein (CP) gene of tobacco mosaic virus (TMV) (Powell-Abel *et al.*, 1986). PDR has been studied and used extensively for a wide variety of viral genes, viruses, and hosts since that seminal report (see Dasgupta *et al.*, 2003 for review). Likewise, tobacco transgenics were used in the discovery that post-transcriptional gene silencing (PTGS) was the result of a diffusible signal. When viral RNA is the elicitor or target of PTGS, the mechanism is referred to as virus-induced gene silencing (VIGS) (see Robertson, 2004 for review). Transgenic tobacco studies (particularly using *N. benthamiana*) have been used extensively to understand the RNA silencing mechanisms at work in VIGS. The other approach

is nonpathogen-derived resistance that uses host resistance genes to confer virus resistance. Once again, tobacco has been a model organism to understand the resistance mechanisms and to generate transgenic virus resistant plants. The classic example is the tobacco N gene that provides resistance to TMV. It is a disease resistance gene (R) member of the toll-interleukin (TIR)-leucine-rich repeat (LRR) family.

2.3.1.3 Fungi—pathogenesis-related proteins

There are five families of PR proteins (PR-1 to PR-5) that have members displaying antifungal activity. In the first report of fungus-resistant transgenic plants, Broglie *et al.* (1991) expressed a bean chitinase gene in tobacco as well as in *Brassica napus* that displayed resistance to *Rhizoctonia solani*. Since that time, transgenic tobacco has been used in numerous studies to examine the antifungal effect of various PR-proteins expressed alone or in combination (e.g., Broglie *et al.*, 1991; Alexander *et al.*, 1993; Nielsen *et al.*, 1993; Vierheilig *et al.*, 1993; Liu *et al.*, 1994; Zhu *et al.*, 1994; Velazhahan and Muthukrishnan, 2003).

2.3.1.4 Bacteria

Magainin is one of the earliest reported antimicrobial peptides isolated from skin secretions of the African clawed frog *Xenopus laevis* and is thought to function as a natural defense mechanism against infection. The engineered magainin analog peptide, *Myp30*, was found to inhibit spore germination of the oomycete, *Peronospora tabacina* (Adam) *in vitro*, and the growth of a bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (Jones). Transgenic tobacco (*N. tabacum* L.) plants expressing *Myp30* were evaluated for resistance to these pathogens. The expression of the peptide only to an extracellular location resulted in significant reduction in sporulation and lesion size due to *P. tabacina* infection. A significant increase in resistance to the bacterial pathogen was also observed regardless of the targeting location of the peptide (Li *et al.*, 2001).

2.3.2 Abiotic stress

Plant growth and productivity are greatly influenced by environmental conditions such as drought, cold, and salinity, which lead to water stress. Different kinds of stresses often trigger similar responses in plants as a result of cellular dehydration, which causes osmotic stress. In addition, the production of reactive oxygen species increases damage to cellular structures and impacts metabolism. Many stress-associated genes have been identified, including those involved in osmolyte biosynthesis, fatty acid metabolism, free radical detoxification, and signal transduction as well as molecular chaperones, ion and water transporters, and transcription factors. Tobacco transformation has played an important part in studies to evaluate the roles of these genes in stress tolerance.

To date, most studies have looked at constitutive overexpression of stress-related proteins using the CaMV 35S promoter. While this strategy allows elucidation of protein function with relative ease, it results in transgene expression in all parts of the plant at all developmental stages, which may have undesirable consequences for plant growth (Holmström *et al.*, 1996; Kumria and Rajam, 2002; Kasuga *et al.*, 2004). The use of stress-inducible promoters has demonstrated the potential to more effectively transfer genes or pathways for stress tolerance into other important plant species (Nelson *et al.*, 1998; Kasuga *et al.*, 2004; Khodakovskaya *et al.*, 2006). Expression of foreign proteins has also been controlled by the addition of targeting sequences (Shen *et al.*, 1997; Nuccio *et al.*, 1998).

2.3.2.1 Biosynthesis of osmolytes and osmoprotectants

Tobacco has provided a good model system for understanding the role of compatible solutes in osmotic stress. In response to a variety of stresses, compatible solutes accumulate in cells at high concentrations, facilitating the retention of water, and thereby stabilizing the structure of macromolecules, without interfering with cytoplasmic functions. Introduction of novel pathways for the biosynthesis of compatible solutes in tobacco has resulted in increased

stress tolerance of transgenic plants. Common nontoxic solutes include quaternary ammonium compounds, such as glycine betaine, amino acids, amino acid derivatives, sugars, and sugar alcohols.

2.3.2.2 Quaternary ammonium compounds—glycine betaine

Glycine betaine (GlyBet) is a quaternary ammonium compound occurring naturally in a variety of plants, animals, and microorganisms (Rhodes and Hanson, 1993). It is a very efficient compatible solute that accumulates in response to stress conditions. Transgenic approaches in tobacco and other higher plants that do not normally accumulate GlyBet have provided evidence for the physiological role of this osmoprotectant. GlyBet is synthesized in higher plants by the two-step oxidation of choline, catalyzed by a ferredoxin-dependent choline monooxygenase (CMO) and a NAD⁺-dependent betaine aldehyde dehydrogenase (BADH) (Rhodes and Hanson, 1993). In mammalian cells and *Escherichia coli*, GlyBet is synthesized by another two-step reaction involving a NAD⁺-dependent choline dehydrogenase (CDH) in combination with BADH (Landfald and Strøm, 1986). By contrast, the soil bacterium *Arthrobacter* is able to synthesize GlyBet directly from choline in a one-step reaction catalyzed by choline oxidase (COD) (Ikuta *et al.*, 1977).

Tobacco has been used extensively as a model for the introduction of GlyBet synthesis into a nonaccumulator. Engineering GlyBet synthesis in tobacco has been achieved by changing the CMO/BADH pathway, the CDH/BADH pathway, or the COD pathway (Sakamoto and Murata, 2000). Transfer of the CMO/BADH pathway from natural GlyBet accumulators to tobacco was attempted by introduction of a BADH cDNA from either spinach (*Spinacia oleracea*) or sugarbeet (*Beta vulgaris*) under control of the CaMV 35S promoter (Rathinasabapathi *et al.*, 1994). BADH was targeted to the chloroplasts of transgenic plants and levels of the enzyme were similar to those in spinach following salt stress. Transgenic plants accumulated GlyBet to high levels, but only in the presence of the exogenously supplied precursor betaine aldehyde, demonstrating that increased

expression of BADH alone was not sufficient for GlyBet synthesis in tobacco (Rathinasabapathi *et al.*, 1994). High levels of BADH expression were also achieved following transformation of tobacco with barley (*Hordeum vulgare*) BADH cDNA (Ishitani *et al.*, 1995). GlyBet accumulation was not reported; however, BADH transcripts accumulated in a stress-responsive manner. Li *et al.* (2003) introduced BADH from the halophyte *Suaeda liaotungensis* into tobacco, under control of the CaMV 35S promoter. Levels of GlyBet were much lower in transgenic plants than in *S. liaotungensis*, which accumulates GlyBet to high levels. Despite not accumulating GlyBet to physiologically relevant levels, some transgenic plants survived on media containing 200 mM NaCl and suffered less membrane damage than wild-type plants (Li *et al.*, 2003). Expression of GlyBet in tobacco has also been shown to increase tolerance to high temperature stress (45 °C) during seedling growth by maintaining the activation of Rubisco, enhancing photosynthesis (Yang *et al.*, 2005). These authors introduced the spinach BADH gene under control of the CaMV 35S promoter and transgenic plants were able to accumulate low levels of GlyBet, up to 4.6 µmol g⁻¹ fresh weight, mainly in chloroplasts.

Since non-GlyBet-accumulating plants show some BADH activity, a spinach CMO gene was introduced into tobacco under control of the CaMV 35S promoter, to investigate the potential for GlyBet production by engineering synthesis of this enzyme alone (Nuccio *et al.*, 1998). CMO was successfully targeted to the chloroplasts, but transgenic plants accumulated levels of GlyBet far below those of natural accumulators, possibly limited by the endogenous choline supply. In a similar approach, Huang *et al.* (2000) introduced the COD gene from *Arthrobacter pascens* into tobacco under control of the CaMV 35S promoter. Transgenic plants accumulated too little GlyBet to significantly affect osmoregulation, despite a moderate increase in salt tolerance (Huang *et al.*, 2000). In contrast to these studies, the COD gene from *Arthrobacter globiformis* (*codA*), targeted to the chloroplasts under control of the CaMV 35S promoter, conferred freezing tolerance (−2 °C for 24 h) on transgenic tobacco plants (Konstantinova *et al.*, 2002). Choline oxidase protein was detected in the homozygous line analyzed, although the expression level remained stable under nonstress or

stress conditions. Transgenic plants were also able to survive freezing stress under field conditions (Konstantinova *et al.*, 2002).

In an effort to remove the constraint placed by insufficient choline on GlyBet synthesis, Mcneil *et al.* (2001) employed a spinach cDNA encoding phosphoethanolamine *N*-methyltransferase (PEAMT), a key enzyme in choline biosynthesis. The PEAMT coding sequence was introduced under control of the strongly constitutive figwort mosaic virus 34S promoter, into tobacco plants already expressing spinach CMO and sugarbeet BADH. Transgenic plants contained up to 50-fold more free choline than control plants and GlyBet synthesis was enhanced up to 30-fold, without affecting plant growth (Mcneil *et al.*, 2001).

Attempts to engineer the bacterial GlyBet biosynthesis pathway into tobacco produced similar results to those targeting the plant pathway. Introduction of the second enzyme of the *E. coli* GlyBet pathway, *betB*, encoding BADH, was not sufficient for GlyBet production in tobacco (Holmström *et al.*, 1994). By comparison, the first enzyme of the *E. coli* GlyBet pathway, *betA*, encoding CDH, under control of the *Arabidopsis RbcS1A* promoter allowed GlyBet accumulation in transgenic tobacco plants (Holmström *et al.*, 2000). Plants expressing *betA* had enhanced salt tolerance, measured as reduction in fresh weight, compared to controls (Lilius *et al.*, 1996; Holmström *et al.*, 2000), and also showed greater resistance to photoinhibition under salt stress and low temperature conditions (Holmström *et al.*, 2000). When transgenic tobacco plants expressing *betA* were crossed with those expressing *betB*, effectively completing the biosynthesis pathway, GlyBet accumulation was two- to threefold higher than in plants only producing CDH (Holmström *et al.*, 2000). Transgenic lines producing both CDH and BADH, however, did not appear to have improved stress tolerance over those producing CDH alone.

2.3.2.3 Amino acids—proline

Proline accumulates to very high levels in plants under stress, protecting them against osmotic and oxidative stresses. In *E. coli*, proline biosynthesis from glutamate is controlled by a three-gene operon, *proB* (γ -glutamyl kinase),

proA (glutamic γ -semialdehyde dehydrogenase), and *proC* (Δ^1 -pyrroline-5-carboxylate reductase); however, in plants, proline can be synthesized directly from glutamate under stress conditions by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) (Delauney and Verma, 1993). An alternative proline biosynthesis pathway exists in plants, involving transamination of ornithine, catalyzed by ornithine- δ -aminotransferase (δ -OAT), to two possible intermediates, both of which can be reduced to proline. Engineering the proline biosynthesis pathway in tobacco has demonstrated that overproduction of proline in plants can confer increased tolerance to osmotic stress.

Kavi Kishor *et al.* (1995) introduced the mothbean (*Vigna aconitifolia*) P5CS gene into tobacco under control of the CaMV 35S promoter. Transgenic plants produced high levels of P5CS and synthesized 10- to 18-fold more proline (830 to 1590 $\mu\text{g g}^{-1}$ fresh weight) than control plants. Osmotic potential was maintained in leaf cells of transgenic plants and wilting was delayed under drought treatment, likely due to constitutive levels of proline (Kavi Kishor *et al.*, 1995). Transgenic plants also showed enhanced biomass and flower development under salt stress conditions (Kavi Kishor *et al.*, 1995). Under nonstress conditions, Konstantinova *et al.* (2002) observed only a threefold higher proline content compared to wild-type plants in tobacco lines transformed with the same mothbean P5CS gene. This level increased to 15-fold, however, during chilling and freezing stresses (-2°C for 24 h) (Konstantinova *et al.*, 2002). The same authors also introduced a P5CS gene from *Arabidopsis* into tobacco. There was no difference in proline content between these transformed lines and wild-type plants under normal conditions, but proline content increased five- to eightfold during chilling and freezing stresses (Konstantinova *et al.*, 2002). Plants harboring either P5CS gene were also able to survive freezing stress under field conditions (Konstantinova *et al.*, 2002). Tobacco plants engineered to express enzymes from the *E. coli* proline biosynthesis pathway are able to accumulate similar levels of proline under salt-stress conditions (Sokhansanj *et al.*, 2006).

Since P5CS is under feedback inhibition by proline, which may be lost under stress conditions, Hong *et al.* (2000) compared proline levels in transgenic tobacco plants expressing a wild-type

mothbean P5CS enzyme and a mutated form, whose feedback inhibition was removed, both under control of the CaMV 35S promoter. Under nonstress conditions, plants expressing the mutated P5CS enzyme accumulated twofold more proline than plants expressing the wild-type P5CS and had higher germination rates on media containing 200 mM NaCl. In addition, higher proline levels were associated with lower malondialdehyde (MDA) levels, a major cytotoxic product of lipid peroxidation used as an indicator of free radical production (Hong *et al.*, 2000).

To investigate the possibility of increasing osmotolerance in plants by manipulating the ornithine-dependent proline biosynthesis pathway, Roosens *et al.* (2002) introduced the *Arabidopsis* ornithine- δ -amino transferase (δ -OAT) cDNA into tobacco, under control of the CaMV 35S promoter. Transgenic plants overexpressing δ -OAT synthesized approximately threefold more proline than control plants and had increased biomass and germination frequency under osmotic stress conditions. In another alternative strategy to increase proline biosynthesis, Yonamine *et al.* (2004) introduced the tobacco *NtHAL3a* gene, involved in the coenzyme A biosynthetic pathway, into cultured tobacco BY2 cells under control of the CaMV 35S promoter. Transgenic plants contained approximately four- to fivefold more proline under nonstressed and salt-stressed conditions and showed improved tolerance to 100 mM and 140 mM NaCl (Yonamine *et al.*, 2004). Kolodyazhnaya *et al.* (2006) introduced an antisense suppressor of proline dehydrogenase from *Arabidopsis* into tobacco, leading to transgenic plants with increased proline content and elevated salt tolerance.

2.3.2.4 Amino acid derivatives—polyamines

Polyamines (PAs) are nitrogenous cellular compounds, formed by the decarboxylation of amino acids, which accumulate under a variety of abiotic and oxidative stress conditions. Examples include putrescine, spermidine, and spermine. The role of PAs in plants has been studied by overexpression of S-adenosylmethionine decarboxylase (*samdc*), a key enzyme in spermidine and spermine biosynthesis, in tobacco. Transgenic tobacco plants expressing a carnation (*Dianthus*

caryophyllus L.) *samdc* cDNA under control of the CaMV 35S promoter accumulated 2.2–3.1 times more soluble total PAs than wild-type plants (Wi *et al.*, 2006). Transgenic plants had an increased number and weight of seeds, increased net photosynthetic weight, and suffered less chlorophyll degradation after salt, cold, acidic stress, and abscisic acid (ABA) treatment. In addition, transcription of antioxidant enzymes was induced more significantly in transgenic plants than controls, following stress treatment (Wi *et al.*, 2006). Increased PA biosynthesis in transgenic tobacco was also achieved by introduction of a human *samdc* gene under control of the CaMV 35S promoter (Waie and Rajam, 2003). Transgenic plants had increased spermidine and putrescine levels and exhibited tolerance to salt stress (250 mM NaCl) and PEG-induced drought stress. Kumria and Rajam (2002) expressed the mouse putrescine synthesis gene ornithine decarboxylase (ODC) in tobacco under control of the CaMV 35S promoter to up-regulate PA metabolism. Transgenic plants had higher mouse ODC activity, but reduced activity of plant ODC and the alternative putrescine synthesis gene arginine decarboxylase (ADC). PA levels were two- to threefold higher in transgenic plants compared to controls and plants were more tolerant to salt stress (300 mM NaCl) (Kumria and Rajam, 2002).

PAs share a common precursor, S-adenosylmethionine (SAM), with ethylene, resulting in metabolic competition between ethylene and PA biosynthesis. Antisense expression of ethylene biosynthetic genes is expected to shift the competition for the SAM precursor in favor of PA biosynthesis. Wi and Park (2002) introduced antisense constructs of carnation cDNAs encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase or ACC oxidase into tobacco. Transgenic lines had higher PA contents and increased number and weight of seeds, in addition to reduced chlorophyll loss following oxidative stress, high salinity, acid conditions, or ABA treatment (Wi and Park, 2002).

2.3.2.5 Sugars and sugar alcohols

Compounds related to sugar metabolism also accumulate in plants during responses to water

stress and osmotic adjustment. These include sucrose, trehalose, fructans, mannitol, and D-ononitol. The role of these osmolytes in protecting against abiotic stress has been investigated by engineering tobacco with enzymes in their biosynthetic pathways.

Sucrose and hexose concentrations in the cytoplasm of plant leaf cells can be increased by inhibition of sugar transportation to the sink organ. Transgenic tobacco plants expressing an apoplastic yeast invertase maintained constant photosynthetic activity and higher turgor pressure under 300 mM salt stress, whereas wild-type plants showed marked photoinhibition and a greater increase in osmotic pressure (Fukushima *et al.*, 2001).

The nonreducing disaccharide trehalose protects biomolecules from environmental stress in many microorganisms. Most plant species, however, do not appear to accumulate trehalose to detectable levels. Increasing trehalose concentrations in plants could therefore enhance drought and salinity tolerance (Penna, 2003). Trehalose is produced from UDP-glucose and glucose-6-phosphate, via trehalose-6-phosphate, catalyzed by the enzymes trehalose-6-phosphate synthetase and trehalose-6-phosphate phosphatase (Goddijn and Van Dun, 1999). Transgenic tobacco plants expressing the yeast trehalose-6-phosphate synthetase (*TPS1*) gene under control of the Rubisco small subunit (*rbcS*) promoter accumulated low concentrations of trehalose, ranging from 0.08% to 0.32% of the dry weight, in leaves and roots (Holmström *et al.*, 1996). Plants exhibited significant growth retardation and detached leaves showed reduced water loss compared to controls, when subject to air-drying. In a similar experiment, transgenic tobacco plants expressing the yeast trehalose-6-phosphate synthetase (*TPS1*) gene under control of the CaMV 35S promoter accumulated trehalose up to 0.17 mg g⁻¹ fresh weight in leaves and showed increased drought tolerance (Romero *et al.*, 1997).

These plants also exhibited phenotypic changes including stunted growth and lancet-shaped leaves, as well as reduced sucrose content. In this case, water loss from detached leaves was not significantly affected by trehalose accumulation. Given also that trehalose concentrations in transgenic plants were too low for a conventional osmoprotectant

effect, these authors suggested that sugar synthesis had resulted in altered sugar metabolism (Romero *et al.*, 1997). Recently, a trehalose-6-phosphate gene from *Arabidopsis* (*AtTPS1*) was introduced into tobacco under control of the CaMV 35S promoter (Almeida *et al.*, 2005). Transgenic lines displayed higher germination frequencies on media containing 0.5 M mannitol or 0.2 M NaCl or under temperature stress at 15 °C or 35 °C. In accordance with earlier experiments, water loss in transgenic plants or detached leaves did not vary significantly between transgenic and wild-type plants (Almeida *et al.*, 2005).

Overexpression of the *E. coli* trehalose-6-phosphate synthetase (*otsA*) and trehalose-6-phosphate phosphatase (*otsB*) genes in tobacco plants also enhanced trehalose synthesis (0.11 mg g⁻¹ fresh weight) with concomitant changes in leaf morphology (Goddijn *et al.*, 1997). Despite the low-level accumulation of trehalose in transgenic tobacco expressing both *otsA* and *otsB*, an increase in dry mass and improved photosynthesis under drought stress was observed (Pilon-Smits *et al.*, 1998). The low levels of trehalose accumulation observed in transgenic plants overexpressing genes of the trehalose biosynthetic pathway may result from the activity of an endogenous trehalase enzyme, which breaks down trehalose to glucose (Goddijn *et al.*, 1997). When the potent trehalase inhibitor validamycin A was added to transgenic plants *in vitro* or growing hydroponically, trehalose accumulation increased up to 0.41 mg g⁻¹ fresh weight in tobacco leaves (Goddijn *et al.*, 1997).

Fructans are soluble, polymers of fructose, used as storage sugars in many plants, but not tobacco. It has been suggested that, due to their solubility, fructans may also play a role in osmotic adjustment (Pilon-Smits *et al.*, 1995). The *SacB* gene encoding levansucrase from *Bacillus subtilis*, fused to the carboxypeptidase Y vacuolar sorting signal from yeast, was introduced into tobacco under control of the CaMV 35S promoter to produce transgenic plants that accumulate bacterial fructans (Pilon-Smits *et al.*, 1995). Fructan-producing plants performed significantly better under polyethylene-glycol-mediated drought stress, exhibiting 55% higher growth rates than wild-type plants resulting in 33% more fresh weight and 59% more dry weight (Pilon-Smits *et al.*, 1995). This difference in weight was most pronounced in the roots. In addition

to polyethylene-glycol-mediated drought stress, Konstantinova *et al.* (2002) found that tobacco plants carrying the *SacB* gene were able to recover from freezing stress (-2°C for 24 h), whereas wild-type tobacco plants were not. Plant survival under freezing field conditions was also increased, allowing earlier planting. Fructan accumulation increased in the transgenic line during freezing stress and decreased after recovery (Konstantinova *et al.*, 2002).

The sugar alcohol mannitol occurs widely in plants and animals, but is not normally synthesized in tobacco. Tarczynski *et al.* (1992) overexpressed the *E. coli* mannitol-1-phosphate dehydrogenase (*mtlD*) gene in tobacco, under control of the CaMV 35S promoter, resulting in biosynthesis of mannitol. Mannitol concentrations were greater than $6\text{ }\mu\text{mol g}^{-1}$ fresh weight in the leaves and roots of some transformed plants (Tarczynski *et al.*, 1992). Transgenic plants showed increased tolerance to prolonged high salt conditions (30 days of 250 mM NaCl) (Tarczynski *et al.*, 1993). Fresh weight loss in plants from *mtlD* lines was less than in control lines and transgenic plants increased in height by an average of 80% compared to 22% in control plants. However, since plants containing mannitol often produced new roots and leaves, the relative increases in fresh weight and height in these plants are likely due to new growth rather than a reallocation of resources (Tarczynski *et al.*, 1993). Karakas *et al.* (1997) introduced the same *mtlD* cassette into tobacco and saw only marginal increases in dry mass under salt stress. Transgenic plants were 20–25% smaller than wild-type tobacco plants, but whereas salt stress decreased the dry weight of wild-type plants by 44%, transgenic plants suffered no reduction in dry weight. Since mannitol was shown to be a relatively minor osmolyte in transgenic tobacco, the slower growth of transgenic plants and not the presence of mannitol *per se*, may have resulted in greater salt tolerance. Shen *et al.* (1997) targeted *mtlD* to the chloroplasts by addition of an aminoterminal transit peptide. Mannitol accumulated at concentrations ranging from 2.5 to $7\text{ }\mu\text{mol g}^{-1}$ fresh weight, with one line accumulating approximately 100 mM mannitol in chloroplasts. These plants had increased resistance to methyl-viologen-induced oxidative stress, as demonstrated by increased retention of chlorophyll in leaves following treatment (Shen

et al., 1997). The location of mannitol in chloroplasts appeared to increase the hydroxyl radical-scavenging capacity of cells, thereby reducing oxidative damage.

Transgenic tobacco plants with increased tolerance to salt and drought conditions were produced by introducing a *myo*-inositol *O*-methyltransferase (IMT1) cDNA from ice plant (*Mesembryanthemum crystallinum*) into tobacco under control of the CaMV 35S promoter (Sheveleva *et al.*, 1997). In the halophytic ice plant, IMT1 methylates *myo*-inositol to form D-ononitol, a cyclic polyol, in a stress-inducible manner, likely resulting in decreased osmotic potential in the cytoplasm and regulation of sodium accumulation in the vacuole (Nelson *et al.*, 1998). Transgenic tobacco plants accumulated D-ononitol in amounts exceeding $35\text{ }\mu\text{mol g}^{-1}$ fresh weight, experienced less inhibition of photosynthetic CO_2 fixation and recovered more quickly from salt or drought stress. One day after rewatering drought-stressed plants, photosynthesis had recovered 75%, compared to 57% in wild-type plants (Sheveleva *et al.*, 1997).

2.3.2.6 Ectoine

Ectoine is a tetrahydropyrimidine that functions as a compatible solute in halophilic bacteria, where it is synthesized from aspartic β -semialdehyde by three successive enzyme reactions. The three ectoine biosynthetic genes, *ectA*, *ectB*, and *ectC*, from *Halomonas elongate* were each placed under control of the CaMV 35S promoter and introduced together into tobacco cv. Bright Yellow 2 (BY2) cells (Nakayama *et al.*, 2000). Transgenic BY2 cells accumulated ectoine to low levels (14 to 79 nmol g^{-1} fresh weight) and displayed tolerance to hyperosmotic shock (620 mM mannitol or 500 mM NaCl for 20 min) (Nakayama *et al.*, 2000). The ectoine biosynthesis genes were used to create transgenic tobacco plants that accumulated ectoine under salt stress conditions (Moghaieb *et al.*, 2006). Transgenic plants showed less reduction in dry weight, photosynthetic rate, and impairment of stomatal conductance than wild-type plants, as well as increased Na^+ concentrations in leaves and roots and increased osmotic adjustment (Moghaieb *et al.*, 2006). Ectoine was suggested to improve maintenance of root function, so

that water is taken up consistently under saline conditions and to increase transpiration and protect Rubisco proteins from salt, thereby increasing photosynthetic rate (Moghaieb *et al.*, 2006).

2.3.3 Protective proteins

2.3.3.1 LEA and LEA-related proteins

Osmotic stress induces late embryogenesis-abundant (LEA) proteins in plant's vegetative tissues, a large and diverse group of stress-responsive proteins conferring dehydration tolerance (Bartels and Sunkar, 2005). Dehydrins are group-2 LEA proteins that respond to water stress in plants. Members of the LEA/dehydrin superfamily have been expressed in tobacco, to investigate their role in dehydration stress, including plant responses to freezing. Kaye *et al.* (1998) introduced cDNA sequences encoding the spinach CAP160 and CAP85 cold-acclimation proteins into tobacco, under control of the CaMV 35S promoter. Transgenic plants containing each sequence were crossed to obtain progeny expressing both proteins. The CAP160 sequence had very limited homology to the Arabidopsis rd29A and rd29B stress-regulated proteins and CAP160 mRNA expression was increased by low temperature and water stress (Kaye *et al.*, 1998). Whilst the temperature at which 50% of cells were killed by freezing stress did not vary between transgenic and wild-type tobacco, plants expressing the spinach proteins suffered slightly less electrolyte leakage, suggesting a small reduction in freeze damage (Kaye *et al.*, 1998). Citrus (Citrus unshiu Markov.) dehydrin (CuCOR19) cDNA was introduced into tobacco under control of the CaMV 35S promoter (Hara *et al.*, 2003). Transgenic plants suffered less electrolyte leakage than control plants following 3 h freezing stress at -4°C and also exhibited better seedling growth and earlier germination at 15°C . MDA content was lower in transgenic lines than control lines following freezing stress, indicating reduced lipid peroxidation (Hara *et al.*, 2003). The CuCOR19 protein prevented peroxidation of soybean liposomes *in vitro*, suggesting that this dehydrin may act as a radical scavenging protein, protecting plant membranes.

2.3.3.2 Osmotin

Osmotin is a protein involved in adaptation to low water potential, but not induced by osmotic shock in cultured tobacco cells (Singh *et al.*, 1989). Synthesis of mRNA encoding osmotin is either induced or stabilized by ABA, but accumulation of the protein is induced by adaptation to low water potentials via a post-translational control mechanism (Singh *et al.*, 1989). The osmotin gene was overexpressed in tobacco plants under control of the CaMV 35S promoter (Sokhansanj *et al.*, 2006). Transgenic plants were able to produce shoots on medium containing 320 mM NaCl and showed comparable chlorophyll *a* content to wild-type plants (Sokhansanj *et al.*, 2006).

2.3.3.3 Heat shock proteins

Maintenance of protein functional conformation and prevention of aggregation is important for cell survival following abiotic stress. Heat shock proteins (HSPs) are synthesized upon exposure to high temperature stress and assist in protein refolding under stress conditions (Wang *et al.*, 2004). Overexpression of HSPs in tobacco has been used to improve understanding of HSP function during temperature stress.

A tobacco class I cytosolic small HSP gene, *TLHS1*, which showed a strong molecular chaperone activity *in vitro*, was overexpressed in tobacco under control of the CaMV 35S promoter (Park and Hong, 2002). Transgenic plants were less affected by heat stress at 40°C for 4 h or 45°C for 1 h, as measured by almost two times higher cotyledon opening rate in transgenic compared to control plants. A mitochondrial small HSP (MT-sHSP) cDNA from tomato (*Lycopersicon esculentum*) was introduced into tobacco under control of the CaMV 35S promoter (Sanmiya *et al.*, 2004). Transgenic plants constitutively produced the MT-sHSP protein, accumulating higher levels after heat stress and were able to survive treatment at 48°C for 2 h, in contrast to wild-type plants. Transgenic tobacco plants, expressing tobacco HSP70 (*NtHSP70-1*) under control of the CaMV 35S promoter, produced significantly higher levels of HSP70 in leaves than control plants, under nonstress conditions (Cho and Hong, 2006). Three-week-old, HSP70

overexpressing seedlings were able to maintain leaf turgidity following 2 weeks drought stress, whereas control plants showed severe wilting and stress symptoms. Expression of the *CaERD15* (early responsive to dehydration) gene was considerably reduced in HSP70 overexpressing lines following drought stress, suggesting increased dehydration stress tolerance (Cho and Hong, 2006).

Alvim *et al.* (2001) transformed tobacco with the CaMV 35S promoter driving expression of the soybean (*Glycine max*) chaperone binding protein (BiP), a constitutively expressed HSP70 (HSC70) homolog and an important component of the endoplasmic reticulum stress response. Transgenic tobacco plants harboring a sense-orientation BiP gene displayed enhanced tolerance to the glycosylation inhibitor tunicamycin, which promotes the accumulation of unfolded proteins in the endoplasmic reticulum. Sense transgenic plants were able to maintain leaf turgidity under progressive dehydration stress, whereas antisense transgenic plants showed increased sensitivity to water stress (Alvim *et al.*, 2001). BiP may act to alleviate oxidative stress, since levels of the antioxidant enzyme superoxide dismutase (SOD) were increased in control and antisense plants, but not in sense transgenic plants (Alvim *et al.*, 2001).

2.3.3.4 Membrane damage—fatty acid metabolism genes

Membrane damage under cold stress may be dependent upon the degree of unsaturated fatty acids present in phosphatidyl-glycerol membranes. Chilling-sensitive plants possess a high degree of saturated fatty acids, whereas cold-tolerant plants contain a high proportion of unsaturated fatty acids. During acclimation to cold temperatures, desaturase enzymes increase the proportion of unsaturated fatty acids allowing membranes to remain fluid (Iba, 2002). Cold stress also increases production of free radicals leading to lipid peroxidation. This causes loss of unsaturated fatty acids, increase in membrane rigidity, and membrane degradation. Transgenic tobacco experiments have contributed to understanding the relationship between low temperature tolerance and membrane rearrangement and synthesis.

The effect of unsaturated membrane lipid levels on chilling tolerance was demonstrated by the

introduction of a cDNA encoding glycerol-3-phosphate acyltransferase from chilling-sensitive squash into tobacco, under control of the CaMV 35S promoter (Murata *et al.*, 1992; Moon *et al.*, 1995). The transgenic plants showed increased saturation of chloroplast thylakoid membrane lipids, resulting in a slightly decreased ability of leaves to recover from low-temperature photoinhibition. Ishizaki-Nishizawa *et al.* (1996) introduced a broad-specificity $\Delta 9$ desaturase gene (*des9*) from the cyanobacterium *Anacystis nidulans* into tobacco under control of the CaMV 35S promoter. This desaturase, which was targeted to plastids, introduces a *cis*-double bond at the $\Delta 9$ position of both 16 and 18 carbon-saturated fatty acids. Transgenic plants had reduced saturated fatty acid contents in most membrane lipids and displayed tolerance to prolonged chilling (11 days at 1 °C or 52 days at 10 °C).

Tobacco was transformed with an *Arabidopsis* chloroplast ω -3 fatty acid desaturase gene (*FAD7*) under control of the CaMV 35S promoter to produce plants with increased levels of trienoic (16:3 and 18:3) fatty acids (Kodama *et al.*, 1994). Transgenic plants displayed enhanced cold tolerance compared to wild-type plants, which showed inhibition of leaf growth after 7 days at 1 °C. Khodakovskaya *et al.* (2006) introduced the *FAD7* gene into tobacco under control of the cold-inducible *Arabidopsis cor15a* promoter. Compared to wild-type plants, transgenic plants showed greater survival under prolonged low temperature stress (44 days at 0.5, 2, or 3.5 °C), more stable levels of trienoic fatty acids and stability of chloroplast membrane ultrastructure (Khodakovskaya *et al.*, 2006). Conversely, transgenic tobacco lines in which the *FAD7* gene was silenced contained lower levels of trienoic fatty acids and were able to acclimate better to higher temperatures (45 days at 36 °C or 3 days at 47 °C) (Murakami *et al.*, 2000). Hamada *et al.* (1996) introduced a tobacco microsomal ω -3 fatty acid desaturase gene (*NtFad3*) in sense and antisense orientations under control of the CaMV 35S promoter. In one of the sense lines, the 18:3 content increased by about 1.5-fold in root tissues and 1.1-fold in leaf tissues, whereas in antisense lines, the 18:3 content was decreased to about 80% in root tissues and 70–80% in leaf tissues, compared to control plants (Hamada *et al.*, 1996). Recently transgenic tobacco plants were produced

that express the transcript of a double-stranded RNA (dsRNA) of the tobacco plastid ω -3 fatty acid desaturase gene *NtFAD7* (Hamada *et al.*, 2006). Compared to control plants, 16:3 and α -18:3 fatty acid contents decreased to less than 2.7% and 7.5–10.4%, respectively, in leaves of transgenic plants. Increased drought tolerance was observed in tobacco plants overexpressing cytosolic *B. napus* *FAD3* or plastidic *Arabidopsis* *FAD8* ω -3 fatty acid desaturase genes under control of the CaMV 35S promoter (Zhang *et al.*, 2005). Plants expressing *FAD3* showed a large increase in the ratio of linolenic (18:3) to linoleic (18:2) acids, whereas in *FAD8* expressing plants, this increase was smaller and mainly associated with plastidic lipids.

2.3.4 Ionic stress

2.3.4.1 Transporter proteins

Maintaining intracellular ion homeostasis is critical to cell survival, affecting enzyme activities and membrane potentials. Exposure to high salt environments imposes sodium toxicity in addition to osmotic stress. Plants employ three mechanisms to prevent excess Na^+ accumulation in cells (Zhu, 2003; Bartels and Sunkar, 2005). Na^+ transporters restrict the entry of Na^+ into cells; intracellular Na^+ is compartmentalized into the vacuole; plasma membrane Na^+/H^+ antiporters transport cytosolic Na^+ back into the external medium or the apoplast. Whilst many of the genes involved in ion homeostasis have been identified and their regulatory mechanisms elucidated in *Arabidopsis*, tobacco has provided a background to compare these genes with those of the halophyte *Thellungiella halophila*.

Gao *et al.* (2006) introduced vacuolar H^+ -pyrophosphatases (PPases) from *T. halophila* (*TsVP*) and *Arabidopsis* (*AVPI*) individually into tobacco, under control of the CaMV 35S promoter. These enzymes function to pump Na^+ into vacuoles. At 300 mM NaCl, transgenic plants had 60% greater dry weight than wild-type tobacco and higher viability of mesophyll protoplasts (Gao *et al.*, 2006). Transgenic lines expressing *TsVP* were able to accumulate 25% more solutes than wild-type plants under nonstress conditions and 20–32% more Na^+ under salt stress. Cell membrane damage and MDA content were

reduced in *TsVP*-expressing plants, suggesting that compartmentalization of Na^+ in vacuoles reduces its toxic effects on cells (Gao *et al.*, 2006). Salt tolerance in tobacco was also improved by overexpression of the *GhNHX1* cDNA, a putative tonoplast Na^+/H^+ antiporter from cotton (*Gossypium hirsutum*), under control of the CaMV 35S promoter (Wu *et al.*, 2004).

2.3.4.2 Calcineurin

Calcineurin (CaN) is a Ca^{2+} - and calmodulin-dependent protein phosphatase (PP2B) that is an integral intermediate of a salt stress transduction pathway in yeast effecting NaCl tolerance through regulation of Na^+ influx and efflux. A truncated form of the catalytic subunit and the regulatory subunit of yeast CaN were co-expressed in tobacco, under control of the CaMV 35S promoter, to reconstitute a constitutively active phosphatase *in vivo* (Pardo *et al.*, 1998). Transgenic lines exhibited increased tolerance to NaCl as seedlings *in vitro* or as plants in hydroponics. The protective effects of CaN in transgenic tobacco appeared to result from the preservation of root integrity during salt shock (Pardo *et al.*, 1998). These results provided evidence that modulation of a signal transduction pathway in plants could be used to improve stress tolerance.

2.3.4.3 Calcium binding protein

Pandey *et al.* (2002) produced transgenic tobacco plants expressing a novel calcium binding protein from *Entamoeba histolytica* (EhCaBP), under control of the CaMV 35S promoter. Seeds of transgenic plants showed enhanced germination rates under nonstress conditions and seedlings produced 20–37% more dry weight than wild-type plants. Transgenic seeds were also able to germinate and grow on 200 mM NaCl and produced 55–100% more dry weight compared to wild-type plants (Pandey *et al.*, 2002).

2.3.5 Oxidative stress—detoxification

Abiotic stress often leads to severe cellular damage in plants as a result of oxidative

stress. Antioxidant enzyme systems protect plant cells by either suppressing the production of, or by scavenging reactive oxygen intermediates (Mittler, 2002). Generation of transgenic tobacco plants overexpressing components of reactive oxygen-scavenging systems has been important in understanding how plants defend themselves against oxidative stress (Allen, 1995).

2.3.5.1 Reactive oxygen intermediate scavenging

SOD acts as a first line of defense against oxidative stress, converting superoxide anion radicals into hydrogen peroxide. Sen Gupta *et al.* (1993) introduced a chloroplastic Cu/Zn SOD from pea (*Pisum sativum*) into tobacco under control of the CaMV 35S promoter. During chilling stress at moderate light intensity, transgenic plants maintained photosynthetic rates approximately 20% higher than wild-type plants and were able to retain almost 90% of their photosynthetic capacity following chilling stress at high light intensity. Transgenic plants also exhibited reduced levels of light-mediated cellular damage following treatment with the active oxygen generator methyl viologen (Sen Gupta *et al.*, 1993). These results demonstrated that SOD is an important component of the active-oxygen scavenging system in plants; however, a petunia chloroplastic Cu/Zn SOD introduced into tobacco under control of an *rbcs* promoter fragment increased SOD levels, but did not increase tolerance to ozone stress (Pitcher *et al.*, 1991).

Mitochondrial manganese superoxide dismutase (MnSOD) from *N. plumbaginifolia* was introduced into tobacco under control of the CaMV 35S promoter, either as a full-length cDNA or with its mitochondrial leader sequence replaced by a chloroplast transit sequence (Bowler *et al.*, 1991). High-level production of MnSOD in chloroplasts significantly reduced oxidative damage induced by methyl viologen in the light, whereas, under dark conditions, transgenic plants expressing either chloroplast- or mitochondria-targeted MnSOD were significantly more resistant to methyl viologen than control plants. In addition, transgenic plants overexpressing MnSOD in chloroplasts displayed enhanced tolerance to

ozone damage (Van Camp *et al.*, 1994). Chloroplastic iron superoxide dismutase (FeSOD) from *Arabidopsis* coupled to a chloroplast-targeting sequence was introduced into tobacco under control of the CaMV 35S promoter (Van Camp *et al.*, 1996). Expression of FeSOD in transgenic plants was able to protect both the plasmalemma and photosystem II against oxidative damage caused by methyl viologen treatment. This is in contrast to overexpression of MnSOD, which only enhanced protection against ion leakage (Slooten *et al.*, 1995).

Hydrogen peroxide resulting from SOD activity is scavenged by ascorbate peroxidases (APX), glutathione peroxidase (GPX) or catalase. APX and GPX require an ascorbate (AsA) or glutathione (GSH) regenerating cycle, involving the oxidation of AsA to monodehydroascorbate (MDA) or GSH to oxidized glutathione (GSSG). The role of APX in protection against oxidative stress was demonstrated through the production of transgenic tobacco plants expressing antisense copies of the tobacco cytosolic APX gene (Örvar and Ellis, 1997). Transgenic plants exhibited reduced APX activity and significantly higher levels of injury following high-level ozone exposure. Torsethaugen *et al.* (1997), however, observed that transgenic tobacco plants overproducing a chloroplast-targeted cytosolic APX isoform from pea showed no increase in protection against ozone-induced stress, compared to wild-type plants. Badawi *et al.* (2004) transformed tobacco with an *Arabidopsis* APX cDNA fused to a chloroplast transit sequence from *Arabidopsis* glutathione reductase (GR), under control of the CaMV 35S promoter. Transgenic plants showed increased tolerance to the active oxygen-generators methyl viologen and sodium sulfite, as well as enhanced tolerance to salt and water stress, as determined by net photosynthesis. Overexpression of the pepper (*Capsicum annum*) APX-like 1 gene (*CAPOA1*) in tobacco, under control of the CaMV 35S promoter, also resulted in transgenic plants with increase in total peroxidase activity (Sarowar *et al.*, 2005). Transgenic plants exhibited increased tolerance to methylviologen-mediated oxidative stress, in addition to significantly increased growth. These results suggest that the tobacco active oxygen scavenging system can be enhanced by overproduction of APX.

Overexpression of a tobacco glutathione S-transferase with glutathione peroxidase activity (GST/GPX) in transgenic tobacco, under control of the CaMV 35S promoter, enhanced seedling growth under stress conditions (Roxas *et al.*, 1997). Transgenic plants contained higher levels of GSSG and AsA and were able to maintain metabolic activity under stress conditions (Roxas *et al.*, 2000).

Yoshimura *et al.* (2004) introduced a *Chlamydomonas* glutathione peroxidaselike protein, targeted to either the chloroplasts or cytosol, into tobacco under control of the CaMV 35S promoter. Transgenic plants showed increased tolerance to methyl-viologen-induced oxidative stress, chilling stress under high light conditions, and salt stress. Lipid hydroperoxidation was suppressed in leaves of transgenic plants compared to wild-type plants, under stress conditions, leading to maintenance of membrane integrity (Yoshimura *et al.*, 2004).

Protection against methyl-viologen-induced oxidative stress could be increased by overexpressing both SOD and APX in chloroplasts of tobacco plants (Kwon *et al.*, 2002). Chloroplast-targeted Cu/Zn SOD, MnSOD, and APX from pea, all under control of the CaMV 35S promoter, were introduced alone or in combination. Tolerance to oxidative stress was only slightly increased in transgenic plants expressing a single SOD isoform; however, the combination of SOD with APX resulted in greater protection (Kwon *et al.*, 2002).

MDA radicals produced by APX are converted back to AsA through reactions with ferredoxin or MDA reductase. MDA can also spontaneously disproportionate to AsA and DHA, which is reduced to AsA in a reaction catalyzed by DHA reductase (DHAR), using GSH as the electron donor. GSSG is converted back to GSH by GR. Transgenic tobacco plants expressing a chloroplast-targeted human DHAR cDNA, under control of the CaMV 35S promoter, exhibited higher levels of AsA and GSSG than wild-type plants (Kwon *et al.*, 2001). In addition to increased DHAR activity, GR activity was also increased in transgenic T₀ and T₁ plants (Kwon *et al.*, 2001, 2003). Leaf discs of transgenic plants showed a reduction in membrane damage following treatment with methyl viologen or hydrogen peroxide compared to wild-type plants as well as enhanced tolerance

to low temperature and salt stress. Eltayeb *et al.* (2006) introduced cytosolic DHAR from *Arabidopsis* into tobacco, under control of the 35S promoter. Transgenic plants exhibited greater tolerance to ozone, drought, salt, and polyethylene glycol (PEG)-induced stress than wild-type plants, manifested by higher net photosynthesis. A GR gene from *E. coli* was introduced into tobacco under control of the CaMV 35S promoter and targeted to the chloroplast (Aono *et al.*, 1993). Increased GR activity in leaves of transgenic plants was correlated with increased tolerance of photooxidative stress induced by paraquat and sulfur dioxide.

Apoplastic ascorbate oxidase (AAO) catalyzes the oxidation of AsA to MDA using oxygen. A tobacco AAO cDNA was introduced into tobacco plants in both sense and antisense orientation, under control of the CaMV 35S promoter (Yamamoto *et al.*, 2005). In sense orientation, overexpression of AAO led to severe inhibition of germination by high salinity. In contrast, germination frequency, photosynthetic activity, root length, and seed yields were higher in antisense plants at high salinity, than in either wild type or sense plants (Yamamoto *et al.*, 2005). The authors suggest antisense suppression of AAO leads to a relatively low level of hydrogen peroxide accumulation and a high redox state of apoplastic and symplastic ascorbate under salt stress.

Antisense technology in tobacco also provided evidence for an alternative defense mechanism that can compensate for the lack of APX and chloramphenicol acetyltransferase (CAT) (Rizhsky *et al.*, 2002). Double antisense plants were less sensitive to oxidative stress than were transgenic plants lacking either APX or CAT. Increased protection against oxidative stress was correlated with reduced photosynthetic activity, the induction of MDAR, metabolic genes belonging to the pentose phosphate pathway and IMMUTANS, a chloroplast homolog of mitochondrial alternative oxidase (Rizhsky *et al.*, 2002).

2.3.5.2 Lipid peroxidation

Free radical-mediated lipid peroxidation is accompanied by the generation of highly reactive aldehyde degradation products. Aldose/aldehyde

reductases reduce a range of aldehydes and ketones to alcohols, whereas aldehyde dehydrogenases are important enzymes in the conversion of toxic aldehydes to less reactive carboxylic forms. Despite their potential roles in detoxification pathways, functional information on both types of enzyme is currently lacking. Transgenic overexpression in tobacco has provided insights into detoxification of reactive aldehyde species.

A stress-activated alfalfa gene (*MsALR*) encoding a novel plant NADPH-dependent aldose/aldehyde reductase was expressed in transgenic tobacco plants under control of the CaMV 35S promoter (Oberschall *et al.*, 2000). Transgenic plants had greater tolerance to paraquat-induced oxidative stress and reduced accumulation of lipid peroxidation-derived reactive aldehydes. Plants expressing this enzyme were also able to recover from prolonged drought stress and showed tolerance to heavy metal treatment (Oberschall *et al.*, 2000). Rodrigues *et al.* (2006) transformed tobacco with a soybean cDNA (*GmTP55*) encoding an ALDH7 aldehyde dehydrogenase, under control of the CaMV 35S promoter. Transgenic plants accumulated *GmTP55* mRNA in leaves and showed tolerance to salinity (200 mM NaCl) during germination and to water stress during plant growth. Transgenic plants also exhibited enhanced tolerance to H₂O₂- and paraquat-induced oxidative stress, associated with reduced concentrations of lipid peroxidation-derived reactive aldehydes (Rodrigues *et al.*, 2006).

Enzymes of the glyoxalase pathway are required for glutathione-based detoxification of methylglyoxal (MG), a potent cytotoxic compound produced during lipid and carbohydrate metabolism and salt stress. Overexpression of glyoxylase I (*glyI*) from *Brassica juncea*, both under control of the CaMV 35S promoter, conferred tolerance to MG and high salinity in transgenic tobacco (Veena *et al.*, 1999). When these plants were further transformed with the glyoxylase II (*glyII*) gene isolated from rice, transgenic plants exhibited even greater tolerance to high MG and NaCl concentrations and were able to grow, flower, and set seed under continuous salinity stress (Singla-Pareek *et al.*, 2003). Increased levels of glutathione-related antioxidative enzymes enabled plants to resist an increase in MG levels under salinity stress, maintaining a higher GSH:GSSG ratio (Yadav *et al.*, 2005).

2.4 Generalities on Plant Mitochondrial Genomes and Function

Mitochondrial biogenesis and functioning depend on both nuclear and mitochondrial DNA (mtDNA) encoded subunits. The mitochondrial (mt) genome in higher plants is larger and more complex than in other eukaryotes (Schuster and Brennicke, 1994). With some exceptions, plant mitochondrial genomes are maternally inherited, as in most animals. According to cosmid mapping, they may be represented as a single circular map, the so-called master molecule (Palmer and Shields, 1984), which can give rise to subgenomic circles by recombination between inverted or direct repeats (Backert *et al.*, 1997). Subgenomic molecules are often in substoichiometric amounts, and can serve as reservoir for mitochondrial genome evolution (Small *et al.*, 1989). Mitochondrial genomes are quite variable, ranging from 218 to 2500 kb (Palmer and Herbon, 1988). Within one family, the Cucurbitaceae, mitochondrial genome sizes vary between 330 kb and 2,500 kb (Ward *et al.*, 1981). However, the numbers of proteins specified by the smallest and largest of the mitochondrial genomes of the family are roughly similar (Stern and Newton, 1985).

In contrast to animal mitochondria, much of the mtDNA in plants appears to be noncoding. Indeed, all of the known genes account for only 10–20% of mtDNA of various species (Palmer *et al.*, 2000). The noncoding mtDNA includes introns, pseudogenes, nonfunctional chloroplast sequences, and retrotransposons of nuclear origin. The coding part of the *Arabidopsis thaliana* mitochondrial genome consists of about 57 open reading frames (Unsel *et al.*, 1997). Known genes found so far in all the sequenced plant mitochondrial genomes include those for ribosomal RNAs, tRNAs, and several mitochondrial proteins. The mtDNA proteins include several subunits of the complexes of the electron transfer chain: Complex I (NAD 1, 2, 3, 4, 4L, 5, 6, 7 and usually NAD9), Complex III (COB), Complex IV (COX 1, 2, 3), and Complex V (ATP 1, 6, 8, and 9). In addition, the mitochondrial genomes include genes for proteins involved in the biogenesis of cytochrome c (*ccmB*, *FN*, *FC*, and usually *ccmC*), as well as those for several ribosomal proteins.

Plant mtDNA mutants are scarce, as mtDNA usually has a slow rate of nucleotide substitution,

although there are some exceptions (Palmer *et al.*, 2000). Thus, most of the well-studied mitochondrial mutations derive from rearrangements and deletions. In some cases, these rearrangements are neutral and do not confer any marked phenotype, as in the *Nicotiana sylvestris* U mutant (Vitart *et al.*, 1992; Albert *et al.*, 2003). In contrast, several mtDNA reorganizations have been reported to be associated with cytoplasmic male sterility (CMS) that is thought to be caused by the expression of mitochondrial encoded chimeric proteins deleterious to pollen development. Nuclear genes, designated restorers of fertility (*Rf*) impair the production of CMS-associated polypeptides (discussed in Schnable and Wise, 1998; Budar *et al.*, 2002; Wise and Pring, 2002). An important class of *Rf* genes consists of PPR transcription factors that are involved in the control of both chloroplastic and mitochondrial gene expression (Lurin *et al.*, 2004). RNA editing in plant mitochondria, which involves the substitution of some C residues present in the initial transcript for U residues has also been associated with CMS in several species (Schuster and Brennicke, 1994). In contrast to CMS, mtDNA deletion mutants are impaired in both vegetative and reproductive development. They are generally heteroplasmic, with cells harboring a mixture of normal and deleted mtDNA molecules, and are not stably transmitted through sexual reproduction. In maize, mtDNA deletion mutations resulting in abnormal growth are termed nonchromosomal stripe (NCS). A number of maize NCS mutants have been analyzed and shown to result from deletions of portions of essential mitochondrial genes (Newton *et al.*, 1989, 1990; Marienfeld and Newton, 1994). The heteroplasmic plants exhibit distorted growth, attributed to the loss of the mitochondrial protein synthesis in mutant sectors (Hunt and Newton, 1991; Roussel *et al.*, 1991; Newton *et al.*, 1996), but they can shed pollen from normal sectors. The *Arabidopsis* maternally inherited distorted leaf (MDL) phenotype has been associated with rearrangements and deletions in *rps3/rpl16* (Sakamoto *et al.*, 1996). In contrast, an abnormal growth mutant of cucumber, designated MSC (mosaic), is paternally transmitted (Malepszy *et al.*, 1996). Near-homoplasmic deletion mutants stable by sexual reproduction have been reported in *N. sylvestris*, conferring growth retardation and

partial male sterility (Li *et al.*, 1988; Lelandais *et al.*, 1998).

A major function of plant mitochondria is oxidative phosphorylation that couples electron transport through the respiratory chain to proton translocation from the matrix to the intermembrane space, generating an electrochemical gradient necessary for ATP synthase (Siedow and Umbach, 1995). In addition to the main electron transport chain consisting of complexes I–IV, mitochondria in plants and some fungi possess nonproton-pumping respiratory enzymes encoded by the nuclear genome: various external and internal NAD(P)H alternative dehydrogenases bypassing Complex I (Rasmusson *et al.*, 1999, 2004; Moller, 2001) and a cyanide-resistant terminal oxidase (AOX) branching at the level of the ubiquinone pool (Lambers, 1982; reviewed in Vanlerberghe and McIntosh, 1997). In contrast to Complexes I, III, and IV, alternative respiratory enzymes are not directly involved in energy production. Hence, it has been proposed that AOX may prevent over-reduction of the respiratory chain, either in situations of excessive NADH supply as a consequence of a high TCA activity or a high ubiquinone reduction level during inhibition of the COX pathway (Lambers, 1982). Another role for alternative respiratory enzymes may be to minimize the formation of reactive oxygen species (ROS) by over-reduced electron transport chain components (Wagner and Krab, 1995; Purvis, 1997; Moller, 2001), particularly at the ubiquinone pool, and to limit oxidative stress (Popov *et al.*, 1997; Maxwell *et al.*, 1999). Accordingly, several studies have revealed new important functions for plant mitochondria, as the control of cell redox homeostasis and their importance for chloroplastic metabolism (Dutilleul *et al.*, 2003a, b). Plant mitochondria, like animal mitochondria seem to be involved in resistance to biotic and abiotic stresses and cell death control (Purvis and Shewfelt, 1993; Ordog *et al.*, 2002; Tiwari *et al.*, 2002); however, experimental evidence is scarce and precise mechanisms remain to be determined.

In addition to oxidative phosphorylation, mitochondria play numerous roles in plant metabolism (Douce and Neuburger, 1989; Mackenzie and McIntosh, 1999). They are directly involved in the synthesis of nucleic acids, pantothenate, and several amino acids as methionine, glycine, serine, and proline and indirectly, through S-adenosyl

methionine, in all methylation reactions. They also contain branched-chain amino acid transaminases (BCATs) catalyzing the last step of the synthesis and/or the initial step of the degradation of leucine, isoleucine, and valine (Aubert *et al.*, 1996; Anderson *et al.*, 1998; Diebold *et al.*, 2002). Plant mitochondria are involved in the C2 oxidative photosynthetic carbon cycle (Mouillon *et al.*, 1999; Rébeillé *et al.*, 2006) and possess all the necessary enzymatic equipment for *de novo* synthesis of tetrahydrofolate and lipoic acid, serving as cofactors for glycine decarboxylase and serinehydroxymethyltransferase functioning. The final steps of biotin and folate synthesis involve several mitochondrial proteins (Picciocchi *et al.*, 2003). Folates are crucial intermediates for a set of reactions that involve the transfer of single-carbon units (C1 metabolism). Plant mitochondria also contain the enzymatic equipment necessary to transform malonate into the two main building units for fatty acid synthesis, malonyl- and acetyl-acyl carrier protein (ACP), a component of complex I (Gueguen *et al.*, 2000), and have been reported to contribute to leaf lipid synthesis in *Arabidopsis* (Wada *et al.*, 1997).

All the above processes are carried out by nuclear-encoded enzymes and thus manipulation of mitochondrial function might involve transformation of both nuclear and mitochondrial genomes. However, the only photosynthetic organism of which the mitochondrial genomes can be easily manipulated is *Chlamydomonas reinhardtii* (Remacle *et al.*, 2006). Thus, in higher plants transgenesis essentially involves nuclear genes. For example, antisense repression of the mitochondrial NADH-binding subunit of complex I in transgenic potato plants affects male fertility (Heiser *et al.*, 1997). In addition, the introduction of mitochondrial genes in the tobacco nuclear genome has been reported (Hernould *et al.*, 1993; Pineau *et al.*, 2005).

2.4.1 *Nicotiana* mtDNA mutants and transgenics

Tobacco species are a valuable model system for investigating the genetic interaction between mitochondria, chloroplasts, and nucleus of the plant cell. Indeed, the tobacco mtDNA is fully sequenced (Sugiyama *et al.*, 2005), and stable

mtDNA deletion mutants have been obtained by protoplast culture (reviewed in Vedel *et al.*, 1999).

2.4.1.1 *N. sylvestris* mtDNA deletion mutants

In *N. sylvestris*, the female ancestor of the cultivated *N. tabacum*, protoplast culture has given rise to stable mutants with either neutral mtDNA rearrangements (Vitart *et al.*, 1992; Albert *et al.*, 2003) or mtDNA deletions (Li *et al.*, 1988; Chétrit *et al.*, 1992). These deletion mutants, designated CMSI and CMSII due to their partial male sterility, do not exhibit the leaf variegation described above for the other abnormal growth mutants, and their morphological defects are stably transmitted through sexual and somatic generations. This probably reflects the near-homoplasmic mutant composition of their mtDNAs that contain large deletions including the *nad7* sequence encoding the NAD7 Complex I subunit (Pla *et al.*, 1995; Gutierrez *et al.*, 1997; Lelandais *et al.*, 1998). Respiration measurements on mitochondria isolated from CMS leaf tissues showed near complete lack of activity of Complex I and impaired oxidation of glycine, a major respiratory substrate in photosynthetic cells of C3 plants (Douce and Neuburger, 1989). In contrast, the oxidation rate of tricarboxylic cycle substrates and exogenous NADH were increased. Total leaf respiration measured by gas exchange experiments (IRGA) was higher in the mutant than in the WT (Duranceau *et al.*, 2000; Sabar *et al.*, 2000; Dutilleul *et al.*, 2003a). Nonproton-pumping respiratory pathways maintain normal *in vivo* respiration levels in leaves of NAD7-deficient CMS I and II, but these rates are decreased in pollen (Sabar *et al.*, 1998). Survival of these plants depends on the activation of nuclear-encoded internal and external alternative NAD(P)H dehydrogenases, which bypass Complex I (Rasmusson *et al.*, 1999, 2004).

Cyanide-resistant respiration and alternative oxidase (AOX) protein levels were shown to be induced in CMS I and II (Gutierrez *et al.*, 1997; Sabar *et al.*, 2000) as in all the maize NCS plants (Karpova *et al.*, 2002). Because alternative oxidase is nucleus encoded and its induction can be seen at the RNA level, the mutant mitochondria must produce a signal to induce AOX gene

expression. Furthermore, because AOX is also induced by electron transport inhibitors, such as antimycin A (Vanlerberghe and McIntosh, 1997), it is apparent that dysfunction of the mitochondrial respiratory chain results in a strong signal for induction of AOX. However, use of oxygen isotope fractionation to measure the *in vivo* AOX activity (Guy *et al.*, 1989) showed that *in vivo* partitioning through the AOX and the cytochrome (COX) pathways was similar in CMSII and wild-type leaves (Priault *et al.*, 2007). This is not related to marked differences in the redox state of the protein, rather to either expression of a specific AOX gene and/or impaired metabolic control (Vidal *et al.*, 2007). The higher respiration of mature CMSII leaves was supported exclusively by enhanced COX activity (Priault *et al.*, 2007; Vidal *et al.*, 2007). Enhanced activity of the proton-pumping COX route in the mutant can thus be viewed as a compensation for the lack of the first coupling site of the respiratory chain.

Complex I activities are also strongly reduced in a *N. sylvestris* nuclear mutant, termed NMS1, deficient for the NAD4 subunit (De Paepe *et al.*, 1990; Brangeon *et al.*, 2000). Alternative oxidase transcripts and proteins were induced, although to a lesser extent than in CMS, and external NAD(P)H dehydrogenase activities were not increased (Sabar *et al.*, 2000). These results show that lack of NAD4 and NAD7 has different biochemical and physiological consequences. The relationships with the phenotype, that is more affected in NMS1 than in CMS plants, remain however to be determined.

In contrast to respiration, photosynthesis is affected in the *N. sylvestris* CMSI, CMSII, and NMS1 mutants (Sabar *et al.*, 2000). It was demonstrated that mitochondrial Complex I activity is required for optimal photosynthetic performance and is necessary to avoid redox disruption of photosynthesis (Dutilleul *et al.*, 2003a). CMSII plants are also impaired in acclimation to high light (Priault *et al.*, 2006a), but have higher photorespiration (Priault *et al.*, 2006b). These studies show the importance of mitochondria for chloroplast biogenesis and function, previously revealed by physiological and inhibitor studies (reviewed in Krömer, 1995; Raghavendra and Padmasree, 2003).

Moreover, the metabolite profile of CMSII leaves is enriched in amino acids with low C/N,

and depleted in starch and 2-oxoglutarate (2-OG). The accumulation of nitrogen-rich amino acids was not accompanied by increased expression of enzymes involved in nitrogen assimilation (Dutilleul *et al.*, 2005). Analysis of pyridine nucleotides showed that both NAD and NADH were increased by twofold in CMS leaves as compared to wild type, providing strong evidence that pyridine nucleotide availability exerts a crucial influence in the integration of ammonia assimilation and the anaplerotic production of carbon skeletons.

The *N. sylvestris* CMSII mutant was further exploited to explore the role of plant mitochondria in the regulation of cellular redox homeostasis and stress resistance. Acclimation in response to loss of Complex I function is associated with marked spatial and temporal reorganization of antioxidant and defense metabolism that affords enhanced protection to oxidative stress (Dutilleul *et al.*, 2003b). The overall cellular redox state is maintained, as evidenced by lower H₂O₂, and ascorbate and glutathione redox states similar to the wild type. The reorganization of the antioxidant system, both inside and outside the mitochondria, is associated with enhanced resistance to ozone and TMV (Dutilleul *et al.*, 2003b) and with altered reaction to harpin, a bacterial elicitor of the hypersensitive response (Boccaro *et al.*, 2001; Garmier *et al.*, 2002).

To test whether *N. sylvestris* CMS defects directly result from the deletion of *nad7*, CMS transgenic plants carrying an edited *nad7* cDNA fused to the CAMV 35S promoter and to a mitochondrial targeting sequence, were generated (Pineau *et al.*, 2005). The *nad7* sequence was transcribed, translated, and the NAD7 protein directed to mitochondria in CMS transgenics therefore termed CMSII*nad7*, which recovered both wild-type morphology and growth features. Blue-native/SDS gel electrophoreses and enzymatic assays showed that a functional complex was present in CMSII*nad7* mitochondria, demonstrating that lack of complex I in CMSII was indeed the direct consequence of the absence of the NAD7 subunit. Hence, NAD7 is necessary for complex I assembly in plants. The reversion of the AOX expression pattern observed in the transgenic CMS*nad7* was another indication for the restoration of the wild type properties of the respiratory chain. Furthermore, CMS*nad7* plants

harbored amino acid contents similar to the wild type (Dutilleul *et al.*, 2005). Taken together, these results also show that allotopic expression from the nucleus can fully complement the lack of a mitochondria-encoded complex I gene.

2.4.1.2 *N. tabacum* mutants for mtRNA editing

Transgenic tobacco plants transformed with an unedited copy of the mitochondrial *atp9* gene were partially male sterile (Hernould *et al.*, 1993), whereas the expression of the antisense *atp9* RNA abolished the effect of the unedited chimeric gene, by strongly decreasing the abundance of edited *atp9* transcripts (Zabaleta *et al.*, 1996). These results clearly indicate the importance of RNA editing for plant fertility.

2.4.2 Tobacco transgenics of nuclear genes for mitochondrial function and biogenesis

2.4.2.1 Respiratory chain enzymes

To date a few studies of plant transgenics for nuclear genes encoding respiratory proteins have been reported, possibly because most genes encoding main chain subunits are not single copy, and that double mutants are lethal. In *Nicotiana* species, the most extensive studies have concerned the alternative respiratory enzymes, AOX and alternative NAD(P)H dehydrogenases.

Alternative oxidase The AOX protein is a homodimer with the two polypeptides linked by a disulfide bridge (Andersson and Nordlund, 1999; Juszczuk and Rychter, 2003). The reduced form is more active than the covalently linked oxidized form in *in vitro* assays (Umbach and Siedow, 1993; Day *et al.*, 1994) and the enzyme is further activated by pyruvate and other α -ketoacids (Millar *et al.*, 1993). However, the AOX activation state *in vivo* is not well understood (Millenaar *et al.*, 2002). AOX genes belong to a multigene family, comprising at least two subfamilies, termed AOX1 and AOX2, with only limited nucleotide homology (Considine *et al.*, 2002)

Sense and antisense DNA constructs of the *N. tabacum* *Aox1* gene were introduced into tobacco, and transgenic plants with both increased and decreased levels of the AOX protein were analyzed (Vanlerberghe *et al.*, 1994, 1995). Antisense cultured cells could not survive in the presence of inhibitors of the cytochrome pathway (Vanlerberghe *et al.*, 1997, 2002), confirming that a critical function of AOX may be to support respiration when the main pathway is impaired.

Tobacco AOX sense and antisense transgenics were further used to demonstrate the critical role of plant mitochondria in response against abiotic and biotic stresses, including cell death (Maxwell *et al.*, 1999; Ordog *et al.*, 2002; Robson and Vanlerberghe, 2002; Gilliland *et al.*, 2003; Amirsadeghi *et al.*, 2006). In particular, antisense AOX transgenics were reported to be more susceptible to cell death inducers. However, subsequent measurement of AOX activity by oxygen isotope discrimination indicated that the *in vivo* AOX activity measured by oxygen isotope discrimination was similar in transgenics with either low or high protein amounts as in the wild type (Guy and Vanlerberghe, 2005), showing that complex regulations of whole mitochondrial metabolism rather than AOX activity *per se* was involved in the previously observed stress responses.

NAD(P)H dehydrogenases Overexpression of the potato *NDB1* gene in transgenic *N. sylvestris* demonstrated that this gene encodes an external dehydrogenase specific for NADPH and dependent on calcium for activity. Transformed plants had increased protein levels for alternative oxidase and uncoupling protein, indicating crosstalk for the different categories of energy-dissipating proteins that bypass oxidative phosphorylation (Michalecka *et al.*, 2004)

2.4.2.2 TCA cycle enzymes

Isocitrate dehydrogenase Transgenic *N. tabacum* plants overexpressing NADP⁺-dependent mitochondrial isocitrate dehydrogenase (mtICDH) displayed a measurable increase in the reductive activation of AOX in comparison with wild type, implicating this enzyme in the redox activation

of AOX (Gray *et al.*, 2004). These results support the hypothesis that mtICDH may be a regulatory switch involved in tricarboxylic acid cycle flux and the reductive modulation of AOX.

Aconitase In animals, aconitase is a bifunctional protein that is involved in both TCA cycle functioning and RNA processing. A similar role was recently demonstrated in *Arabidopsis* and *N. benthamiana* transgenics. Aconitase-silenced plants displayed a delayed hypersensitive response (HR), suggesting that aconitase might play a role in mediating oxidative stress and regulating cell death (Moeder *et al.*, 2007).

2.4.3 Other mitochondrial functions in relation with stress tolerance

Transgenic tobacco cells accumulating free proline by silencing proline dehydrogenase expression were tolerant to osmotic stress (Tateishi *et al.*, 2005). Transgenic tobacco plants overexpressing the tomato mitochondrial small heat-shock protein exhibited normal morphology and growth rates, but showed higher tolerance to heat stress, whereas antisense plants were more susceptible (Sanmiya *et al.*, 2004).

2.4.4 Concluding remarks

Nicotiana mtDNA mutants and nuclear transgenics for mitochondrial enzymes have been invaluable tools for the study of plant respiration and of the involvement of mitochondrial metabolism in cell redox homeostasis, stress resistance, and cell death, in plants as in animals (Desagher and Martinou, 2000). However, in photosynthetic cells, the crosstalk between mitochondrial and chloroplast metabolism seems to be of crucial importance and needs further investigation.

2.5 Plastid Transformation

N. tabacum was the first higher plant to receive an engineered plastome (Svab *et al.*, 1990) and

all basic principles of plastid transformation technology are derived from the work in this species. Tobacco was chosen mainly due to its performance in tissue culture and to the fact that the tobacco plastome was the first to be fully sequenced (Shinozaki *et al.*, 1986; last update Yukawa *et al.*, 2005)

A query to PubMed for plastid or chloroplast transformation results in more than 25 review articles. Some of these are compiled in Table 1. This chapter does not attempt to give a fully comprehensive overview on all data published on tobacco plastid transformation. Rather, it presents aspects that complement the aforementioned reviews, such as significant findings or those, which have not been fully discussed previously. Almost all of the review articles stress the potential advantages of plastid versus nuclear transformants, i.e., high expression level, engineering and co-expression of several genes in polycistronic operons, transgene containment due to maternal inheritance, lack of gene silencing, and unknown position effects and precision of engineering via homologous recombination. The emphasis on potential benefits combined with limited discussion of problems or failures has generated very high expectations with respect to the value of the technology as a platform for recombinant protein expression or generation of agronomically important traits. It should be kept in mind, however, that expectations are not always met. Expression levels of recombinant proteins may indeed be extremely low if the protein of interest is efficiently degraded in the organelle (Leelavathi and Reddy, 2003; Birch-Machin *et al.*, 2004). Also, the lack of post-translational modifications such as glycosylation in plastids severely limits the value of this expression platform where higher order modification of the end product (e.g., a human protein) is of importance for function and/or regulatory aspects.

2.5.1 Vector design

Plastome transformation is either based on the activity of the organelle's own endogenous recombination capacity or the use of a heterogeneous recombination system, such as the phiC31 phage integrase.

Table 1 Selected reviews on plastid transformation

Authors	Year	Title
Maliga <i>et al.</i>	1993	Toward plastid transformation in flowering plants
Kofer <i>et al.</i>	1998a	PEG-mediated plastid transformation in higher plants
Hager and Bock	2000	Enslaved bacteria as new hope for plant biotechnologists
Heifetz	2000	Genetic engineering of the chloroplast
Bock	2001	Transgenic plastids in basic research and plant biotechnology
Heifetz and Tuttle	2001	Protein expression in plastids
Maliga	2003	Progress toward commercialization of plastid transformation technology
Bock and Khan	2004	Taming plastids for a green future
Maliga	2004	Plastid transformation in higher plants
Daniell <i>et al.</i>	2005a	Chloroplast-derived vaccine antigens and other therapeutic proteins
Daniell <i>et al.</i>	2005b	Breakthrough in chloroplast genetic engineering of agronomically important crops
Maliga	2005	New vectors and marker excision systems mark progress in engineering the plastid genome of higher plants
Nugent and Joyce	2005	Producing human therapeutic proteins in plastids
Dhingra and Daniell	2006	Chloroplast genetic engineering via organogenesis and somatic embryogenesis
Lutz <i>et al.</i>	2006a	Construction of marker-free transplastomic tobacco using the Cre-loxP site-specific recombination system
Bock	2007	Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering, and molecular farming
Koop <i>et al.</i>	2007	The genetic transformation of plastids

2.5.1.1 Use of the endogenous recombination system

Plant plastid transformation has mainly been achieved through the use of sequences on an engineered transformation vector containing sufficient homology to the target plastome to allow for homologous recombination mediated by the organelle's recombination system. These so-called "homologous flanks" are generally approximately 1 kbp in size assuming that shorter flanks would reduce recombination efficiency while significantly longer flanks make cloning more difficult. Expression cassettes in plastid transformation vectors must take into account that regulatory elements, such as promoters, 5' untranslated regions (UTRs), ribosome binding sites, and 3' UTRs need to be compatible with the plastid gene expression machinery. A heterologous RNA polymerase can also be used for transcription, if the expression cassette is equipped with a suitable promoter (McBride *et al.*, 1995). Further modifications may include a "downstream box" for enhanced translation efficiency (Kuroda and Maliga, 2001; Herz *et al.*, 2005), fusion, and/or purification tags for enhanced protein stability and facilitation of protein extraction (Leelavathi and Reddy, 2003), and proteolysis recognition sites

if authentic starting amino acids are required for a processed protein end product (Staub *et al.*, 2000). A sample plastid transformation vector for the insertion of a dicistronic operon is depicted in Figure 2. A selectable marker gene is certainly also required for the transformation process. If the second cistron is used for this purpose, it is safe to assume that the protein encoded by the first cistron is expressed in the recovered transformants. Alternatively, a selection marker cassette could also be positioned elsewhere on the same transformation vector or on a different transformation vector and used in a co-transformation approach, which works efficiently in plastid transformation (Carrer and Maliga, 1995; Herz *et al.*, 2005). Up to four genes combined in a single operon were successfully introduced into the tobacco plastome (Lossel *et al.*, 2003; Quesada-Vargas *et al.*, 2005), and a principle limitation of the number of cistrons that can be co-introduced into and co-expressed in a plastome cannot be seen.

Note that not all elements depicted in Figure 2 are indispensable. Thus, separate promoters are not required, if transcription is mediated by endogenous transcription start signals (Staub and Maliga, 1995). Such "operon extension vectors" are described in detail by Herz *et al.*

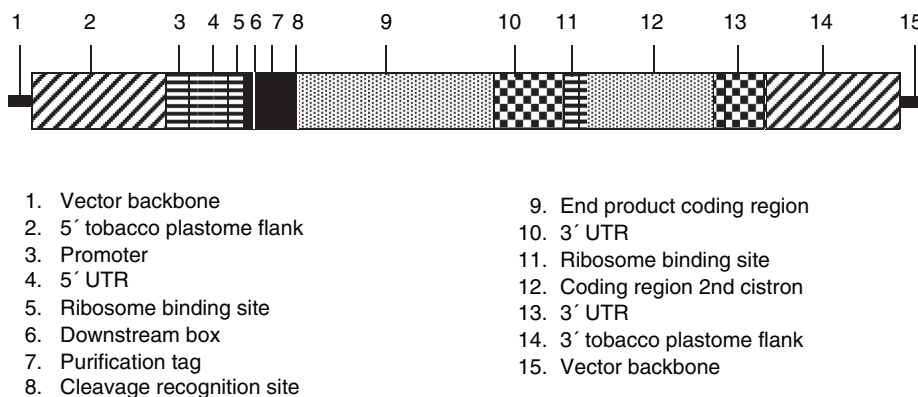


Figure 2 Elements of a dicistronic tobacco plastid transformation vector

(2005). It is also possible to use incomplete expression cassettes on co-transforming, separate transformation vectors, since complete and functional expression cassettes can be assembled by homologous recombination inside the plant after transformation treatment (Herz *et al.*, 2005).

2.5.1.2 Use of the *phiC31* phage integrase

It has been speculated that difficulties in transferring the plastid transformation methods developed for tobacco to other species might be related to differences in the efficiencies of the endogenous recombination systems among different species. It is therefore useful to investigate the suitability of heterogeneous systems. Lutz *et al.* (2004) demonstrated that the integrase of phage *phiC31* can be used for integrating foreign DNA into the tobacco plastome if supplied via expression from a nuclear gene and targeted to the plastid or—although less efficiently—if supplied through a transiently expressed plastid vector. While the approach is certainly interesting, its value remains to be demonstrated. Integration using this recombination system requires the presence of a suitable recognition sequence (*attB*) at the desired integration locus of the target plastome. This, however, must be generated through the endogenous recombination system first, which may be difficult, if indeed the efficiency of the endogenous recombination system was the limiting factor in a particular species.

2.5.2 Target tissues

The larger size of chloroplasts in comparison with proplastids made leaf explants the target tissue of choice for plastid transformation treatments. Indeed, green tissue or protoplasts prepared thereof were the targets for the first successful plastid transformations in tobacco (Svab *et al.*, 1990; Golds *et al.*, 1993) and other species (*N. plumbaginifolia*: O'Neill *et al.*, 1993; *Arabidopsis thaliana*: Sikdar *et al.*, 1998; *S. tuberosum*: Sidorov *et al.*, 1999; *L. esculentum*: Ruf *et al.*, 2001; *B. napus*: Hou *et al.*, 2003; *Lesquerella fendleri*: Skarjinskaia *et al.*, 2003; *Petunia hybrida*: Zubko *et al.*, 2004; *Lactuca sativa*: Lelivelt *et al.*, 2005; *Populus alba*: Okumura *et al.*, 2006). However, nongreen tissues, such as in tobacco, protoplast-derived microcolonies (Huang *et al.*, 2002), albino leaves (Klaus *et al.*, 2003) or cell suspensions (Langbecker *et al.*, 2004), and cell cultures of other species (*Oryza sativa*: Khan and Maliga, 1999; *Daucus carota*, *G. hirsutum*: Kumar *et al.*, 2004a, b; *Glycine max*: Dufourmantel *et al.*, 2004) have also been used successfully. It is therefore evident that plastid transformation does not require fully developed chloroplasts. This is of significance since in important crops, such as the cereals, regeneration from green tissues is not yet possible.

2.5.3 Methods of gene delivery

There are two methods to deliver transforming DNA into tobacco plastids, which make it possible

to recover stably transformed lines, the particle gun-mediated biolistic process and treatment of isolated protoplasts with PEG in the presence of suitable transformation vectors. For detailed protocols the reader is referred to the reviews listed in Table 1 (see e.g., Dhingra and Daniell, 2006 and Lutz *et al.*, 2006a, for particle bombardment and Kofer *et al.*, 1998a for PEG treatment). The mechanism of entry of the transforming DNA is assumed to be by mechanical impact in the case of the biolistic procedure: microprojectiles supposedly penetrate the organelle's envelope thus carrying the DNA inside. It is not known whether or how a chloroplast envelope would reseal after penetration. With PEG-treatment the mechanism of DNA entry into a cell and then through the two envelope membranes is even less clear. The assumption derived from transient expression assays with nuclear reporter constructs is that PEG produces transient discontinuities or "holes", in the plasma membrane through which DNA can enter into the cell (Paszkowski *et al.*, 1984). Such a process would lead to entry of plasmids into the cytosol, and it remains completely unknown how, subsequently, the DNA could reach the inside of an organelle. If, however, there is transfer of DNA from the cytosol into organelles that have an envelope consisting of two membranes, then it is also conceivable that, through particle bombardment plasmids are primarily delivered into the cytosol and enter the organelle afterward. Experiments that would elucidate the mechanisms of DNA uptake are difficult to conceive. In tobacco, plastid transformation is highly efficient irrespective of the method used for DNA delivery and the precise pathway of DNA entry.

It is important to note that a femtosyringe-based microinjection procedure was used to deliver reporter genes into plastids (Knoblauch *et al.*, 1999; Van Bel *et al.*, 2001), and transient reporter gene expression was clearly achieved; however, stable transformants were not generated. When species, closely related to tobacco, prove recalcitrant to plastid transformation, an interesting approach can be used exploiting the fact that plastids in tobacco can be transformed. Kuchuk *et al.* (2006) transformed the plastomes of five different recalcitrant solanaceous species after generating cytoplasmic hybrids with tobacco supplying the nuclear genome and the other species donating the cytoplasmic genomes.

2.5.4 Selection systems

Selection systems for higher plant plastid transformation need to fulfil several highly demanding requirements. Selective advantage must be generated on two levels, that of the single plastid and that of the individual cell. It is assumed that the initial transformation event involves a single or few of the high number—up to 10 000 per cell in a fully developed leaf (Bendich, 1987)—of plastid chromosomes only. The kinetics of the increase of the transplastome copy number during selection is not known. It has been speculated that the presence of an origin of replication on the transformation vector might allow for multiplication of the vector inside the organelle, which increases the number of transformation events (Dhingra and Daniell, 2006). However, there is no experimental evidence supporting this assumption. Moreover, the origin of replication used by the Daniell laboratory was shown to be dispensable for replication (Muhlbauer *et al.*, 2002). While prolonged presence of vector plasmid molecules has indeed been observed, it is more likely that such molecules are derived from recombination rather than replication events (Staub and Maliga, 1994; Klaus *et al.*, 2004). Replication of the plastome itself, possibly in combination with gene conversion events (Khakhlova and Bock, 2006), therefore, appears to be the cause of increase of transplastome copy number in an organelle. Although molecules are exchanged between individual plastids of a cell (Kohler *et al.*, 1997), there is no evidence that this process also involves plastid DNA. Therefore, the assumption is that plastid DNA replication and plastid division are both necessary processes leading to the increase of numbers of organelles containing transformed DNA copies inside a cell. Selection systems need to favor those plastids that contain the highest proportion of transformed plastomes and/or those cells that contain the highest proportion of plastids with transformed plastomes. At the same time, nontransformed plastids and cells without transformed plastids need to stay viable. Selection pressure, therefore, has to be adjusted carefully.

It is noteworthy that transmittance of different plastome copies to the products of organelle division and transmittance of organelles to the products of cell division are both random processes. Segregation, i.e., the generation of cells,

which are homozygous with respect to their plastomes, termed “homoplastomic”, from cells containing different types of plastomes, termed “heteroplastomic”, does not require selective pressure. Segregation is a statistical process (Michaelis, 1966) and cannot be avoided, unless there is counter selection, for example, in the case of the disruption or deletion of an essential gene (Drescher *et al.*, 2000).

Different schemes of selection were used in tobacco plastid transformation. Selection using an antibiotic always comprised the initial step of primary selection, and in some schemes this was followed by secondary selection using a different inhibitor or a pigmentation phenotype. Screenable markers, such as the GFP were also used to assist detection of transformed sectors of tissues in tobacco (Khan and Maliga, 1999) and other species (Sidorov *et al.*, 1999; Skarjinskaia *et al.*, 2003).

2.5.4.1 Primary selection using antibiotic inhibitors

Different selection markers have been used successfully, all of which are based on tolerance toward aminoglycoside antibiotics. Tolerance is either based on mutations of the ribosomal RNA target site (Svab *et al.*, 1990) or on the expression of detoxifying enzymes (Carrer *et al.*, 1993; Svab and Maliga, 1993; Huang *et al.*, 2002). Aminoglycoside antibiotics are inhibitors of protein biosynthesis on prokaryotic/organelle ribosomes and should, therefore, also inhibit mitochondrial protein biosynthesis. Detoxifying enzymes should reduce the concentration of the antibiotic in question in the whole cell and should, therefore, also reduce the effect of the inhibitors on mitochondria. However, in the case of insensitive plastid ribosomal RNA target sites it is unclear why the supposed inhibition of protein biosynthesis in the mitochondria is tolerated by

the cell. Possibly, growth and development *in vitro*, i.e., under heterotrophic conditions, can occur with reduced mitochondrial protein biosynthesis.

Aminoglycoside antibiotics that have been used successfully for the selection of plastid transformants in tobacco are listed in Table 2.

A report that was published under the interesting title “Marker free transgenic plants:” received considerable attention. The plant nuclear gene *badh* encoding BADH, in combination with betaine aldehyde as the selective agent, was described as an alternative selection system and was claimed to be far superior to those listed in Table 2 (Daniell *et al.*, 2001). Certainly, irrespective of its functionality as a selection marker, the *badh* gene can be expressed in higher plant plastids (Kumar *et al.*, 2004a, b). However, the conclusion presented in Daniell *et al.* (2001) is curious in that the vectors used for transformation maintained other selectable markers in addition to a *badh* selection cassette (compare Maliga, 2005). Furthermore, neither the Daniell group nor any other laboratory has since reported successful selection using betaine aldehyde, and a number of laboratories failed to independently reproduce the results. In the absence of reproducibility, attempts to select plastid transformants via the betaine aldehyde system should be avoided. Chloroplast engineering requires a substantial investment of human and laboratory capital and other methods of selection have proven to be effective.

Alternatives to the antibiotic resistance genes listed in Table 2 have not proven reproducibly effective. Herbicides could not be used for selection, although expression of herbicide resistance genes is possible in plastids and can lead to considerable levels of tolerance (Daniell *et al.*, 1998; Iamtham and Day, 2000; Lutz *et al.*, 2001; Ye *et al.*, 2001, 2003). A similar situation was found for other marker genes, which have been successful in selection of nuclear transformants, such as hygromycin phosphotransferase (Dhingra

Table 2 Selective agents and selection marker genes in tobacco plastid transformation

Antibiotic agents	Tolerance conferring genes	References
Spectinomycin/streptomycin	16S rRNA	Svab <i>et al.</i> , 1990
Spectinomycin/streptomycin	<i>aadA</i>	Svab and Maliga, 1993
Kanamycin	<i>nptII</i>	Carrer <i>et al.</i> , 1993
Kanamycin	<i>aphA6</i>	Huang <i>et al.</i> , 2002

and Maliga, personal communication), sulfadiazin insensitive dihydropteroate synthase, and blasticidin deaminase (U.-H. Koop Lab, personal communication).

2.5.4.2 Secondary selection

Herbicide tolerance can be used to accelerate segregation once a sufficient number of transformed plastid genome copies have accumulated (Iamtham and Day, 2000). As an alternative, pigmentation can help detect sectors containing transformed plastome copies and at the same time considerably accelerate segregation toward homoplasmy. Klaus *et al.* (2003) used targeted inactivation of pigmentation-related plastid genes to generate lines with pale to white phenotypes. In a second round of transformation, the inactivated genes were reintroduced. Transformation events re-established the wild-type green phenotype, which made it possible to easily detect such transformed regenerants. Regreening could not be achieved by spontaneous mutations. Thus, the appearance of green regenerants clearly indicated true plastid transformation events. In addition, PCR analysis showed that the first regenerated shoots were already homoplastomic for the second transformation, therefore the usual repetitive cycles of regeneration in the presence of selection was no longer required. Evidently, under the culture conditions chosen, the green tissues had a strong selective advantage over the pigment deficient ones.

2.5.5 Strategies for removal of selectable marker

Public concern, whether scientifically justified or not, requests removal of antibiotic resistance marker genes from transgenic plants intended for human consumption or animal feed. Marker removal approaches are of benefit when the number of available selection markers is low and multiple consecutive transformation steps are required for generating a desired end product. Furthermore, expression of marker genes, not required in established transplastomic lines, constitutes an unnecessary metabolic burden on transplastomic plants. In plastid transformation, four different strategies are available and have been successfully

applied to tobacco: use of direct repeats included in the transformation vector, use of separate transformation vectors harboring selection marker and gene of interest in a co-transformation approach, use of site-specific recombinases with segregation of different plastomes, and use of a transformation vector architecture, which leads to co-integrate formation and subsequently to automatic marker elimination.

2.5.5.1 Direct repeats for marker removal

Higher plant plastids have a highly active recombination system. This needs to be considered, when designing transformation vectors to avoid undesired rearrangement through direct repeat-mediated loop-out recombination of introduced sequences (Maliga *et al.*, 1993; Zou *et al.*, 2003). On the other hand, direct repeat-mediated loop-out recombination can also be used for marker removal (Iamtham and Day, 2000). Since the timing of loop-out recombination cannot be controlled, and since transformants cannot be distinguished from wild-type lines, this system requires a secondary selection system, e.g., herbicide resistance (Iamtham and Day, 2000). Interestingly, the same approach can be applied for targeted gene inactivation in chloroplasts (Kode *et al.*, 2005, 2006).

2.5.5.2 Co-transformation and segregation

Ye *et al.* (2003) used two different vectors and a selection scheme, initially based on spectinomycin as the selective inhibitor and subsequently on herbicide selection. The rationale behind this scheme is that initial selection with herbicides is not possible in plastid transformation. However, after enrichment for transplastomic plastome copies, the level of herbicide tolerance might be sufficient to, in a heteroplastomic situation, allow for segregation of lines, which carry the herbicide but not the antibiotic resistance genes. Indeed, 20% of the recovered lines fulfilled this criterion. As with direct repeat-mediated loop-out recombination, the approach requires a secondary marker, i.e., the resulting lines are not “marker-free”, and the expression of the secondary marker might constitute an unnecessary metabolic burden.

2.5.5.3 Use of site-specific recombinases

Hajdukiewicz *et al.* (2001) and Corneille *et al.* (2001) independently and simultaneously introduced CRE recombinase-mediated marker removal from transplastomic tobacco. The expression of the CRE protein-encoding gene, derived from the P1 bacteriophage, leads to insertion or excision of sequence elements, provided that recognition elements, *loxP* sites, are present on the recombination substrate molecules. Marker gene removal thus requires directly repeated *loxP* elements flanking the marker gene in the plastome. CRE recombinase can be expressed from a nuclear expression cassette, translated in the cytosol and then introduced into the plastid through the organelle's import machinery. Surprisingly, not only the desired excision events are observed, but additional plastome rearrangements that are not necessarily only due to “cryptic” *lox* sites in the plastome (Corneille *et al.*, 2003) but are either based on short direct repeats or on recombination “hot spots”. CRE recombinase seems to generally increase recombination activity in the plastome. It is not understood why the frequency of plastome rearrangements depends on the way, in which the recombinase is introduced into the transplastomic lines. This can either be by *Agrobacterium*-mediated stable or transient (Lutz *et al.*, 2006a, b) nuclear transformation or by crossing a transplastomic line with a suitable nuclear transformant. Marker removal through *Agroinfiltration*-based transient expression (Lutz *et al.*, 2006a, b) is efficient and clearly is preferable since removal of stably integrated expression cassettes from the nuclear genome is not necessary. In addition, plastome rearrangements are not occurring once CRE recombinase is no longer present.

2.5.5.4 Automatic marker removal through co-integrate formation

Integration of foreign DNA into the plastome mediated by two homologous flanking regions requires two events of reciprocal recombination. If these recombination events occur more or less simultaneously, the resulting transformed plastome contains the sequences flanked by the homologous regions. Klaus *et al.* (2004) observed that occurrence of the recombination events at

different time points is the rule rather than the exception, and that both homologous flanks participate in the initial recombination event with the same probability. Recombination via—initially—a single flank only leads to the formation of a co-integrate, i.e., the whole vector plasmid will be integrated and the homologous flanks will be duplicated to generate direct repeats. This is shown for integration via the 5' homologous flank in Figure 3a. Note that the process occurs with the same frequency via the 3' flank. Because of the presence of direct repeats, which present substrates for loop-out recombination, co-integrates are inherently unstable and either reversal to wild-type plastomes (recombination between elements “2” in Figure 3a) or generation of molecules identical

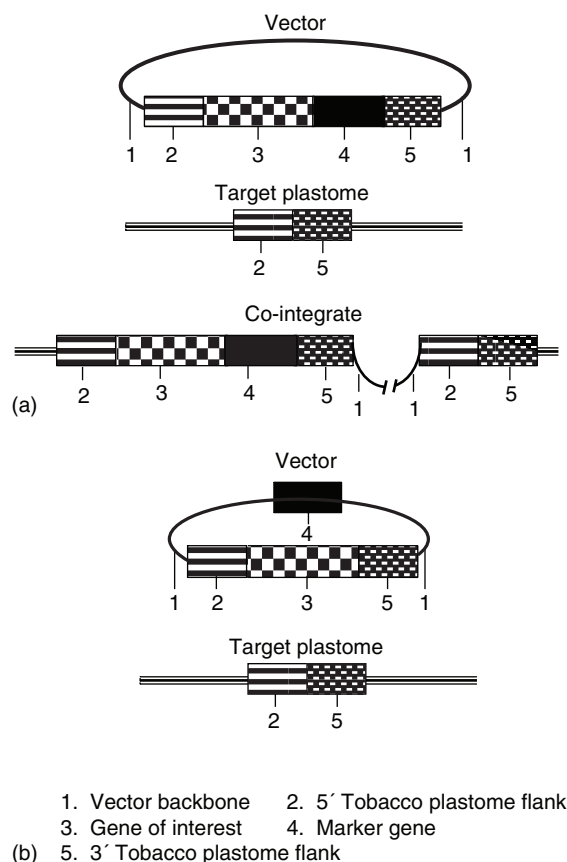


Figure 3 Marker-removal via co-integrate formation. (a) conventional transformation vector and co-integrate resulting from recombination via the 5' flank; (b) novel transformation vector with the selection marker cassette outside the homologous flanks

to those formed through two simultaneous recombination events (recombination between elements “5” in Figure 3a) will occur.

Klaus *et al.* (2004) took advantage of the fact that the vector backbone is initially integrated and is removed by the organelle’s recombination system later by positioning the selection marker expression cassette outside the sequences between the homologous flanks (Figure 3a). As long as the presence of a selection inhibitor is maintained there will be a selection against wild-type plastomes and the number of plastomes containing co-integrates will increase. On removal of the selection pressure co-integrates are resolved resulting in plastomes identical to those of the acceptor lines and marker-free plastomes containing the gene of interest. Phenotypical selection using pigmentation or other visible markers assists in detecting the regenerates containing the gene of interest. However, it has also been shown that PCR screening easily identifies the transformed regenerants. Thus, the approach does not depend on the availability of pigment mutant lines. A simple change in vector design leads to automatic marker removal without the need for any other genes or markers.

2.5.6 Chloroplast biology and engineering of important traits

After the first successful demonstration of stable plastid transformation in tobacco (Svab *et al.*, 1990), the methodology has been utilized for understanding basic chloroplast biology and expressing desirable traits. It is noteworthy to mention that advances in the understanding of chloroplast gene expression using plastid transformation in tobacco have led to more efficient vector designs and thus better expression of introduced genes.

2.5.6.1 Understanding chloroplast biology

Chloroplast gene expression is regulated at transcriptional, translational, and post-translational levels. All these stages of gene expression regulation have been investigated in transplastomic tobacco. Understanding of chloroplast gene function and its relation to chloroplast

biology was achieved via targeted inactivation of individual genes. Availability of the tobacco chloroplast genome sequence was very beneficial in this exercise, as the sequence and identity of individual genes were already known (Shinozaki *et al.*, 1986; Yukawa *et al.*, 2005). Only the crucial plastid transformation experiments that contributed toward advancing our understanding of chloroplast biology are discussed here.

The chloroplast genetic machinery is prokaryotic in nature and very strong biochemical evidence existed for a functional plastid genome-derived RNA polymerase activity (Hu and Bogorad, 1990; Hu *et al.*, 1991). This fact was not only confirmed via plastid transformation but individual function of the RNA polymerase subunit genes was also ascertained using the transplastomic lines where the subunit genes had been knocked out (Allison *et al.*, 1996; Hajdukiewicz *et al.*, 1997; Serino and Maliga, 1998; De Santis-Maciossek *et al.*, 1999; Krause *et al.*, 2000). Further, the basal plastid promoter and its constituents were identified using a series of promoter deletions in transplastomic tobacco (Allison and Maliga, 1995; Shiina *et al.*, 1998).

The chloroplast genome contains light-regulated photosynthesis-related genes and their expression generally responds positively to light and anterograde signals. Specifically, red and blue light are involved in upregulation of gene expression (Sexton *et al.*, 1990; Grover *et al.*, 1999). The blue light-regulated promoter region of the *psbD*-C operon was initially investigated in barley and *in vitro* experiments clearly defined the elements responsible for blue light perception (Christopher *et al.*, 1992). Function of the blue light-regulated *psbD*-C promoter was confirmed and specific elements characterized using plastid transformation (Allison and Maliga, 1995). It has recently been reported that green light, thought to be a benign part of the light spectrum, negatively regulates plastid gene expression (Dhingra *et al.*, 2006). It would be interesting to identify DNA elements that participate in this unique phenomenon using plastid transformation.

Perhaps one of the most important discoveries that resulted from plastid transformation experiments in tobacco was the identification of second transcription machinery in the plastids (Allison *et al.*, 1996; Hajdukiewicz *et al.*, 1997; Kapoor *et al.*, 1997). The beta subunit of plastid-encoded

RNA polymerase was disrupted and that resulted in the detection of the additional polymerase activity (Allison *et al.*, 1996). The information regarding two polymerase plastid transcription machineries is now being incorporated into plastid transformation vector designs.

The chloroplast genome sequence of tobacco revealed the presence of *ndh* genes based on homology with mitochondrial respiratory chain complex I subunit genes coding for proton pumping NADH:ubiquinone oxidoreductase (Shinozaki *et al.*, 1986; Fearnley and Walker, 1992), but the existence of a functional respiratory chain complex I in chloroplasts was a matter of debate. Individual *ndh* subunits were disrupted in tobacco and existence of a functional *Ndh* complex in mature chloroplasts was still demonstrated. It was also shown that the complex is dispensable under normal growth conditions but may be essential under stress (Burrows *et al.*, 1998; Kofer *et al.*, 1998b).

Other targeted inactivation studies include *rbcL* knockout to facilitate Rubisco engineering (see Section 2.6; Kanevski and Maliga, 1994). The *sprA* gene was proposed to participate in maturation of 16S rRNA but transplastomic plants with an inactivated *sprA* were normal (Sugita *et al.*, 1997). In another study attempts were made to disrupt the *ycf9* gene but were unsuccessful. Although actual function of *ycf9* was not ascertained, it was clear that it had a vital role in plant survival (Maenpaa *et al.*, 2000). Another open reading frame coded by *ycf5* was targeted for deletion to study its function. Null mutants of *ycf5* were pale green and electron flow around photosystem II (PS II) was found to be affected indicating a role of this gene product in the generation of functional PS II units (Tsuruya *et al.*, 2006). It was revealed that the *rps18* gene is indispensable in higher plants, whereas this gene is absent in plastids of nongreen unicellular organisms (Rogalski *et al.*, 2006). Targeted inactivation of *psaJ* gene had no apparent effect on plant growth but under limiting light conditions it is essential for efficient photosystem I excitation (Schottler *et al.*, 2007). There are other open reading frames (ORFs) in the chloroplast genome of higher plants whose function remains to be understood. Chloroplast transformation approach will continue to expand our understanding of chloroplast biology.

2.5.6.2 Recombinant proteins

Several recombinant proteins have been expressed via plastid transformation in tobacco. Some of the recent reviews provide a list of these proteins with other pertinent details (Daniell *et al.*, 2005c; Grevich and Daniell, 2005). In order to avoid redundancy only recent reports are mentioned here. Four new reports add to the expanding list of biopharmaceutical proteins being expressed via tobacco plastid transformation. Foot-and-mouth disease virus (FMDV) *VP1* gene was expressed at 2–3% of total soluble protein in tobacco chloroplasts (Li *et al.*, 2006a). Li *et al.* (2006b) also expressed a partial spike (S) protein of SARS-CoV to generate a vaccine against the severe acute respiratory syndrome (SARS) corona virus in tobacco. Authors indicate this as a first step toward creating an oral vaccine against the SARS virus. Another report details successful expression of anti-Epstein-Barr Virus viral capsid antigen (VCA) using tobacco plastid transformation at 0.04% tsp (Lee *et al.*, 2006). For all these reports immunogenicity remains to be tested. More recently vaccine against amoebiasis has been generated in transplastomic tobacco. The *LecA* gene used for generating the antigen is expressed at 6.3% tsp and is able to elicit an immunogenic response in mice (Chebolu and Daniell, 2007). These reports present exciting evidence that transplastomic engineering may produce important products for human benefit. However, successful implementation of such technologies will depend on plant cultivation at an agricultural scale. Another recurring theme is the mention of “oral vaccines”, yet these proteins are being expressed in a nonfood and nonfeed crop, tobacco. If oral vaccines are to become a reality, the protein will need to be extracted from tobacco and then administered orally or expressed in other edible crops like tomato.

Other important recombinant proteins can be grouped under the biomaterials, enzymes, and amino acids categories. Two biopolymers have been successfully expressed in transplastomic tobacco. These include p-hydroxybenzoic acid and polyhydroxybutyrate (Lossel *et al.*, 2003; Viitanen *et al.*, 2004). If the production can be increased to industrial levels, plant-based generation of biopolymers will be extremely beneficial in the health care sector. Xylanase (an enzyme) and

tryptophan (an amino acid) have also been reported to be well expressed when biosynthesis genes were integrated into the tobacco plastid genome (Zhang *et al.*, 2001; Leelavathi *et al.*, 2003).

2.5.6.3 Agronomic traits

The tobacco plastid genome has been engineered for conferring biotic and abiotic stress tolerance. Disease and insect resistance have been engineered by expressing antimicrobial peptide and the Cry family of genes (see De Cosa *et al.*, 2001; Daniell *et al.*, 2005c; Grevich and Daniell, 2005; Chakrabarti *et al.*, 2006). Drought tolerance was conferred in tobacco by the expression of trehalose phosphate synthase gene (Lee *et al.*, 2003). Effective phytoremediation of mercury was also reported in transplastomic tobacco expressing the bacterial *merA* and *merB* genes (Ruiz *et al.*, 2003).

2.5.6.4 Inducible expression

There are several reasons, why inducible expression in plastids is desirable. Metabolic drain during growth and development could be avoided, if an economically feasible preharvest induction was available. Likewise, negative effects of gene product(s) or metabolic changes caused by the function of introduced genes might be a problem, if expression was constitutive. Furthermore, presence of pharmaceutical gene products or metabolites throughout the whole growth phase of a plant is more demanding in terms of biosafety and regulatory control than precisely controlled short-term presence. Finally, for basic research it would be valuable if plastid gene expression could be switched on and off at desired time points.

Expression of plastid genes is mediated by regulated promoters, which supply transcriptional control depending on physiological, developmental, or tissue specificity parameters. Therefore, inducible expression in plastids can only be achieved using heterologous control elements.

The first system that was described used a plastid transgene under the control of the phage T7 promoter in combination with import of the T7 polymerase encoded by a nuclear transgene (McBride *et al.*, 1995). Although using this system-

controlled expression is achieved to a certain extent (Magee *et al.*, 2004) and negative effects on fertility observed during constitutive expression of PHB genes were abolished, when these genes were transcribed by an ethanol induced T7 polymerase (Lossel *et al.*, 2005), the system is not optimal. Plastid gene expression is altered in the presence of T7 polymerase even if they do not contain a T7 promoter (Magee and Kavanagh, 2002), and the low level of expression typical for most nuclear inducible systems even in the noninduced state may be sufficient to cause an undesirable phenotype (Magee *et al.*, 2004).

An alternative approach uses constitutive repression of a transgene by the lac repressor and induction by application of isopropyl- β -D-galactopyranoside (IPTG), which leads to an increase of the protein in question by about 20-fold (Mühlbauer and Koop, 2005). While this system is attractive since it uses regulatory elements located in the plastid only and does not require additional nuclear transgenes, it is also not optimal, since, probably due to read-through transcription from a different promoter, there is low level expression in the noninduced state and spraying IPTG in greenhouses or even on open fields does not seem economically feasible. This problem might be at least partially overcome by performing postharvest induction (Mühlbauer and Koop, 2005).

Buhot *et al.* (2006) reported using the *E. coli* *groE* heat shock promoter, a eubacterial promoter, which is not recognized by the nuclear (NEP)- and plastid (PEP)-encoded transcription systems present in plastids. Controlled expression is achieved through a chimeric transcription factor that mediates interaction of NEP and the eubacterial promoter. The system was tested and shown to function via transient expression of the transcription factor, and the performance in stably transformed plants remains to be seen. Like in the case of T7 polymerase/T7 promoter, induction is indirect, i.e., via a protein that is encoded by a nuclear transgene.

Recently, a further approach was described by Tungsuchat *et al.* (2006). It is based on CRE recombinase-mediated rearrangement of a transplastome. In this case, a gene of interest lacking an AUG translation start codon is linked to the nonexcised start codon of the marker gene through excision of the selection marker gene. The

advantage of the system lies in the fact that it is not sensitive to read-through transcription. Control is by generating a translatable open reading frame. Prior to excision there is no detectable gene product (GFP) of the gene of interest, while accumulation of GFP is found up to be 0.3% of the total cellular protein after excision. Again, a transgene expressed from the nucleus is required to trigger plastid expression: primary transplastomic lines harboring an inactive gene of interest were transformed in their nuclear genome using *Agrobacterium*-mediated gene transfer. It remains to be seen, how the approach can be adapted for practical purposes.

While all the approaches toward inducible plastid gene expression may be valuable for basic research and for lab-scale expression studies, none of these systems is suitable for production-scale or field applications. Further improvements of the described systems and/or development of alternatives are required. Thus, inducible expression remains being a prominent challenge in plastid transformation technology.

2.6 Understanding Rubisco Function in Higher Plants for Improving Photosynthesis

Plants are remarkable factories: efficient green assembly lines that convert inert atmospheric gas and water into complex chemical compounds using solar energy. The foundation of this feat is the capture of atmospheric carbon dioxide through specialized structures and then its integration into higher-order structures starting with Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco is a unique chloroplast stromal enzyme that in higher plants is composed of eight plastid genome-derived large subunits and eight small subunits encoded in the nucleus and imported from the cytosol (Houtz and Portis, 2003). The efficiency of carbon dioxide fixation by Rubisco is negatively impacted by its oxygenase activity (Ogren, 2003). Although an efficient system, Rubisco is an attractive target for engineering added capacity to fix atmospheric carbon. At a time where man seeks to exploit the energy locked in organic bonds present in recent tissues rather than fossil fuels, and when alleged greenhouse gases may negatively shape climate change, research into

integration of more atmospheric carbon into usable forms is timely and important. Research endeavors to understand Rubisco action with an aim of improving it has been carried out mainly in tobacco. Most have involved suppression/deletion of individual subunits or expression of both native and heterologous subunits in the nuclear or plastid compartment.

In 1988, the first transgenic Rubisco mutant was reported (Rodermeil *et al.*, 1988). This transgenic tobacco mutant was generated to understand coordinated expression of nuclear and organellar genes in the biosynthesis of Rubisco. The mutant is severely depleted in photosynthesis and Rubisco enzyme has been extensively studied to understand the role of the small subunit and the impact of Rubisco depletion on photosynthesis and carbon–nitrogen flux, as well as plant growth and development (Hudson *et al.*, 1992; Jiang and Rodermeil, 1995; Fritz *et al.*, 2006).

Most experiments pertaining to Rubisco engineering described so far have attempted to establish an experimental system to assemble a chimeric Rubisco. One of the strategies employed for engineering of Rubisco was to relocate the large subunit to the nuclear genome (Kanevski *et al.*, 1999). Transgenic tobacco plants were generated where the plastid resident large subunit was deleted using plastid transformation. This transgenic mutant plant was used as a host to engineer the large subunit in the nuclear genome. Although expression of the large subunit in the nucleus and its subsequent import into the chloroplast was normal, Rubisco enzyme content and activity were approximately 10% and 3% of the wild-type levels. Thus, the transgenic plants were severely depleted in Rubisco enzyme and its activity (Kanevski *et al.*, 1999). Experimental simulations predict that Rubisco derived from the photosynthetic bacteria *Chromatium vinosum* should exhibit superior performance compared to higher plant Rubisco (Bainbridge *et al.*, 1995). With this premise, the large subunit gene derived from *C. vinosum* was engineered in the nuclear genome of Rubisco deficient tobacco mutant SP25. The bacterial large subunit gene was transcribed in the cytosol but no large subunits or Rubisco activity was detectable (Madgwick *et al.*, 2002). From the preceding reports one can discern that integration and allotopic expression of the large subunit of Rubisco is possible but functionality

remains a problem. One can only speculate on the reasons for this failure. Post-translational modifications, import or different codon bias of the introduced genes may be responsible for lack of functionality.

Plastid transformation is routinely performed in tobacco (Dhingra and Daniell, 2006). As an alternate approach, plastid transformation was employed to express heterologous or native subunits of Rubisco in the plastid genome of tobacco. The native *rbcL* gene in tobacco was replaced via homologous recombination with the *rbcL* gene derived from *Synechococcus* or *Helianthus annuus*. Chloroplast transgenic tobacco plants expressing the algal *rbcL* gene lacked photoautotrophic growth and the large subunit protein was completely absent. Plants expressing the *Helianthus rbcL* gene were compromised in growth, as the carboxylase activity of the hybrid Rubisco was 20% of the wild-type levels (Kanevski *et al.*, 1999). Other reports have attempted to engineer Rubisco genes from algae that exhibit superior enzymatic properties relative to the higher plant enzyme. In these instances, Rubisco is produced from a dicistronic unit comprised of the large and small subunit gene. Expression of *rbcLS* operons from the rhodophyte *Galdieria sulphuraria* and the diatom *Phaeodactylum tri-cornutum* resulted in abundant foreign Rubisco expression but enzyme assembly remained an issue (Whitney *et al.*, 2001). Perhaps the assembly machinery is specific to the native subunits; thus foreign subunits remain unassembled or incorrectly assembled. A homodimeric form of the Rubisco encoding gene *RbcM* derived from proteobacterium, *Rhodospirillum rubrum* was integrated into the chloroplast genome of tobacco replacing the native *rbcL* gene. This enzyme is unique as it has no small subunit and thus has no assembly issues for activity. Growth of transgenic tobacco plants was supported in carbon dioxide enriched atmosphere and the catalytic properties were similar to the algal enzyme (Whitney and Andrews, 2001b, 2003).

The native tobacco small subunit gene has also been integrated into its own plastid genome. A His-tagged version of the *RbcS* gene was introduced into the wild-type background. A very low level of plastid-derived small subunit protein was detected (~1% of total small subunit protein) in the chloroplasts of transgenic plants (Whitney

and Andrews, 2001a). This continuum of reports reveals the lack of a system to appropriately express and assemble chimeric Rubisco proteins. One recent report used a creative approach of using the antisense *RbcS* tobacco plant to engineer the small subunit gene in the plastid genome. Although the introduced *RbcS* gene was well expressed it was not able to complement the deficiencies generated due to silencing of the small subunit (Zhang *et al.*, 2002). Importantly, this report became a prelude to the successful demonstration of a functional chloroplast-derived Rubisco in a following report. The plastid genome of the *RbcS* antisense mutant was used as a host to express tobacco *RbcS* gene with enhanced translation (Dhingra *et al.*, 2004). From this report it seemed that translation was a limiting factor that was overcome by the use of specific 5' and 3' untranslated regions that have been previously shown to result in improved expression of chloroplast expressed genes (Eibl *et al.*, 1999). The report by Dhingra *et al.* (2004) demonstrated that it was feasible to assemble Rubisco entirely in the chloroplast but it remains to be seen if heterologous subunits will be efficiently assembled using the expression enhancements that were described.

3. CONCLUSIONS

Twenty-five years ago tobacco was a rapidly regenerating system stemming from a crop with economic impact that is closely related to other high value crops. For these reasons it was held up as the system of choice for pilot transformation studies of both the nucleus and plastid. Upon review of the literature it is clear that these original studies set a stage for the flurry of work soon to follow in *Arabidopsis*—a laboratory-scale system with easier transformation, a smaller genome, and much faster life cycle. Recent studies in *N. benthamiana* have brought the genus back to the limelight as an outstanding system for transient protein expression. Overall, it is always important to understand the utility of this system, its advantages and limitations, as it still may be relevant to facets of discovery in plant biology. As a nonfood and nonfeed crop tobacco retains a remarkable potential for use as a biofactory. Ironically, this genus with a notorious health

reputation may prove to be indispensable for the production of medically relevant compounds.

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Coffee

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1. INTRODUCTION

1.1 History, Origin, and Economic Importance

Coffee is one of the world's most valuable export commodities, ranking second on the world market after petroleum products. The total retail sales value exceeded US \$70 billion in 2003 and about 125 million people depend on coffee for their livelihood in Latin America, Africa, and Asia (Osorio, 2002). Commercial production relies on two species, *Coffea arabica* L. and *Coffea canephora* Pierre. The cup quality (low caffeine content and fine aroma) of *C. arabica* makes it by far the most important species, representing 70% of the world production.

C. arabica has its primary center of genetic diversity in the highlands of southwest Ethiopia and the Boma Plateau of Sudan. Wild populations of *C. arabica* have also been reported in Mount Imatong (Sudan) and Mount Marsabit (Kenya) (Thomas, 1942; Anthony *et al.*, 1987). Cultivation of *C. arabica* started in southwestern Ethiopia about 1500 years ago (Wellman, 1961). Modern coffee cultivars are derived from two base populations, known as Typica and Bourbon, which were spread worldwide in the 18th century.

Historical data indicate that these populations were composed of progenies of very few plants, i.e., only one for the Typica population (Chevalier and Dagron, 1928) and the few plants that were introduced to the Bourbon Island (now Reunion) in 1715 and 1718 for the Bourbon population (Haarer, 1956).

1.2 Botanical Description

Coffee species belong to the Rubiaceae family, one of the largest tropical angiosperm families. Variations in cpDNA classified the Coffeae tribe into the Ixoroideae monophyletic subfamily, close to Gardenieae, Pavetteae, and Vanguerieae (Bremer and Jansen, 1991). Two genera, *Coffea* L. and *Psilanthus* Hook. f., were distinguished on the basis of flowering and flower criteria (Bridson, 1982). Each genus was divided into two subgenera based on growth habit (monopodial vs. sympodial development) and type of inflorescence (axillary vs. terminal flowers). All species are perennial woody bushes or trees in intertropical forests of Africa and Madagascar for the *Coffea* genus, and Africa, Southeast Asia, and Oceania for the *Psilanthus* genus. More than 103 *Coffea* species have been described so far and about 70%

of those species are threatened with extinction as a result of a combination of decline in quantity and quality of habitat (Davis *et al.*, 2006). They differ greatly in morphology, size, and ecological adaptations. Some species like *C. canephora* and *Coffea liberica* Hiern are widely distributed from Guinea to Uganda. Other species display specific adaptations, e.g., *Coffea congensis* Froehner to seasonally flooded areas in the Zaire basin and *Coffea racemosa* Lour. to very dry areas in the coastal region of Mozambique. All species are diploid ($2n=2x=22$) and generally self-incompatible, except for *C. arabica*, which is tetraploid ($2n=4x=44$) and self-fertile (Charrier and Berthaud, 1985).

Molecular phylogeny of *Coffea* species has been established based on DNA sequence data. The internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA (Lashermes *et al.*, 1997) as well as the chloroplast-DNA variation (Lashermes *et al.*, 1996; Cros *et al.*, 1998) were successfully used to infer phylogenetic relationships of *Coffea* species. No major difference between coffee and other plants was observed in the arrangement of the chloroplast genome and in the structure of the ITS2 region in nuclear rDNA. Furthermore, the results suggest a radial mode of speciation and a recent origin in Africa for the genus *Coffea*. Several major clades were identified, which present a strong geographical correspondence (i.e., Madagascar, East Africa, Central Africa, Central and West Africa). In addition, *Psilanthus* species did not differ from *Coffea* species, suggesting that the present division into two genera should be revised.

1.3 Speciation and Genome Features of *C. arabica*

The genome constitution and mode of speciation of *C. arabica* have been subjected to several investigations. Based on cytological observations and fertility of interspecific hybrids, *C. canephora* and *C. congensis* were suggested to have a common ancestor with *C. arabica* (Carvalho, 1952; Cramer, 1957). Furthermore, *C. congensis* can be considered as an ecotype of *C. canephora* in the light of the fertility of interspecific hybrids (Louarn, 1993) and the genetic diversity detected by molecular markers (Prakash *et al.*, 2005). These

phylogenetic assumptions were consolidated by a high homology found in the ITS2 region sequences of *C. arabica*, *C. canephora*, and *C. congensis* (Lashermes *et al.*, 1997). Regarding cpDNA that exhibited a maternal inheritance in coffee (Lashermes *et al.*, 1996), *C. arabica* appeared to be similar to two species, *Coffea eugenioides* Moore and *Coffea* sp. "Moloundou" (Cros *et al.*, 1998).

Restriction fragment length polymorphism (RFLP) markers in combination with genomic *in situ* hybridization (GISH) were used to investigate the origin of *C. arabica*. By comparing the RFLP patterns of potential diploid progenitor species with those of *C. arabica*, the source of the two sets of chromosomes, or genomes, combined in *C. arabica* was specified. The genome organization of *C. arabica* was confirmed by GISH using simultaneously labeled total genomic DNA from the two putative genome donor species as probes (Lashermes *et al.*, 1999). These results clearly suggested that *C. arabica* is an amphidiploid (i.e., C^aE^a genomes) resulting from the hybridization between *C. eugenioides* (E genome) and *C. canephora* (C genome) or ecotypes related to those diploid species (Figure 1). Results also indicated low divergence between the two constitutive genomes of *C. arabica* and those of its progenitor species, suggesting that the speciation of *C. arabica* took place very recently. Precise

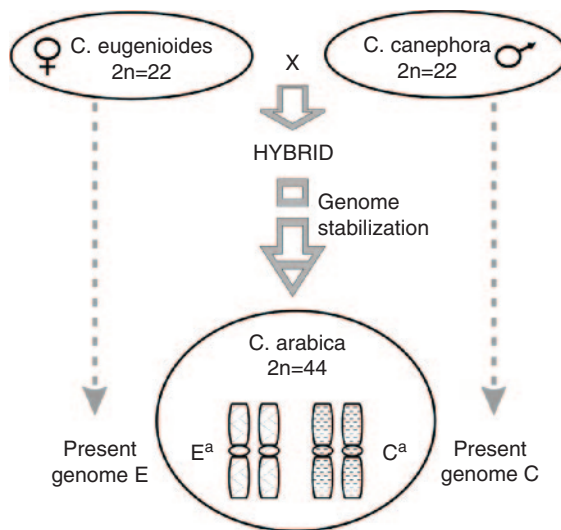


Figure 1 Origin of the allotetraploid species *Coffea arabica* (C^aE^a genomes)

localization in Central Africa of the speciation process of *C. arabica* based on the present distribution of the coffee species appeared difficult since the constitution and extent of tropical forest varied considerably during the late quaternary period.

Furthermore, in spite of the close relationship among the two constitutive genomes, bivalents are observed in meiosis and *C. arabica* is considered to display a diploidlike meiotic behavior (Krug and Mendes, 1940). Investigations suggest that homologous chromosomes do not pair in *C. arabica*, not as a consequence of structural differentiation, but because of the functioning of pairing regulating factors (Lashermes *et al.*, 2000a).

The nuclear DNA content of several coffee species has been estimated by flow cytometry (Cros *et al.*, 1995). The DNA amount (2C values) varies between diploid coffee species from 0.95 to 1.8 pg (picogram). In comparison to other angiosperms (Bennett and Leitch, 1995), the genomes of coffee species appear to be of rather low size (i.e., 800 and 1300 Mb for *C. canephora* and *C. arabica*, respectively). These variations in DNA amount, other than variation due to ploidy level (e.g., *C. arabica*), are probably due almost entirely to variation in the copy number of repeated DNA sequences. Differences may correspond to genomic evolution correlated with an ecological adaptation process. Furthermore, reduced fertility of certain interspecific F₁ hybrids appears to be associated with significant differences in nuclear content of parental species (Barre *et al.*, 1998).

1.4 Traditional Breeding: Breeding Objectives, Tools, and Strategies

C. arabica is characterized by very low genetic diversity, which is attributable to its allotetraploid origin and evolution. It is believed that the agromorphological variation observed, which gave rise to so many named varieties during the 20th century, results from spontaneous mutations in a few major genes conditioning plant, fruit, and seed characters (Carvalho, 1988). The cultivars present, therefore, a homogeneous agronomic behavior characterized by a high susceptibility to many pests and diseases, and very low adaptability (Bertrand *et al.*, 1999). Spontaneous accessions collected in

the primary center of diversity as well as wild relative *Coffea* species constitute a valuable gene reservoir for breeding purposes.

Transfer of desirable genes in particular for disease resistance from diploid species like *C. canephora* and *C. liberica* into tetraploid Arabica cultivars without affecting quality traits has been the main objective of Arabica breeding (Carvalho, 1988; Van der Vossen, 2001). To date, *C. canephora* provides the main source of disease and pest resistance traits not found in *C. arabica*, including coffee leaf rust (*Hemileia vastatrix*), coffee berry disease (*Colletotrichum kahawae*) and root-knot nematode (*Meloidogyne* spp.). Likewise, other diploid species present considerable interests in this respect. For instance, *C. liberica* has been used as source of resistance to leaf rust (Srinivasan and Narasimhaswamy, 1975) while *C. racemosa* constitutes a promising source of resistance to the coffee leaf miner (CLM) (Guerreiro Filho *et al.*, 1999). Exploitation of such genetic resources has so far relied on conventional procedures in which a hybrid is produced between an outstanding variety and a donor genotype carrying the trait of interest, and the progeny is backcrossed to the recurrent parent. Undesirable genes from the donor parent are gradually eliminated by selection. In so doing, conventional coffee breeding methodology faces considerable difficulties. In particular, strong limitations are due to the long generation time of coffee tree (5 years), the high cost of field trial, and the lack of accuracy of current strategy. A minimum of 25 years after hybridization is required to restore the genetic background of the recipient cultivar and thereby ensure good quality of the improved variety.

Occurrence of spontaneous hybrids between *C. arabica* and diploid relative species such as *C. canephora* and *C. liberica* is reported from time to time, especially when these species grow in direct proximity (Mahé *et al.*, 2007). Those exceptional natural hybrids have been intensively used in coffee breeding programs as the main source of resistance to pests and diseases. In particular, the Timor Hybrid, a natural hybrid originating from an interspecific cross between *C. arabica* and *C. canephora* (Bettencourt, 1973; Lashermes *et al.*, 2000b) is being extensively used as the main source of resistance to pests and diseases, including coffee leaf rust (*H. vastatrix*), coffee berry disease (CBD) caused by *C. kahawae*, and

resistance to root-knot nematode (*Meloidogyne exigua*). Alternatively, genes from diploid coffee could be transferred into the *C. arabica* genome through the production of triploid ($2n=33$) or tetraploid ($2n=44$) interspecific hybrids (Herrera *et al.*, 2002a, b, 2004). Triploid hybrids are derived from a direct cross between the diploid progenitor and *C. arabica* while tetraploid hybrids are obtained by crossing both the species after chromosome duplication of the diploid coffee. The use of triploid hybrids is more difficult than tetraploid hybrids because of low fertility. Nevertheless, selection of high producing lines in early generations derived from triploid hybrids (i.e., first backcross to *C. arabica* or second generation by selfing) has been reported (Orozco Castillo, 1989).

The use of molecular techniques has opened up new possibilities for genetic analysis and provides new tools for efficient conservation and use of coffee genetic resources. For instance, a whole range of different DNA molecular marker techniques has been used to study the genetic diversity in *C. arabica*. The base populations of cultivated coffee appeared to derive from wild coffee collected in southwestern Ethiopia. In addition, spontaneous accessions collected in the primary center of diversity seemed to constitute a valuable gene reservoir. These results should increase interest in wild coffee for the purpose of broadening the genetic base of the cultivars. Moreover, heterosis has been reported in F_1 hybrids resulting from crosses between subspontaneous Ethiopian accessions and improved cultivars (Bertrand *et al.*, 2005). Furthermore, the recent development of molecular markers closely linked to resistance genes against *M. exigua* (Noir *et al.*, 2003) and leaf rust (Prakash *et al.*, 2004) opened up the possibility of molecular marker-assisted selection.

1.5 Toward the Identification and Characterization of Coffee Genes

The recent development of large coffee expressed sequence tag (EST) sequencing projects (Lin *et al.*, 2005; Viera *et al.*, 2006) as well as the development of coffee bacterial artificial chromosome (BAC) genomic libraries (Noir *et al.*, 2004; Leroy *et al.*, 2005) is leading to an enormous production

of information in the area of coffee genomics. To take advantage of these resources, a key issue is the development and use of methods to define gene functions at the molecular and organism level. The identification of specific genes involved in significant coffee biological processes is a prerequisite for interpretation of molecular information on the structure and organization of the coffee genome. Tools are, therefore, required that allow both forward and reverse genetics in coffee as well as the elucidation of gene function, regulation and interaction of genes.

The overall benefit of the application of genomics to coffee will be to increase the precision of genetic improvement and coffee growing practices by establishing refined correlation between molecular characters (i.e., genes, proteins, and metabolites) and important agricultural traits. With the advent of such knowledge, the development of improved cultivars in terms of quality and reduced economic and environmental costs can be achieved with an increased probability of success.

2. DEVELOPMENT OF TRANSGENIC COFFEE

Coffee genetic engineering emerged during the last decade as a potential tool with two objectives: (i) to elucidate the function, regulation, and interaction of agronomically interesting genes through functional genomics approach, and (ii) to serve as a tool to introduce desirable traits into commercial genotypes.

However, these objectives were available only after the establishment of protocols for *in vitro* regeneration through somatic embryogenesis for both the principal commercial species. Two types of somatic embryogenesis processes have been described using leaf sections as explants that enable efficient regeneration of plantlets from different tissues: (i) *direct somatic embryogenesis*: somatic embryos are obtained quickly (approximately 70 days) on only one medium with the production of limited callusing. This procedure is particularly suited to *C. canephora* and (ii) *indirect somatic embryogenesis* based on the use of two media: an induction medium for primary callogenesis, and a secondary regeneration medium to produce friable embryogenic callus regenerating several hundred

thousand somatic embryos per gram of callus on both *Coffea* sp. (Etienne, 2005).

2.1 Donor Gene

2.1.1 Gene source

The recent development of high throughput methods for analyzing the structure and function of genes represents a new paradigm with broad implications for agricultural production. With the complete sequencing of the first model organisms and the development of centers of excellence in genomics, the knowledge of genomes and their expression increases exponentially, inclusive for woody plants like coffee. During the last decade, important efforts were made in coffee to develop EST databases. The Brazilian government funded an ambitious Coffee Genome Program with the objective to establish a data bank of more than 200 000 ESTs, which could lead to the identification of more than 30 000 genes. Recently, a collaborative Nestlé-Cornell University team published an EST database of around 47 000 cDNA clones, corresponding to 13 175 unigenes (Lin *et al.*, 2005). In parallel, BAC libraries of both coffee species, *C. arabica* and *C. canephora*, were established (Noir *et al.*, 2004; Leroy *et al.*, 2005). Such maps are of central strategic importance for marker-assisted breeding, for straightforward positional cloning of agronomical important genes, and analysis of gene structure and function.

As a consequence of the recent efforts on coffee genomics, a lot of coffee gene candidates have been identified and some of them are currently under cloning. Among them, are pathogen resistance genes including *Mex-1* gene to *M. exigua* root-knot nematode (Noir *et al.*, 2003), *Sh-3* gene to race 3 of coffee leaf rust (*H. vastatrix*) (Prakash *et al.*, 2004), and *Ck-1* gene to CBD (*C. kahawae*) (Gichuru *et al.*, 2006) and others involved in physiological functions such as sucrose synthase (Leroy *et al.*, 2005), caffeine synthase (Ogita *et al.*, 2004; Satyanarayana *et al.*, 2005), seed's oil content (Simkin *et al.*, 2006), and osmotic stress response (Hinniger *et al.*, 2006). For the mentioned intrinsic genes and for the ones to be discovered, the availability of efficient genetic transformation procedures becomes necessary in order to validate

their function and to analyze their structure. On attendance, the majority of transformation events done until now in coffee correspond principally to *uidA* and/or *gfp* (green fluorescent protein) reporter genes inserted between T-DNA borders in pBIN or pCambia type binary backbone (see Table 1). Exceptions to the previous was done by Leroy *et al.* (2000), who achieved stable transfer of *cry1Ac* gene (from *Bacillus thuringiensis* (Bt) and encodes an endotoxin active against CLM *Perileuoptera coffeela*) and of *csr1-1* gene (conferring resistance to chlorsulfuron herbicide).

In addition, Ogita *et al.* (2003) and Kumar *et al.* (2004) reported the production of transformed plants with suppression of the expression of the genes that encode the enzymes theobromine synthase (*CaMXMT1*) and N-methyl transferase (*NMT*) respectively, both involved in caffeine biosynthesis. In particular, Ogita *et al.* (2003) achieved production of doubled stranded RNA interference (RNAi) method. RNAi sequences homologous to the *CaMXMT1* mRNA have given as a result that leaves of one-year-old transformed trees exhibited reduced theobromine and caffeine content (30–50% compared with the control).

2.1.2 Promoters

With few exceptions, the 35S promoter, derived from the cauliflower mosaic virus (CaMV), has been the most common component of transgenic constructs used with either direct or indirect *Agrobacterium*-mediated coffee transformation (see Table 1). Surprisingly, apart of van Bostel *et al.* (1995) and Rosillo *et al.* (2003) who tested different promoters controlling *uidA* gene and compared its expression in endosperm, somatic embryos, and leaf explants of *C. arabica*, none other research group has done this type of research in coffee. van Bostel *et al.* (1995) found that EF-1 α promoter (from *Arabidopsis thaliana* EF-1 α translation elongation factor p35S driven) revealed the most efficient *GUS* gene transient expression; however, the use of this promoter has been further restricted to this group (Leroy *et al.*, 2000; Perthuis *et al.*, 2005). Rosillo *et al.* (2003) compared the efficacy of transfer and expression of *GUS* gene driven by 35S with respect to two coffee promoters

Table 1 Summary of transformation studies in *Coffea* sp.^(a)

Gene transfer method	<i>Coffea</i> species	Explant used	Strain	Binary vector	Promoter	Selection marker	Donor gene	Main results	Reference	Country
Direct transfer	<i>C. arabica</i>	Protoplast	—	ND	pGA472	<i>nptII</i>	<i>uidA</i>	TE	Barton <i>et al.</i> , 1991	USA
	<i>C. arabica</i>	SE	—	pCambia 3201	CaMV35S	<i>bar</i>	<i>uidA</i>	GI	Fernandez-Da Silva and Menéndez-Yuffia, 2003 ^(b)	Venezuela
Biolicistic delivery	<i>C. arabica</i>	L	—	pPIGK	EF-1a	<i>bar</i>	<i>uidA</i>	TE	van Bortel <i>et al.</i> , 1995 ^(b)	France
	<i>C. arabica</i>	ET	—	pCambia 2301	CaMV35S	<i>nptII</i>	<i>uidA</i>	GI	Rosillo <i>et al.</i> , 2003	Colombia
	<i>C. arabica</i>	ET	—	pBI-426	CaMV35S	<i>nptII</i>	<i>uidA</i>	GI, PR	Cunha <i>et al.</i> , 2004	Brazil
	<i>C. canephora</i>	ET	—	pCambia 3301	CaMV35S	<i>bar</i>	<i>uidA</i>	GI, PR	Ribas <i>et al.</i> , 2005a	Brazil
	<i>C. arabica</i>	SE	—	pUBC	Ubiquitin	none	<i>CryIac</i>	GI	De Guglielmo and Menéndez ^(c)	Venezuela
Indirect transfer	<i>A. tumefaciens</i>	Protoplast	ND	pGV 2260	CaMV35S	<i>hpt</i>	<i>uidA</i>	TE	Spiral and Pétiard, 1991	France
	<i>C. canephora</i>	ET	EHA 101	pIG121-Hm	ND	<i>hpt</i>	<i>uidA</i>	GI, PR	Hatanaka <i>et al.</i> , 1999	Japan
	<i>C. arabica</i>	SE	LBA 4404	pBIN19	EF-1	<i>csr1-1</i>	<i>cryIac</i>	GI, PR	Leroy <i>et al.</i> , 2000	France
	<i>C. canephora</i>	HE	LBA 4404	pSB1	pGSD2	<i>hpt</i>	<i>uidA</i>	GI, PR	Naveen <i>et al.</i> , 2002	India
	<i>C. arabica</i>	ET, SE	EHA 101	pHIB1-IG	CamV35S	<i>hpt</i>	<i>GFP</i> , <i>CaMXMT1</i>	RNAi, PR	Ogita <i>et al.</i> , 2004	Japan
	<i>C. canephora</i>	ET, L	C58	pER10W-35SRed	CaMV35S	<i>nptII</i>	<i>DsRFP</i>	GI, PR	Canche-Moo <i>et al.</i> , 2006	Mexico
	<i>C. canephora</i>	ET	EHA 105	pCambia 3301	CaMV35S	<i>ppt</i>	<i>uidA</i>	GI	Cruz <i>et al.</i> , 2004	Brazil
	<i>C. canephora</i>	H	EHA 101	pBECKS	CaMV35S	<i>hpt</i>	<i>uidA</i>	GI, PR	Mishra and Sreenath, 2004	India
	<i>C. canephora</i>	SE	LBA 4404	pBIN19	EF-1	<i>csr1-1</i>	<i>cryIac</i>	Field test	Perthuis <i>et al.</i> , 2005	France
	<i>C. canephora</i>	SE	A4 & EHA 101	pCambia 1381	CaMV35S	<i>hpt</i>	<i>NMT</i>	RNAi	Kumar <i>et al.</i> , 2004	India
<i>A. rhizophora</i>	<i>C. arabica</i>	ET	EHA 105	pCambia 3300	CaMV35S	<i>bar</i>	<i>ACC-oxidase</i>	antisense expression	Ribas <i>et al.</i> , 2005b	Brazil
	<i>C. canephora</i>	ET	EHA 105	pCambia 3301	CaMV35S	<i>bar</i>	<i>uidA</i>	GI, PR	Ribas <i>et al.</i> , 2006a	Brazil
	<i>C. arabica</i>	SE	A4	pBIN19	CaMV35S	<i>hpt</i>	<i>uidA</i>	GI, PR	Spiral <i>et al.</i> , 1993	France
	<i>C. canephora</i>	L	IFO 14554	ND	ND	ND	ND	GI, PR	Sugiyama <i>et al.</i> , 1995	Japan
	<i>C. canephora</i>	SE	A4	pBIN19	CaMV35S	<i>csr1-1</i>	<i>uidA</i> , <i>cryIac</i>	GI, PR	Leroy <i>et al.</i> , 1997	France
	<i>C. canephora</i>	SE	A4	pCambia 1301	CaMV35S	<i>hpt</i>	<i>uidA</i>	GI, PR	Kumar <i>et al.</i> , 2006	India
	<i>C. arabica</i>	H	A4	pBIN19	CaMV35S	<i>visual</i>	<i>uidA</i>	GI, CP	Alpizar <i>et al.</i> , 2006a ^(b)	France
	<i>C. arabica</i>	H	A4	pCambia 2300	CaMV35S	<i>visual</i>	<i>gfp</i>	GI, CP	Alpizar <i>et al.</i> , 2006b	France

^(a) Et, embryogenic tissue; SE, somatic embryos; H, hypocotyl; L, leaf; GI, reporter gene integration; PR, successful plant regeneration; TE, transient expression; CP, composite plants; ND, no data available

^(b) In these studies, several *Agrobacterium* strains or different tissues were tested, but only best results are shown

^(c) Unpublished results

(α -tubulin and α -arabacin); under their protocol conditions, all promoter constructs resulted in similar transient expression of *GUS* gene. It opens the possibility that pCaMV 35S could be replaced with a coffee promoter.

Not less important is the lack of studies about the characterization and specific localization of p35S-driven-*GUS* activity in transformed tissues of coffee. Sreenath and Naveen (2004) carried out a histochemical survey of p35S-*GUS* activity comparing various tissues in untransformed and transformed embryogenic calli and somatic embryos, and demonstrated that coffee possesses an endogenous *GUS* activity that is expressed at different levels in different tissues, but differs from introduced bacterial *GUS* activity in optimal pH requirement and sensitivity to methanol. Hatanaka *et al.* (1999) demonstrated that leaves and roots of p35S-*GUS* transformed plants exhibited a deep blue color in reaction with X-gluc, while nontransformed plantlets did not react with X-Gluc. Leroy *et al.* (2000) found that 50% of the embryogenic callus transformed with pEF-1 α -*GUS* driven promoter and growing on culture media containing a high dose of chlorsulfuron (80 $\mu\text{g l}^{-1}$) did not exhibit a blue staining. This work clearly demonstrated that increasing the selective marker concentration does not alleviate the problem of escapes and decreased the rate of transformation events. Therefore, this research team used *GUS* histochemical test as a secondary screening of whole plantlets. Recently, Alpizar *et al.* (2006a), found that distribution of p35S-*GUS* expression exhibits the same pattern of expression within the same root system but this pattern can be variable between root systems. In all situations, the strongest p35S-*GUS* expression was always observed in the root tip and in the central cylinder.

Ogita *et al.* (2004) demonstrated that epifluorescence of p35S-*gfp* gene was constitutively detected throughout the entire coffee somatic embryo. Similar results were reported in coffee by Canche-Moo *et al.* (2006) in somatic embryos and Alpizar *et al.* (2006b) in roots, using p35S-*DsRFP* and p35S-*gfp* genes, respectively. Satyanarayana *et al.* (2005) recently achieved cloning of the first promoter for the *NMT* gene involved in caffeine biosynthesis, which will be very useful in near future for studying the regulation of caffeine biosynthesis genes.

2.2 Methods Employed

2.2.1 Direct gene transfer

After the first report from Barton *et al.* (1991) of transformation of coffee embryos by electroporation using the *nptII* (neomycin phosphotransferase II) (kanamycin resistance) gene, this method remained unmentioned until the recent works of Fernandez-Da Silva and Menéndez-Yuffá (2003) (see Table 1) that described improved conditions to regenerate transformed *C. arabica* somatic embryos and plantlets expressing the *GUS* and *bar* genes (Figure 2e). Their experiments showed that the electroporation of somatic embryos at torpedo stage can be promising as a method for coffee transformation since they showed the best transient *GUS* expression and regeneration through secondary somatic embryogenesis. The expression of *gus* and *bar* genes was confirmed by polymerase chain reaction (PCR) reaction in the regenerated plants several months after the electroporation of the tissues (De Guglielmo and Menéndez-Yuffá, unpublished results).

Biolistic delivery method has been improved considerably since the first report of *GUS* transient expression in coffee using a powder driven gun by van Bostel *et al.* (1995). Rosillo *et al.* (2003) studied diverse interactions between osmotic preconditioning of *C. arabica* suspension cells and physical parameters using helium gun device. They determined that a short period of endosperm pretreatment with two nonmetabolized agents (mannitol and sorbitol) increased the number of cells expressing *GUS* gene without causing cell necrosis after bombardment. The authors discussed the advantage of using this type of osmotic agents instead of sucrose in order to reduce the turgor pressure of cells and increase cell survival by avoiding the leakage after the shock wave created during bombardment. Ribas *et al.* (2005a) described a protocol for transformation of embryogenic callus and somatic embryos of *C. canephora* using a helium gun, associated with subculturing onto medium containing mannitol before and after bombardment. Their protocol allowed 12.5% of transformed callus expressing *GUS*-positive reaction to histochemical assay.

De Guglielmo and Menéndez-Yuffá (unpublished results), using a pistol at low helium pressure evaluated the efficacy of this technique in



Figure 2 *A. tumefaciens*-mediated transformation of coffee tree. (a) Selective proliferation of cream colored transgenic embryogenic calli of *C. arabica* in the presence of 100 mg l^{-1} hygromycin. The nontransformed calli have turned brown. Transgenic calli were obtained from embryogenic calli co-cultivated with *Agrobacterium tumefaciens* carrying a binary plasmid with *hpt* gene driven by CaMV35S promoter. (b) Transgenic somatic embryos of *C. arabica* differentiated from transgenic calli selected in presence of 100 mg l^{-1} hygromycin. (c) Transgenic plant of *C. canephora* cv. C \times R derived from *A. tumefaciens*-mediated transformation and selected in presence of hygromycin. (d) Transgenic plantlet of *C. arabica* showing blue color indicating strong *gus* gene expression. (e) Coffee Catimor plantlet regenerated from somatic embryos electroporated with pCambia3201

transformation efficacy of torpedo and globular somatic embryos, embryogenic callus, and *in vitro* plant leaves of *C. arabica*. On the basis of GUS transient expression, survival and regeneration of the tissues, the authors determined that torpedo embryos were the best material for biolistic gene delivery. The same authors applied the improved conditions to transform *C. arabica* also with the complete pUBC plasmid (Sardana *et al.*, 1996; Cheng *et al.*, 1998) and the genetic cassette Ubi-*cry*-Nos (promoter-coding sequence-terminator). Such modification of the transformation procedure avoids or eliminates the negative effects attributed to the backbone of the vector of transformation that could be responsible for transgenic silencing, besides the number of foreign sequences introduced in the plant genome, including genes of resistance to antibiotics, which is controversial from the point of view of biosafety.

2.2.2 Indirect gene transfer

Hatanaka *et al.* (1999) achieved the first successful *A. tumefaciens*-mediated transformation of *C. canephora* plants exhibiting strong GUS stable expression. Leroy *et al.* (2000) also reported transformation and efficient regeneration of coffee plants of both *Coffea* sp. containing *uidA* and *cryIac* genes. Ribas *et al.* (2006a) also transformed *C. canephora* explants submitted to sonification during immersion in a suspension of an *A. tumefaciens* strain encoding *uidA* and *bar* genes and regenerated transformed plants. Moreover, Canche-Moo *et al.* (2004) transformed leaf explants through *A. tumefaciens*-mediated transformation involving a vacuum infiltration protocol in a bacterial suspension, followed by a step of somatic embryogenesis induction.

A. tumefaciens-mediated transformation has also served to induce stable gene silencing through RNAi technology of genes encoding theobromine synthase (Ogita *et al.*, 2004) and *NMT* (Kumar *et al.*, 2004) respectively, both genes involved in caffeine biosynthesis, in both *C. arabica* and *C. canephora* species. Ribas *et al.* (2005b) achieved inhibition of ethylene burst in *C. arabica* by means of introducing the transgene in antisense orientation.

Agrobacterium rhizogenes-mediated transformation in both *C. canephora* and *C. arabica* species was first reported by Spiral *et al.* (1993) and Sugiyama *et al.* (1995), respectively. Leroy *et al.* (1997) also achieved stable transformation of somatic embryos of both *Coffea* sp. following the protocol developed by Spiral *et al.* (1993). Kumar *et al.* (2006) described an adapted method for *A. rhizogenes* sonication-assisted embryo transformation. Their technique allowed the transformation and direct regeneration of transformants through secondary embryos by-passing an intervening hairy root stage. Alpizar *et al.* (2006a) developed an *A. rhizogenes*-mediated transformation protocol that enables efficient and rapid regeneration of transformed roots from hypocotyls of zygotic embryos and subsequent production of composite plants (transformed roots induced on nontransformed shoots) (Figures 3a, b, and c). This methodology was specifically developed for functional analysis of genes involved in resistance to root-specific pathogens like nematodes and/or in plant morphology and development. The authors demonstrated that the *M. exigua* root-knot nematode could normally proliferate in transgenic roots (Figures 3d, e, and f) and that consequently this transformation methodology could efficiently be applied for the functional analysis of the *Mex-1* resistance gene.

2.3 Selection of Transformed Tissue

Although in most coffee transformation reports, T-DNA transfer has been done through the use of binary vectors containing both the genes of interest and one or various selection marker(s) genes, few works have compared different concentrations and timings of antibiotic selective markers (*hpt* hygromycin-R, *nptII* kanamycin-R) or herbicide (*csr1-1* chlorsulfuron-R, *bar* ammonium glufosinate-R, *ppt* phosphinothricine-R) in order to find that allow best rates of transformed plant regeneration.

The first works in coffee transformation were done using kanamycin. However, this antibiotic has exhibited contradictory results as potential selection agent for transformed coffee embryos. Barton *et al.* (1991) and Spiral *et al.* (1993) concluded that kanamycin possesses poor selective capacity since nontransformed somatic embryos

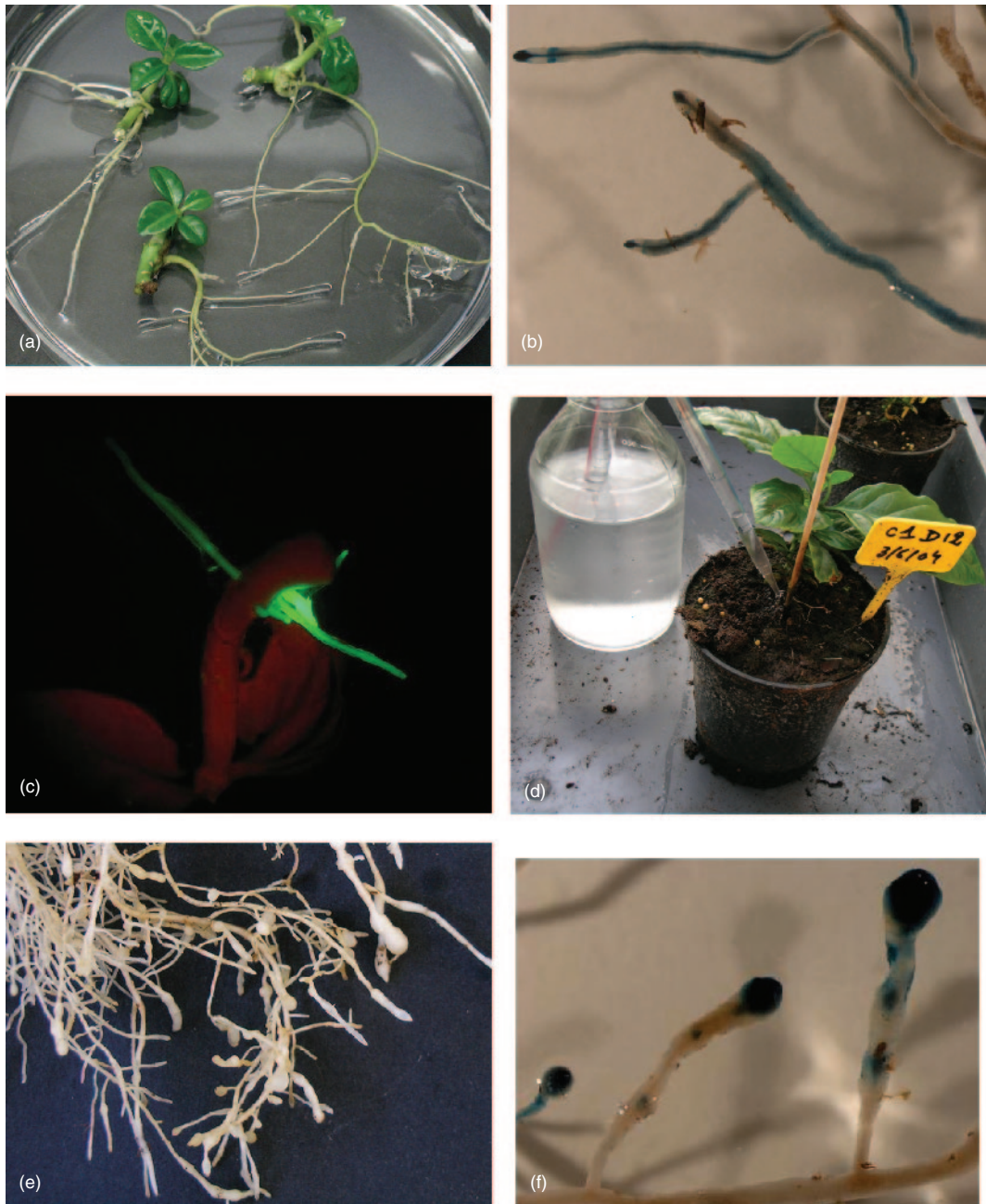


Figure 3 *A. rhizogenes*-mediated transformation of coffee roots. (a) Aspect of branched transgenic roots on nontransgenic stems (composite plants) 12 weeks after *A. rhizogenes* infection on hypocotyl. (b) Histochemical localization of β -glucuronidase (GUS) gene expression in transgenic roots of *C. arabica* transformed with the p35S-gusA-int gene construct. The different tissues actively expressing the GUS gene are stained in blue. (c) Selection of transformed roots using GFP epifluorescence; the green fluorescence is observed specifically on transformed roots. (d) *C. arabica* composite plants in soil substrate ready for nematode inoculation in resistance tests. (e and f) Proliferation of the *M. exigua* root-knot nematode on *C. arabica* composite plants obtained after transformation with *A. rhizogenes*. (e) Gall symptoms caused by *M. exigua* on transformed roots of susceptible *C. arabica* var. Caturra 4 months after nematode inoculation. (f) GUS expression in feeding sites induced by *M. exigua* in transformed roots (var. Caturra)

could regenerate even at high concentration doses (400 mg l^{-1}), whereas Giménez *et al.* (1996) found that secondary somatic embryogenesis was inhibited at 50 mg l^{-1} of kanamycin. Giménez *et al.* (1996) and van Boxtel *et al.* (1997) agreed that embryogenic suspension cultures showed tolerance to 400 mg l^{-1} of kanamycin. Despite the previous warnings, kanamycin was recently used at 400 mg l^{-1} by Cunha *et al.* (2004) and at 100 mg l^{-1} by Canche-Moo *et al.* (2006), both achieving acceptable regeneration of transformed embryos. Hatanaka *et al.* (1999), Naveen *et al.* (2002), Mishra and Sreenath (2004) and Ogita *et al.* (2004) observed that hygromycin at $50\text{--}100 \text{ mg l}^{-1}$ allowed an acceptable regeneration frequency of *A. tumefaciens*-mediated transformed somatic embryos (Figures 2a, b, c, and d). Kumar *et al.* (2006) using *A. rhizogenes*-mediated transformation reported that 20 mg l^{-1} hygromycin interfered with secondary embryos growth and therefore suboptimal levels of selection pressure (5 mg l^{-1}) were employed during early embryogenesis development in order to retain nature of the transformed cell. Because of the low selection efficiency of antibiotics in coffee along with biosafety concerns about transferring of bacterial antibiotic resistance markers to plants and from these to animals or humans, coffee transformation strategy turned into the utilization of other types of selection markers as herbicide selection or positive selection in order to avoid this potential biohazard.

van Boxtel *et al.* (1997) proposed a “hybrid” protocol for selection of coffee transformed tissues, where kanamycin should be used only during early phase of callus development (100 to 200 mg l^{-1}) with subsequent use of the nonselective pre-emergence herbicide glufosinate ammonium (3 mg l^{-1}) on later developmental stages of somatic embryogenesis. van Boxtel *et al.* (1997) and Fernandez-Da Silva and Menéndez-Yuffá (2004) showed that low concentration of glufosinate (6 mg l^{-1}) were enough to inhibit nontransformed callus growth. Subsequent works carried by Ribas *et al.* (2005a, 2006a) confirmed the reliability of *bar* gene (which inactivates the herbicide ammonium glufosinate) as a selection marker to regenerate only transformed somatic embryos in both *C. canephora* and *C. arabica*. Leroy *et al.* (2000) have used the herbicide chlorsulfuron as selectable marker with gene *csr1-1* from

A. thaliana, which was effective in the same cultivated species at a concentration of $80 \mu\text{g l}^{-1}$. Later works also succeeded in regenerating transformed plantlets containing *ppt* gene, which confers resistance to phosphinothricin on selective medium containing $10 \mu\text{M}$ of the herbicide (Cruz *et al.*, 2004).

Other marker genes like phosphomannose isomerase (*pmi*) (Joersbo *et al.*, 1998) and xylose isomerase (*xylA*) (Haldrup *et al.*, 1998) have started to gain attention for their use in coffee genetic transformation. They differ from conventional (antibiotic or herbicides) selection markers as they are based on supplementing the transgenic cells with a recovery metabolic advantage rather than killing transgenic shoots after transformation (Joersbo, 2001). In coffee, Samson *et al.* (2004) tested the regeneration of somatic embryos in presence of mannose or xylose in media devoid of glucose and sucrose, and demonstrated that they were able to regenerate on explants cultured in the presence of mannose as sole carbohydrate source, but not in different concentrations of xylose, indicating thus the potential use of *XylA* gene as a positive selection marker in coffee transformation.

Recently, following environmental concerns about transferring herbicide resistance through natural pollination to nontransformed related plant species growing near the transgenic crop fields, along with the aim of simplifying transformation procedures, visual markers started to replace those based on herbicide selection. In that direction, Ogita *et al.* (2004) and Canche-Moo *et al.* (2006) used *gfp* and *DsRFP* reporter genes respectively for visual selection of somatic embryos of *C. canephora* following *A. tumefaciens*-mediated transformation. First report of complete selection of coffee transformed tissues without using any marker gene was achieved by Alpizar *et al.* (2006a), after selection of putative *A. rhizogenes* transformed roots through histochemical GUS assay (Figure 3b). The selection protocol was recently optimized using GFP epifluorescence and transformed roots were easily selected observing the green fluorescence on living roots (Alpizar *et al.*, 2006b) (Figure 3c). Transformed plants bombarded with the plasmid pUBC or the genetic cassette (Ubi-cry-Nos) were detected by PCR for the gene *cryIac* applied to all the regenerated plants, avoiding completely the use of reporter

or marker genes (De Guglielmo and Menéndez-Yuffá, unpublished results).

2.4 Regeneration of Transformed Whole Plants

The long life cycle of coffee tree calls for stability of the transgenes over several years and, until now, regeneration of stable transgenic coffee has scarcely been studied and seems still far from suitable to be used in routine for the different *Coffea* species. First regeneration of transformed *C. canephora* was achieved by Spiral *et al.* (1993) and for *C. arabica* by Sugiyama *et al.* (1995). In both these studies, transformed plantlets were regenerated from *A. rhizogenes*-mediated transformed somatic embryos and roots respectively; however, in these studies the regeneration protocol was laborious and plants showed a “hairy” phenotype with short internodes and stunted growth. Since the works of Yasuda *et al.* (1985) van Bostel and Berthouly (1996) who developed optimized protocols for direct and indirect somatic embryogenesis from coffee leaves, efficient regeneration of somatic embryos and plantlets was available for genetic transformation. The direct somatic embryogenesis is preferably used for *C. canephora* and the indirect one for *C. arabica*. The use of embryogenic tissues is often preferred to somatic embryos and become the most common way for coffee transformation (see Table 1). Such technique was later used by Leroy *et al.* (2000); however, embryogenic callus was induced only in 1.0% and 0.1% from total *A. tumefaciens*-mediated transformation events for *C. canephora* and *C. arabica*, respectively. From this callus, only 30% (*C. canephora*) and 10% (*C. arabica*) developed into secondary embryos, and from these, only 50% regenerated into plantlets. The previous results clearly demonstrated the genotype effect on coffee transformation, which is more efficient with *C. canephora*.

Ogita *et al.* (2004) developed an advantageous method that consisted in the production of direct somatic embryos directly from the epidermal tissues of the initial embryos (without callus formation and where somatic seedlings stage is reached within 3–6 months). Kumar *et al.* (2006) reported that regeneration of coffee plantlets following *A. rhizogenes*-mediated transformation

of somatic embryos was barely efficient (only 3% of total transformation events). They also described some plantlets exhibiting a “hairy root” phenotype with abnormal elongation and brittle and wrinkled leaves phenotype. However, the percentage of plantlets in the previous study with aberrant phenotype was significantly low compared with the results previously described by Sugiyama *et al.* (1995), who reported that all regenerated coffee plantlets showed a “hairy” phenotype. Such abnormal phenotype is stable as demonstrated by Perthuis *et al.* (2005) who showed that four out of the nine independently transformed *C. canephora* clones obtained with *A. rhizogenes* were still displaying this phenotype in field conditions, however, all these plants died rapidly after planting.

Although a considerable number of publications have reported the regeneration of transformed coffee plants, all plants from such experiments were maintained in transgenic greenhouses. Perthuis *et al.* (2005) reported the sole work of successful establishment of a batch of transformed coffee plants in field condition. Alpizar *et al.* (2006a, b) developed an efficient protocol for rapid regeneration of transformed roots from hypocotyls of zygotic embryos, and subsequent production of composite plants (transformed roots induced on nontransformed shoots) (Figures 3c and d). Nematode infection of such roots done in nursery conditions revealed no difference in the number of extracted nematodes between the transformed and nontransformed roots within two *C. arabica* varieties, resistant or susceptible to root-knot nematode, *M. exigua* (Figures 3e and f). The authors also demonstrated that transformed roots did not exhibit “hairy” disturbed phenotype and retained the resistance/sensibility phenotype of varieties from which they are derived.

2.5 Testing the Expression of Transgenes

2.5.1 Herbicide resistance

Leroy *et al.* (2000) regenerated transformed explants expressing resistance to chlorsulfuron. However, not all exhibited amplification of *csr1-1* gene, demonstrating the important occurrence of escapes during herbicide selection. Apart from chlorsulfuron, selection of transgenic

coffee plant has also been conducted by means of regenerating somatic embryos encoding *bar* gene on selective medium containing ammonium glufosinate (Ribas *et al.*, 2006a). Regenerated plants supported up to eight times the herbicide doses recommended for field applications. Transgenic plants were sprayed with the herbicide ammonium glufonate under greenhouse conditions and showed no phytotoxicity symptoms.

2.5.2 Pathogen resistance

Reports about coffee genetic engineering approach to insect resistance had increased considerably during the last few years, but till date no resistance to coffee berry borer (CBB) or CLM has been reported in *Coffea* sp. In the case of CLM, research has focused primarily on the use of *cry1Ac* gene from *Bt*, which possesses the most efficient δ -endotoxin against leaf miner (*Perileucoptera coffeella*) (Guerreiro *et al.*, 1998). Leroy *et al.* (2000) described achieving transformed plants in *C. canephora* with the synthetic version of this gene and expressing resistance to the insect in greenhouse conditions. Perthuis *et al.* (2005) reported that such resistance was stable and operationally effective after six releases of a natural population of *P. coffeella* during 4 years of field assessments. On other hand, production of transformed coffee plants with resistance to CBB was actually conducted by Cruz *et al.* (2004) with the α -*All* gene from common bean. The authors achieved transformation of *C. canephora* plants with this gene and bioassays with the insect are underway to confirm functional validation of its proteins in coffee (Cruz *et al.*, 2004). Following recent achievements in other crops, gene pyramiding could be envisaged in coffee in order to introduce a large number of resistance genes to diverse races of one pathogen or combined different pathogens.

2.5.3 Physiological traits

Ribas *et al.* (2005b) achieved inhibition of the ethylene burst by introducing the 1-aminocyclopropane-1-carboxylic acid (*ACC*) oxidase gene in antisense orientation; this technique would permit the understanding of

genes involved in fruit maturation and ethylene production. Satyanarayana *et al.* (2005) reported the cloning of the promoter of *NMT* gene involved in caffeine biosynthesis pathway. The authors mentioned that current efforts are focused on the use of this promoter sequence for down-regulation of *NMT* gene through transcriptional gene silencing. The recent cloning of the first promoter for the gene involved in caffeine biosynthesis (Satyanarayana *et al.*, 2005) together with the near identification of genes involved in sucrose and drought tolerance metabolism (Pot *et al.*, 2006) opens up the possibility for coffee plant transformation to validate and study the molecular mechanisms that regulate the production of these important targets for *Coffea* sp. cultivation.

2.6 Regulatory Measures

International agreements concerning genetically modified (GM) coffee do not exist in the coffee industry. However, there is a large consensus (ICGN for instance) within the coffee industry to avoid the commercialization of GM coffee and encourage to some extent the research and the knowledge in relation with coffee genomics and genetics including genome functional analysis using transgenics approach.

3. FUTURE ROAD MAP

3.1 Expected Products

Biotechnology offers new opportunities and technologies applicable to coffee agriculture and provides the means for addressing many constraints placed upon crop productivity by biotic and abiotic stresses. Possible applications include, engineering insect-pest resistance, developing male sterility for production of F₁ hybrids, incorporating pathogen resistance, drought and frost resistance, and gene manipulation for producing varieties with caffeine-free beans, uniform ripening, and improved cup quality characteristics. Several new applications have been suggested for the transgenic coffee and in some cases preliminary investigations have been initiated.

Availability of characterized genes is the driving force for developing biotech crops. Till recently, the complete sequences of only a few coffee genes were reported and even fewer genes were characterized (Stiles, 2001). Research programs are currently being implemented in coffee to identify and isolate genes of interest for agronomic, as well as, cup quality characteristics and the situation is changing rapidly. International Network on Coffee Genomics (ICGN) was launched in 2005 to facilitate exchange of genomic resources. The recent incorporation of coffee in the SOL (Solanaceae) genomics network project (Mueller *et al.*, 2005; Tanksley, 2006) is also likely to accelerate the identification of agronomically interesting genes. Coffee, as a member of the family Rubiaceae is distantly related to the model species *Arabidopsis*. A computational comparison of the coffee EST-derived unigene set with the sequence databases for *Arabidopsis* and Solanaceous species (e.g., tomato, pepper), indicate that Solanaceae crops are much better genomic models for coffee than *Arabidopsis* (Lin *et al.*, 2005). These results are consistent with the fact that coffee and Solanaceous species share very similar chromosome architecture and are closely related, both belonging to the Asterid I clade of dicot plant family. Moreover, the ability to identify orthologous genes between coffee and tomato opens up the door to eventually developing detailed comparative maps and to sharing of genomic and biological tools/discoveries (Lin *et al.*, 2005). Comparative genomics is forging a new identity for coffee breeding/improvement and opening the door to an unparalleled rate of gene discovery (Tanksley, 2006). Progress made in coffee in several research labs are likely to yield gene sequences of interest from *C. arabica* and *C. canephora* for resistance to leaf rust, nematodes, beverage quality, etc. By screening for homology, it is hoped to access genes coding for beneficial traits from wild relatives of coffee.

3.1.1 Resistance to insect pests and nematodes

The major pests attacking coffee include CBB (*Hypothenemus hampei*) (Coleoptera, Scolytidae), leaf miner (*Leucoptera* spp.), white stem borer (*Xylotrechus quadripes*), root nematodes (*Meloidogyne* spp. and *Pratylenchus* spp.) (Crowe, 2004; Castillo

and Wintgens, 2004; Lan and Wintgens, 2004). The CBB is present worldwide and considered to be the most devastating and economically important insect pest of coffee. If uncontrolled, the pest can reach at very high infestation levels. Leaf miner is an economically important pest in East Africa and Brazil and is responsible for leaf degradation and subsequent yield decrease. White stem borer (WSB) is a serious pest of arabica coffee in India, China, and other East Asian countries. Developing arabica selections with resistance to WSB is the top most research priority for India.

Both CBB and WSB belong to the order Coleoptera (beetles). There appears to be no naturally occurring resistance gene in coffee gene pool for CBB. The robusta is generally resistant to WSB but the pure arabica as well as robusta-introgressed arabica selections are susceptible to WSB. Although chemical and biological control mechanisms are adapted to combat these insect pests, the fight against insects represents the priority for numerous producing countries and the introduction of effective resistance through biotechnology is envisaged as a serious solution to improve the lives of coffee farmers. While pesticides are very effective in combating the immediate problem of pest attack on crops and have been responsible for dramatic yield increases in crops subject to serious problems, nonspecific pesticides are harmful to beneficial organisms, including predators and parasitoids of the target pest species. The drawbacks of unconstrained and indiscriminate insecticides usage have become apparent, not only in respect to the development of pest resistance, but also in terms of ecological impact. Some of the pesticides used to control coffee pests have entered the banned list.

For insect resistance, several different classes of bacterial plant and animal-derived proteins have been shown to be insecticidal toward a range of economically important pests from different orders (Ferry *et al.*, 2004). Of these, *Bt* toxins are the most commercially relevant, since *Bt*-expressing crops are the only insect-resistant transgenic crops to have been commercialized to date. *Bt*, a soil dwelling bacterium, colonizes and kills a large variety of host insects, but each strain has a high degree of specificity. *Bt* strains and their insecticidal crystal (*Cry*) proteins (ICPs) produced during sporulation have been used extensively as eco-friendly biopesticides in agriculture all over the

world for the past four decades. With the advent of molecular biology and genetic engineering it has become possible to introduce ICPs of *Bt* in crop plants to impart insect resistance. More than 40 different classes of *Bt* toxins have been identified on the basis of amino acid sequence homology (www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt). This database could be a valuable resource for identifying candidate *Bt* genes against target pests of coffee.

Using *A. tumefaciens*-mediated transformation, Spiral *et al.* (1999) successfully transferred the *cry1Ac* gene into *C. canephora* and *C. arabica* genotypes for imparting resistance to leaf miner. Molecular characterization of transformed plants showed that 69% of them carried a unique copy of T-DNA and Cry1Ac protein expression in leaves was obtained in 18 of 23 plantlets tested (Leroy *et al.*, 2000). Three different levels of resistance could be measured with some highly resistant plants, some highly susceptible and fully susceptible plants. Transformed plants highly resistant to leaf miner under greenhouse conditions were tested under field conditions in French Guyana for 4 years for field resistance (Perthuis *et al.*, 2005, 2006) in an experiment with 54 transformation events. Approximately 70% of the events tested were resistant to leaf miner. They also observed that the transformed plants presented similar growth and development compared to control plants. It was the first time that an important agronomic trait was introduced into a coffee plant and also field tested. Unfortunately, the field trial was vandalized and the experiment had to be suspended (Montagnon, 2005).

Mendez-Lopez *et al.* (2003) demonstrated that the *Bt* serovar *israelensis* was highly toxic to CBB during the first instar of the larvae. Protection against coleopteran pests has been achieved in corn and potato using *Bt* genes. This is an important example of the potential utilization of the genetic transformation technology, since to date, there is no reported source of resistance to CBB in *Coffea* species and the chemical control of the insect is difficult due to its life cycle, spent most of the time inside the berry. In another approach to control CBB, an α -amylase inhibitor from *Phaseolus vulgaris* has been considered as a target gene for introduction in coffee plants. *In vitro* assays demonstrated that this protein can inhibit CBB growth and development (Grossi de

Sa *et al.*, 2004). An international collaborative project implemented by Central Coffee Research Institute (CCRI) from India and CIRAD from France identified two new *Bt* Cry36-like toxins to be active against CBB and WSB larvae.

Control of CBB using transformed plants could represent an additional health benefit. In *Bt* corn, a decrease in both *Fusarium* and the mycotoxin fumosin has been reported (Munkvold *et al.*, 1999). Since CBB infestation could facilitate fungi penetration in coffee, lesser CBB infestation could reduce amount of *Aspergillus flavus* and *Aspergillus ochraceus* and consequently the amount of ochratoxin produced in the coffee, which is of major concern in the most important coffee markets in Europe and the United States (Ribas *et al.*, 2006b).

Cultivation of coffee with insect resistant transgenes has to be accompanied by intense insect management studies. The constant selection pressure of the plants on the insect population will probably favor the emergence of insect resistant populations. Since coffee plantations can remain productive for as long as 30 years without replantation of the trees, strategies for pest management must be implemented together with the introduction of insect-resistant transgenic coffee plants, such as the use of cultivated lines of nontransgenic coffee, biological control with natural predators and other agronomic practices to keep resistant insect populations under control (Leroy *et al.*, 2000; Ribas *et al.*, 2006b).

The main *C. arabica* cultivated varieties were shown to be susceptible to the sedentary endoparasitic root-knot nematodes (*Meloidogyne* spp.) (Campos *et al.*, 1990). In many production regions, *Meloidogyne* is a major agricultural constraint and can result in significant drops of production. So far, more than 15 species have been reported as parasites of coffee. Sources of resistance specific to root-knot nematodes have been identified in coffee trees (Bertrand *et al.*, 2001) and the *Mex-1* gene conferring resistance to *M. exigua* in *C. arabica* is in the process of being isolated (Noir *et al.*, 2003).

Genetically improving plants for resistance to nematodes requires increase in knowledge regarding resistance genes and associated mechanisms. Molecular dissection of resistance genes and better understanding of basis and evolution of resistance specificity would contribute to an enhanced

durability and utility of the identified resistance genes. For this purpose, the development of a genetic transformation procedure and additional tools for functional analysis that enable rapid validation and study of resistance genes to nematodes would be extremely useful.

Hairy roots were proposed as an easy system for testing nematode resistance in crop plants and were successfully used to study function of *Mi* and *Hs1pro-1* gene in tomato and sugar beet, respectively (Cai *et al.*, 1997; Remeus *et al.*, 1998; Kifle *et al.*, 1999; Hwang *et al.*, 2000). Nevertheless, difficulty in maintaining axenic culture conditions in a system with three organisms, i.e., plant/nematode/*A. rhizogenes* (Narayanan *et al.*, 1999) and the problematic difference between natural root environment and the use of sterilized nematodes and plant material devoid of aerial system when studying plant–nematodes interactions have been reported. An alternative strategy was developed in coffee for studying the function of genes in roots. It consists of using “composite” plants that can be efficiently and rapidly generated by inducing transformed roots on nontransformed shoots after inoculation with *A. rhizogenes* (Alpizar *et al.*, 2006a, b). These authors described (i) the development of a convenient *A. rhizogenes*-mediated transformation protocol for *C. arabica* enabling efficient and rapid regeneration of transformed roots from the hypocotyls of germinated zygotic embryos, and the subsequent production of composite plants without selection with antibiotics or herbicides (Figures 3a, b, and c); and (ii) the successful infection of transformed roots belonging to composite plant expressing the *gus* and *gfp* genes with the root-knot nematode, *M. exigua* (Figures 3d, e, and f). Composite plants offer the major advantage over axenic hairy root cultures of generating information at the whole plant level and give the possibility to realize functional analysis studies in nonaxenic conditions. To our knowledge, composite plants have never been used to study plant–nematode interaction.

A localized genetic map of the chromosome carrying the major dominant *Mex-1* gene conferring resistance to *M. exigua* in *C. arabica* was constructed (Noir *et al.*, 2003) and the physical mapping of the *Mex-1* region is currently pursued. We will soon apply this plant composite technique to validate *in vivo* the *Mex-1* gene

by functional complementation. This technique could also be applicable to functional analysis studies of coffee genes involved in the resistance to other agronomically important nematode species (*M. paranaensis*, *M. arabicida*, *M. incognita*), in root development or in mycorrhizal symbiotic associations.

3.1.2 Decaffeinated coffee

Caffeine is considered as the most widely used psychoactive substance in the world. It is present in leaves, seeds, and fruits of more than 60 plant species, of which coffee, tea, kolanuts, guarana seeds, cocoa are the most popular ones. Most of the caffeine consumed comes from dietary sources such as coffee, tea, cola drinks, and chocolate. In humans, sleep seems to be the physiological function most sensitive to caffeine. Caffeine prolongs sleep latency, shortens total sleep duration, but preserves the dream phases of sleep. An average concentration of 60–85 mg of caffeine per cup has been assumed for instant, roasted, and ground coffees, respectively (Schilter *et al.*, 2001). The most notable behavioral effects of caffeine occur after low to moderate doses (50–300 mg); these are increased alertness, energy, and ability to concentrate. Higher doses of caffeine rather induce negative effects.

An area of considerable interest for coffee biotechnology is caffeine biosynthesis. Caffeine (1,3,7-trimethylxanthine) belongs to the category of plant alkaloids called methylxanthines. The first step, unique to caffeine biosynthesis pathway, is the methylation of xanthosine at N⁷ position by xanthosine N⁷ methyl transferase. Sequence of gene coding this enzyme is reported (Moisyadi *et al.*, 1999). Molecular cloning and functional characterization of three distinct N-methyltransferases involved in the caffeine biosynthetic pathway in coffee plants are also reported (Ogawa *et al.*, 2001; Uefuji *et al.*, 2003).

The average content of caffeine is 1.2% in arabica and 2.2% in robusta showing a marked interspecific difference as well as high intraspecific variability. Naturally decaffeinated arabica coffee plant was discovered recently (Silvarolla *et al.*, 2004). Coffee is primarily used as a stimulating beverage with characteristic organoleptic properties. There is a sizeable demand for decaffeinated

coffee because caffeine can adversely affect sensitive individuals by triggering palpitations, increased blood pressure, and insomnia. Market for decaffeinated coffee has increased to about 10% of coffee consumption worldwide. The chemical and physical methods used by the industry to produce decaffeinated coffee are expensive and affect the cup quality due to loss of key flavor compounds. Consequently, research in the biological realm for identifying a suitable decaffeinating agent has gained momentum.

The transgenic technology can be used to down-regulate the genes encoding enzymes responsible for caffeine biosynthesis. Recently, transgenic coffee plants were regenerated in which the gene encoding theobromine synthase was repressed by RNAi. Ogita *et al.* (2002, 2003, 2004) reported construction of transgenic coffee plants (*C. canephora*) in which expression of the gene encoding theobromine synthase (*CaMXMT1*) was repressed by RNAi. The caffeine content (measured in the leaves) of these plants was reduced by up to 70% indicating that it should be feasible to produce coffee beans that are genetically deficient in caffeine. Current evaluation in field trials should soon confirm the reduced caffeine content of the seeds. The main psychoactive component in coffee is caffeine. Caffeine content does not seem to have any direct effect on sensory quality (Illy and Viani, 1995). Hence, flavor of the genetically decaffeinated coffee is likely to be the same as regular coffee. However, doubts persist on the quality of this coffee because the accumulation of theobromine in the seeds could lead to adverse effects on coffee quality. Caffeine is also known to give resistance against insect pests. In view of this, it would be necessary to evaluate the reaction of decaffeinated coffee plants to insect pests. Instead of turning off the caffeine biosynthesis in the entire plant, the research can be focused to produce only caffeine-free beans for retaining the beneficial effects of coffee for plant protection.

3.1.3 Resistance to abiotic stresses

Abiotic stresses, such as freezing and drought, are the major climatic limitations for coffee production. As explained recently in a review by DaMatta and Ramalho (2006), these limitations are expected to become increasingly important

in several coffee growing regions due to the recognized changes in global climate, and also because coffee cultivation has spread toward marginal lands, where water shortage and unfavorable temperatures constitute major constraints to coffee yield. For example, periodic freezes, experienced in certain production areas of Brazil disrupt commodity markets. The introduction of frost tolerance in coffee through genetic transformation would be of great importance in alleviating the disruption that a frost can cause to coffee industry. Identification of drought tolerance genes by genetic engineering may be a way forward for marginal environments, complementing the breeding work and marker-assisted selection for tolerance that explores the natural allelic variation at genetically identifiable loci.

In most arabica coffee growing areas, the annual rainfall is in the range of 1500 to 2500 mm. Ideally, rainfall should be well distributed over a period of about 9 months with a single dry season of about 3 months coinciding with the harvest season. *C. canephora* (robusta coffee) is indigenous to West and Central African regions characterized by abundantly distributed rainfall and atmospheric humidity frequently approaching saturation (Charrier and Eskes, 2004). Arabica is generally more tolerant to water stress than robusta, at least partly as a result of a more extensive and deeper root system. *C. racemosa* is mentioned as a good source for drought tolerance.

Drought is an environmental factor that produces water deficit or water stress in plants. Inadequate and uneven distribution of rainfall causes drought condition in coffee, which affects the vegetative growth, induces floral abnormalities, and results in poor fruit-set and less crop. In Brazil, with a major area of production, robusta coffee has been largely cultivated in regions where water availability constitutes the major environmental constraint affecting crop production. Even short periods of drought can substantially decrease coffee yields and consequently irrigation is indispensable for production (Pinheiro *et al.*, 2005). A similar situation exists in India where robusta contributes around 60% of its production (Sreenath and Prakash, 2006). There is a prolonged drought of around 4 months in coffee production areas in India.

The causes of the differences in clonal tolerance to drought in robusta coffee still remain largely

unknown. Pinheiro *et al.* (2005) conducted studies to better understanding of the physiological and morphological basis of drought tolerance in robusta coffee and found that drought tolerance is associated with rooting depth and stomatal control of water use in clones of *C. canephora*. Some naturally occurring drought-tolerant plant species seem to employ deep and dense root systems to maximize water uptake. In the Brazilian Coffee Functional Genomics Program, research is being carried out by several groups aiming at identifying genes involved in biotic, as well as, abiotic (drought, salinity, and cold) stresses, apart from genes involved in flowering, fruit development and quality (Andrade *et al.*, 2006). Increasing knowledge of drought stress adaptation processes has been key to develop genotypes with improved tolerance to dehydration. The most successful or promising cases of genetic engineering for drought tolerance include the use of functional or regulatory genes, as well as, promising technologies such as transfer of transcription factors. In recent years, physiological and molecular bases for plant responses to drought and concurrent stresses such as high temperature and irradiance have been the subject of intense research (Chaves *et al.*, 2003; Chaves and Oliveira, 2004; Coraggio and Tuberosa, 2004). However, such studies are still very limited in coffee. Some studies are done in Brazil and India to understand the molecular basis of drought tolerance in coffee.

The recent progress in gene discovery and knowledge of signal transduction pathways is raising the hope of engineering important traits by manipulation of one single gene, downstream of signaling cascades with putative impact on more than one stress type (Chaves and Oliveira, 2004). Moreover, in genetic engineering, it is important to mimic nature and activate, at the correct time, only the genes that are necessary to protect the plants against stress effects. This may be achieved by using appropriate stress-inducible promoters and will minimize effects on growth under nonstress conditions, which is essential for agricultural crops.

Among the genes that are known to respond to drought stress and which are being manipulated by genetic engineering, some encode enzymes involved in metabolism (for example, linked to detoxification or osmotic response), others are active in signaling, in transport of metabolites

(for example, the proline transporter), or in regulating plant energy status (Chaves and Oliveira, 2004; Coraggio and Tuberosa, 2004). The molecular understanding of stress perception, signal transduction, and transcriptional regulation of these genes, may help to engineer tolerance to multiple stresses. Advances in the molecular biology of stress response in tolerant organisms are leading to a number of possibilities concerning regulatory genes that may be used in agricultural programs, not only to ensure survival under water deficit, but also to guarantee a reasonable productivity under reduced water availability.

3.1.4 Pathogen resistance

Coffee diseases are often classified as root diseases, diseases of stems and branches, foliage diseases, and diseases of berries and beans. Coffee diseases are mostly caused by fungi. The best-known disease of coffee is leaf rust (caused by the fungus *H. vastatrix*) which was first recorded in Sri Lanka in the 1860s and has since spread around the world (Muller *et al.*, 2004). The only satisfactory long-term solution to leaf rust is to plant resistant cultivars. The relevance of durable host resistance to coffee leaf rust (CLR) can be deduced from its economic damage to world arabica coffee production, which has been estimated at US\$ 1–2 billion per year due to crop losses (20–25%) and the need to apply cultural and chemical control measures (10% of production costs) (Van der Vossen, 2001). Resistance to CLR is conditioned primarily by a number of major (S_H) genes and coffee genotypes are classified in resistance groups according to their interaction with physiological races of the rust pathogen (Van der Vossen, 2001). India has a long history of arabica coffee breeding dominated by repeated occurrence of new physiological races of CLR, probably due to the warmer and wetter climatic conditions in major coffee growing areas. Resistance to CLR in robusta coffee is usually a secondary character of selection and based on individual plant, clone, or family scores for field infection (Van der Vossen, 2001).

The CBD caused by the fungus *C. kahawae* can be a devastating anthracnose of developing berries in arabica coffee in Africa. The impact of the disease on productivity is stronger in high altitude plantations over 1500 m in the tropics,

but occasionally it takes hold in plantations at lower altitudes, which have specifically cold and damp microclimate (Muller *et al.*, 2004). CBD may cause crop losses of 50–80% in years favorable to a severe disease epidemic (prolonged wet and cool weather). Economic damage to arabica coffee production in Africa due to CBD alone (crop losses plus costs of control) is estimated at about US\$ 300 to 500 million per year (Van der Vossen, 2001). Breeding programs initiated about 30 years ago in Kenya and Ethiopia have been successful in developing new cultivars with high and apparently durable resistance to CBD (Van der Vossen, 2001).

Several other fungal and bacterial diseases may affect coffee (Muller *et al.*, 2004; Sreenath and Prakash, 2006), but only a very few of them have been targeted in breeding programs, notwithstanding considerable economic damage in certain coffee producing countries, because useful host resistance could not be detected in available coffee germplasm. A number of other fungal diseases attack the branches of arabica coffee causing dieback and sometimes death of the branch. These include *Koleroga*, thread blight or black rot (*Pellicularia koleroga* = *Koleroga noxia*) and pink disease (*Corticium salmonicolor*) (Muller *et al.*, 2004). The less virulent strains of *Colletotrichum coffeanum* occur in most coffee growing areas in the world and cause brown blight (anthracnose) on the ripening cherry and lesions on leaves and green wood. Other fungi that can attack the berries include *Cercospora coffeicola* causing warty disease and various species of *Fusarium*. Various microorganisms attack coffee beans and cause them to rot or develop off-flavors. This occurs either because of storage in damp conditions or as a result of damage by insects.

Arabica is far more susceptible to diseases than robusta coffee and in many countries diseases are the limiting factor to production. Most diseases can now be controlled by chemical means, but often with difficulty and considerable cost. The most satisfactory long-term solution is the selection and breeding of disease-resistant cultivars, which is a major aim of coffee breeding programs (Etienne *et al.*, 2002). Spontaneous accessions collected in the primary center of diversity appeared to constitute a valuable gene reservoir. To date, *C. canephora* provides the main source of disease and pest resistance traits not found in *C. arabica*, including CLR (*H. vastatrix*),

CBD (*C. kahawae*), and root-knot nematode (*Meloidogyne* spp.). Likewise, other diploid species present considerable interests in this respect. For instance, *C. liberica* has been used as source of resistance to leaf rust (Sreenivasan, 1989), while *C. racemosa* constitutes a promising source of resistance to the CLM (Guerreiro Filho *et al.*, 1999).

There are a number of distinct signaling pathways that induce a coordinated expression of sets of antifungal proteins and/or compounds (Thomma *et al.*, 2001). These pathways are commonly dominated by the small signal molecules that play a central role in the induction of the defense proteins: salicylic acid, jasmonic acid, ethylene, or certain reactive oxygen species (Feys and Parker, 2000; Grant *et al.*, 2000). Several other pathways inducing defense exist in addition. Induction of each of these signaling pathways leads to the induction of various defense compounds, including sets of antifungal proteins, phytoalexins, and enzymes involved in plant cell reinforcement (Stuiver, 2006). Understanding the specific induction of targeted pathways and which pathway is causing resistance to which fungus in coffee is important to exploit the strategy. The recent findings of early expressed genes in specific hypersensitive reaction (HR) in resistant coffee plants in response to a virulent races of leaf rust pathogen (Fernandez *et al.*, 2004) could give an insight into the defense pathways operating in coffee.

Resistance to leaf rust pathogen in coffee by S_H genes is reported to be due to hypersensitive reaction. Recognition of the fungal elicitor was determined genetically to occur through a resistance (R) gene product made by the plants. The recognition is highly specific and depends on the presence of the exact R gene and its cognate elicitor. In coffee at least nine S_H resistance genes are recognized that confer resistance to different physiological races of leaf rust pathogen. Stacking or pyramiding of “R” genes is considered as a strategy for providing durable resistance. In coffee, this strategy can be applied by molecular assisted selection.

Efforts have been concentrated on cloning coffee resistance genes toward achieving durable resistance. Using degenerate primers based on conserved motifs of nucleotide binding site (NBS) region of NBS-LRR resistance genes, coffee R-

genelike sequences (i.e., resistance gene analogs or RGAs) representing substantial diversity have been identified (Noir *et al.*, 2001; Sreenath *et al.*, 2002). Most coffee RGAs appear to be closely related by sequence to at least one known R-gene and may encode resistance gene products of as yet unknown specificity. Recently, the genetic and physical maps of two resistance genes have been established, i.e., *Mex-1* gene conferring resistance to the nematode *M. exigua* (Noir *et al.*, 2003) and the *S_H3* gene conferring resistance to rust (Prakash *et al.*, 2004). The genetic map of the *Ck1* gene conferring the resistance to the *C. kahawae* (CBD) has also been recently established (Gichuru *et al.*, 2006). After cloning and functional validation, all these resistance genes could represent good candidates for molecular marker-assisted selection.

3.1.5 Improvement in cup quality characteristics

The distinctive flavor of coffee is certainly the principal reason for the high acceptability of the beverage prepared from the roasted and ground beans. Cup quality has gained importance as a result of the growth of specialty coffee sector. Biotechnology will undoubtedly play a progressively more important role in increasing the knowledge on the chemical constituents and metabolic ways involved in the elaboration of quality.

Chemical constituents of coffee bean have been analyzed thoroughly in recent years (Flament, 2002). While 300 flavor compounds are detected in green coffee, 850 are detected in roasted coffee. New analytical techniques are giving better insight into the fundamental chemistry of specific precursors and pathways leading to key flavor compounds. The constituents of coffee beans include minerals, carbohydrates, proteins, caffeine, chlorogenic acids (CGA), glycosides, lipids, and those transformed or formed at roasting such as trigonelline and the volatile constituents that create the unique flavor. In recent years, large-scale genomic studies have been speeding up the process of gene identification. Screening of fruit specific EST libraries may lead to identification and characterization of key genes responsible for cup quality. Because of the complexity of the chemical composition of coffee beans there

could be several strategies for improving the cup quality characteristics. The role of three major constituents influencing the coffee flavor and taste, namely, trigonelline, CGA, and sucrose are considered in the following paragraphs.

While all coffee beans have a high trigonelline alkaloid content, *C. arabica* cultivars have a higher content (0.79–1.06% on dry matter basis, dmb) than *C. canephora* cultivars (0.66–0.68% dmb) (Stennert and Maier, 1994). At roasting temperature (240 °C) this alkaloid produces pyridines and pyrrole derivatives (De Maria *et al.*, 1994, 1996), which are important volatile coffee flavor components (Flament, 2002). Trigonelline is considered to be important for both taste and nutrition. Consequently, one objective of coffee breeding research programs is to increase trigonelline content in robusta green beans (Ky *et al.*, 2001a).

The CGA are ubiquitous in the plant kingdom. They are a family of esters of quinic acid with several hydroxycinnamic acids particularly caffeic, ferulic, coumaric acids. In green coffee beans, three classes represent about 98% of the CGA content (Morishita *et al.*, 1989): caffeoyl quinic acids (CQAs), dicaffeoyl quinic acids (di CQA), and feruloyl quinic acids (FQA). They are present in relatively large quantities in the coffee bean, as the CGA content varies from 6% to 7% in arabica beans and is about 10% in robusta beans. CGA in green beans are major precursors of phenolic compounds in roasted beans. They are mainly responsible for coffee bitterness (Leloup *et al.*, 1995). The difference in CQA content in *C. arabica* and *C. canephora* is a major element explaining different flavor characteristics between these coffees (Clifford, 1985a; Ky *et al.*, 2001b). CGA is a crucial parameter of breeding programs considering the high CGA content variations in the *Coffea* genus associated with their effects on organoleptic quality and disease resistance (Campa *et al.*, 2003). Contrary to the negative attributes imparting bitterness in the cup, recent research is revealing the beneficial effects of CGA due to their antioxidant properties. In view of this, there could be demand for both increased and reduced CGA in coffee depending on the consumer perception. Genetic manipulation of genes involved in CGA biosynthesis can serve either of the purposes by up- or down-regulating the pathway.

CGA are products of phenylpropanoid metabolism, one branch of the phenolic pathway. Phenylalanine ammonia-lyase (PAL) catalyzes the first step of the phenylpropanoid pathway leading to the synthesis of a wide range of chemical compounds including flavonoids, coumarins, hydroxycinnamoyl esters, and lignin (Hahlbrock and Scheel, 1989). Caffeoyl quinic acid (5-CQA) is the major soluble hydroxycinnamoyl ester accumulated in plants. Formed by esterification between a caffeic acid and a quinic acid, it is grouped with other hydroxycinnamoyl quinic acids (HQA), under the generic name “chlorogenic acids” (Clifford, 1999). The 5-CQA cell content was found to increase in transgenic tobacco leaves overexpressing the *P. vulgaris* PAL2 gene, indicating that PAL is a key enzyme for 5-CQA synthesis (Howles *et al.*, 1996). Recently, Mahesh *et al.* (2006) isolated and characterized a full-length *C. canephora* PAL cDNA and its corresponding genomic sequence. This achievement has opened up the possibility of manipulating the key control enzyme PAL in coffee, which in turn will be useful to regulate several traits like cup taste, antioxidant property, pathogen and pest resistance, etc.

The sucrose content of coffee beans is an important component of coffee flavor, the higher the sucrose content in green beans, the more intense will be the coffee cup flavor (Clifford, 1985b; De Maria *et al.*, 1996). Coffee flavor is developed during the roasting process from aroma precursors present in green beans. The aroma formation is very complex and includes Maillard and Stucker's reactions and flavor precursor degradation (De Maria *et al.*, 1994). Among them, sucrose, which is degraded to furans, is one of the most important (Clifford, 1985b; Dart and Nursten, 1985; De Maria *et al.*, 1996). In addition, the sucrose also reacts with amino acids producing pyrazines and carboxyl compounds also involved in flavor. The sucrose/amino acids ratio in green beans widely determines the profile of volatile compounds. In *C. arabica* green bean, sucrose content is higher than in *C. canephora*: 8.2–8.3% versus 3.3–4% on dmb. Recent success in cloning and sequencing of *C. canephora* sucrose synthase gene (*CcSUS2*) (Leroy *et al.*, 2005) has provided a tool for manipulating the sucrose content in coffee leading to improved cup quality characteristics.

3.1.6 Fruit ripening

Uniformity during fruit ripening is decisively related to cup quality in coffee, and consequently to the value of the product. Fruits at correct ripened stage produce the best organoleptic characteristics for coffee. The presence of green fruits and over-ripened fruits changes the acidity, the bitterness, and consequently the cup quality. There is a possibility to achieve uniformity in fruit ripening by controlling the action of genes involved in the last steps of the maturation process, which is triggered by ethylene. In coffee, as well as other climacteric fruits, a dramatic increase in ethylene biosynthesis promotes the subsequent steps of fruit ripening, with biochemical and physiological changes (Pereira *et al.*, 2005). To control coffee fruit maturation, two of the main genes involved in ethylene biosynthesis, namely, ACC synthase and ACC oxidase have been cloned (Neupane *et al.*, 1999; Pereira *et al.*, 2005). Introduction of the ACC oxidase gene in the antisense orientation is reported in *C. arabica* and *C. canephora* (Ribas *et al.*, 2003, 2005b). The effect of the transgene on fruit maturation and ethylene production are yet to be reported. The inhibition of genes downstream to the initial ethylene burst is also an option to control fruit maturation (Ribas *et al.*, 2006b).

3.2 Expected Technologies

3.2.1 Improved transformation systems

Development of efficient, genotype-independent transformation systems is the ultimate aim of all transgenic crop programs, but remains elusive due to variations in *in vitro* morphogenic response between even closely related cultivars. Despite significant advances over the last 15 years, coffee transformation is still very laborious, with bottlenecks in the methodology that makes it far from a routine laboratory technique. In order to achieve many of the transformation goals, genetic transformation technologies of coffee must continue to be improved. There are two possible applications for coffee genetic transformation: (1) as a tool for gene validation (functional genomics), (2) eventually on the long term, as a way to regenerate new improved transgenic varieties. For the two objectives, the current protocols have to be

improved, but depending on the type of utilization, the requirements can be different (for example, the absolute need of a cleaner process for transgenic varieties through elimination of marker genes).

3.2.2 Elimination of marker genes

Production of genetically transformed crop plants without marker genes is desirable, but remains technically demanding. At present, the regeneration system of coffee is tedious and not very efficient. As transgenic technologies are improved for the crop, progress will be made toward genetically transformed marker-free coffee plants. Recently, Alpizar *et al.* (2006a, b) with an *A. rhizogenes*-mediated transformation system, have reported the possibility to regenerate transgenic roots and composite plants without using marker genes. The visual identification of transgenic roots with *gus* and more efficiently with *gfp* reporter genes allowed an efficient selection. Such a system is currently investigated to regenerate whole transformed plants with an *A. tumefaciens*-mediated transformation protocol.

3.2.3 Control of transgene expression

Transgene products must be expressed in relevant tissues at levels that impart agronomically useful traits. This may be in the stem for *Bt* protection against stem borers, in the fruits against berry borer and CBD, in the leaves against leaf miner and leaf rust, in the roots against nematodes, and in the beans for modification of carbohydrate, protein, caffeine, or CGA for improved cup quality and postharvest characteristics. Levels of transgene expression must be maintained stably in the coffee plant over several years because of the perennial nature of the crop. Access to tissue-specific and inducible promoters is required for successful development of coffee functional analysis and transgenic coffee. Promoter for N-methyltransferase gene associated with caffeine biosynthesis was isolated from *C. canephora* (Satyanarayana *et al.*, 2005). GUS assays proved that the isolated promoter was able to direct the expression of the reporter gene in transgenic tobacco. Marraccini *et al.* (1999) cloned a complete 11S seed storage protein gene and carried out

promoter analysis in transgenic tobacco plants. They obtained about 1 kb of the promoter region and about 0.9 kb of the 3' region in addition to the coding region. Sequence analysis of the promoter region indicated several motifs that occur in other seed storage protein genes that are responsible for both temporal and spatial regulation. The seed specificity of the 11S storage protein promoter may make it a useful tool for coffee biotechnology. By varying the length of the promoter it should be possible to impart the desired level of expression in a tissue specific manner to coffee seeds. This technology might be used for expressing disease and pest resistance genes specifically in the seed at the desired amount. The promoter could also be used to express genes that affect quality factors involved in cup quality or soluble solids important to the soluble coffee market (Stiles, 2001).

Recently, ESTs for two major seed storage proteins of coffee (2S and 11S) have been identified and demonstrated that these proteins are expressed at different times during seed development (Lin *et al.*, 2005). A number of highly expressed genes that show high specificity for different stages of seed development as well as for the pericarp tissue that surrounds the seed have been identified (Lin *et al.*, 2005). These genes could lead to the promoters that can potentially be used to drive gene expression in specific stages/tissues of the coffee plant. Many of these genes are specific to defined periods of seed and/or pericarp development—both critically important for insect/pathogen resistance and determining the quality of the coffee bean with respect to commercial coffee products. Availability of these promoters and additional control elements developed through similar research programs are providing important new tools for improving transgene expression and producing coffee with beneficial traits. It is proposed to create a collection of promoters in the ICGN as a resource for functional analysis of coffee genes.

3.2.4 Transfer of multigene DNA fragments and modifying more complex traits

In many cases, the objective will be to transfer large segments of DNA such as BAC clones and cosmids or in the case of metabolic engineering to simultaneously integrate several genes into the

coffee genome and express them in a coordinated manner. Gene isolation by positional cloning frequently requires several rounds of transformation. Even in well-studied crops for which multiple DNA markers are available, it remains difficult to narrow down the chromosomal region of interest to a manageable size for gene identification (Tao *et al.*, 2002). To minimize the resources invested and to accelerate the process, recent research has shown that it is possible to use large DNA fragments in transformation experiments followed by analysis of transgenic plants to assess functional complementation. Vectors like BIBAC (binary bacterial artificial chromosome) (Hamilton *et al.*, 1996) and TAC (transformation-competent artificial chromosome) (Liu *et al.*, 1999), which are functionally analogous, were designed to have the characteristics of both BACs and binary vectors for *Agrobacterium*-mediated transformation. Another application can be the transfer of clusters of genes (naturally occurring or synthesized) into the plant nuclear genome. Consequently, several disease resistance coffee genes or different genes belonging to a same biochemical pathway can be inserted in one transformation step and subsequently inherited as a single locus. While such capacities are established for the other major crops, further research is required to develop transgenic systems in coffee to facilitate these aims.

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Cocoa

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1. INTRODUCTION

Cacao, *Theobroma cacao* L., is a small understory tree believed to have evolved in the lowland rainforests of the Amazon basin (Wood and Lass, 1985; Bartley, 2005). Today, cacao is grown throughout the humid tropics, often in agroforestry ecosystems with other fruits and commodity crops. The production of dried seeds of cacao (cocoa beans) supports an international market of approximately 3 million tons, with two-thirds being processed into cocoa powder and cocoa butter, and the remaining one-third used for cocoa liquor (the flavor and color component of chocolate) (Wood and Lass, 1985). Cocoa is the major export commodity of several countries in West Africa (68% of world production), including Ivory Coast, Cameroon, Nigeria, and Ghana. Other major cocoa exporters include Ecuador, Venezuela, Brazil, Panama, Costa Rica, Dominican Republic, Malaysia, and Indonesia. Worldwide, approximately 5–6 million small-holder farmers grow 95% of the world's production providing an important source of cash to otherwise primarily subsistence farmers. World cocoa export commerce is \$5 to \$6 billion/year and in the United States alone, the use of cocoa and cocoa butter in chocolate manufacturing, cosmetics, and other products drives an approximately \$70 billion dollar market providing over 60 000 jobs (Morais, 2005). In addition, US chocolate production also uses large amounts of sugar, nuts, and milk valued

at approximately \$3 billion per year in receipts to American farmers.

Cacao is grown in some of the world's most important biodiversity hotspots (Piasentin and Klare-Repnik, 2004). With a long life cycle, predominantly cultivated under shade trees, there are many environmental benefits of cacao cultivation such as watershed and soil conservation, provision of habitat for birds and other animals, and provision of buffering habitat for protected wild rainforest regions (Rice and Greenberg, 2003; Ruf and Zadi, 2003). With these benefits in mind development agencies such as United States Agency for International Development (USAID), Conservation International (CI), The World Wildlife Federation (WWF), and the World Cocoa Foundation have formed alliances to promote sustainable cacao production (Guyton *et al.*, 2003).

Overwhelmingly, plant pathogens present the major challenge to sustainable cacao farming throughout the world. It is estimated that pests and diseases reduce the potential crop by an estimated 810 000 t annually (30% of world production) and individual farm losses can approach 100% (Keane, 1992; Bowers *et al.*, 2001). In one example, Witches' broom disease resulted in a decrease of production in Southern Bahia, Brazil, from 300 000 t in 1989 to 130 000 t 10 years later, for an estimated loss of \$220 million each year (Pereira *et al.*, 1990). This caused a cascade of widespread social disruption among small-holder growers

including loss of land and home ownership. Widely dispersed cacao pathogens include several species of *Phytophthora* (Appiah *et al.*, 2003, 2004; Chowdappa *et al.*, 2003). *Phytophthora megakarya*, the most aggressive species, is reported to have entered Ivory Coast, the world's leading cocoa producer (Nyasse *et al.*, 2002; Opoku *et al.*, 2002; Appiah *et al.*, 2003; Risterucci *et al.*, 2003; Efombagn *et al.*, 2004). Other important diseases and pests include frosty pod in Central America (Evans *et al.*, 2003), the cocoa pod borer (CPB) in Asia (Day, 1986; Santoso *et al.*, 2004), and cocoa swollen shoot virus in West Africa (Hanna, 1954; Hervé *et al.*, 1991; Muller and Sackey, 2005).

The fields of cacao genetics, breeding, and biotechnology have been the subject of a number of relatively recent review articles (Bartley, 1994; Hughes and Ollennu, 1994; Bennett, 2003; Figueira and Alemanno, 2005; Guiltinan, 2007); two books (Dias, 2001; Bartley, 2005) and a conference proceedings (Eskes, 2005). Large genetic variation has been identified in wild populations throughout the Amazon, but this diversity has not yet been widely incorporated into cultivated varieties (Bartley, 2005). Conventional cacao breeding programs were started in the 1920s (Toxopeus, 1969). In the 1930s and 1940s, valuable germplasm was collected from the Amazon regions of Brazil, Ecuador, and Peru (Pound, 1940), and these accessions are maintained as live trees in present day germplasm banks (Lockwood and Gyamfi, 1979; Engels, 1981; Iwaro *et al.*, 2003). Cacao breeding is slow and only recently molecular markers have been used to identify quantitative trait loci (QTLs) to potentially speed up breeding programs (Lanaud *et al.*, 1996; Motilal *et al.*, 2000; Flament *et al.*, 2001; Clement *et al.*, 2003; Risterucci *et al.*, 2003; Queiroz *et al.*, 2003). The current genetic map contains approximately 465 markers, 268 of which are simple sequence repeats (SSRs) (Pugh *et al.*, 2004).

Important traits for cacao include yield efficiency, flavor characteristics, cocoa butter content (% seed lipid content) and quality (fatty acid saturation), tolerance to abiotic stress, and various horticultural traits such as precocity, rootstock/scion interactions, plant height, and stature. However, disease resistance is currently the primary trait targeted by cacao breeders. It is thought that most important cacao plant pathogens did not co-evolve with the species, making it highly unlikely that true

resistance genes will be available in the existing germplasm. For this reason, transgenesis holds an important potential to contribute to cacao genetic improvement. This review will cover the development of tissue culture-based regeneration and genetic transformation systems for cacao. A brief presentation of the legal and societal issues surrounding this technology follows.

2. DEVELOPMENT OF TRANSGENIC CACAO

2.1 Somatic Embryogenesis of Cacao: A Prerequisite for Development of an *Agrobacterium*-Mediated Genetic Transformation System

One of the first steps in developing a successful genetic transformation system for cacao was the development of a highly efficient protocol for *de novo* regeneration of plants from a wide variety of cacao genotypes. Research has been focused mainly on developing somatic embryogenesis (SE) systems from different plant organs and tissues. No success has been reported on the development of organogenesis-based regeneration systems for cacao, such as a leaf explant method. Early attempts reported on the initiation of cacao SE from immature zygotic embryo tissue (Esan, 1977; Pence *et al.*, 1979; Aguilar *et al.*, 1992), leaves (Litz, 1986), nucellus (Sondahl *et al.*, 1989; Chatelet *et al.*, 1992; Figueira and Janick, 1993), and floral explants, including petals and staminodes (Lopez-Baez *et al.*, 1993; Alemanno *et al.*, 1996; Alemanno *et al.*, 1997). However, all of these studies described partial success in producing somatic embryos from a small number of genotypes with no or low rates of embryo to plant conversion.

More recent studies have focused on optimizing the SE system for cacao using floral organ explants (staminodes and petals). The following section reviews the studies completed at The Pennsylvania State University concerning the development of a highly efficient system for somatic embryo-derived cacao plants from many different cacao genotypes (Li *et al.*, 1998; Traore, 2000; Maximova *et al.*, 2002, 2005). This system was consequently successfully utilized for *Agrobacterium*-mediated genetic transformation (Maximova *et al.*, 2003, 2006). It consists of

multiple steps/protocols that involve specific media formulations and periods of culture. The steps include: primary somatic embryogenesis (PSE; from staminode explants), secondary somatic embryogenesis (SSE; from somatic embryo explants), somatic embryo conversion to plants, and plant acclimation to greenhouse conditions. Detailed, complete protocols of the system were published by Maximova and co-workers in 2005 (Maximova *et al.*, 2005) and are available at <http://guiltinlab.cas.psu.edu/Research/Cocoa/protocols.htm>.

Here we present only the main findings along the developmental process.

The first report on PSE by this group was published by Li *et al.* (1998). The publication described the production of primary somatic embryos from 19 different cacao genotypes using staminodes as explants. The study built upon works published by others as reviewed above. Several hormone regimes were tested to increase embryogenic induction. The highest regeneration frequencies (percentage of regenerating explants) were recorded after supplementing primary callus growth medium (PCG) with 22.7 nM thidiazuron (TDZ) and 9 μ M 2,4-dichloro-phenoxyacetic acid (2,4-D). Another important factor for the success of this system was the substitution of the more traditionally used Murashige and Skoog (MS) salts with the Driver and Kuniyuki walnut basal salts (DKW) (Driver and Kuniyuki, 1984). It was found that MS media does not support cacao tissue culture and is nearly lethal to the cells. The study reported wide variation among the different genotypes evaluated in regard to the percentage of regenerating explants as well as the average number of embryos produced per explant. The percentages of regenerating explants varied from 0.8% to 100% and the average number of somatic embryos per explant ranged from 1 to 45.7. The genotype with the highest performance was PSU-Sca 6 with 100% regenerating explants and an average of 45.7 embryos per explant. The lowest regenerating genotype was EET400 with 0.8% regeneration rate and an average of one embryo per explant. It was obvious from the results that this protocol was most favorable for PSU-Sca 6 but there were no apparent correlations between the differences in the response relative to the three different major cacao groups—Forastero, Criollo, and Trinitario. Consequently, PSU-Sca 6

was selected as a potential candidate for future development of a transformation system.

A major increase in the efficiency above the method reported by Li *et al.* (1998) included the development of a protocol for secondary embryogenesis using cotyledon explants from primary somatic embryos (Maximova *et al.*, 2002). For initiation of SSE, cotyledon pieces (approximately 4 mm²) were cultured for 14 days on modified secondary callus growth medium (SCG) containing 2.4 μ M 2,4-D and 1.4 μ M 6-benzylaminopurine (BA) (Li *et al.*, 1998), followed by transfer and maintenance on hormone-free embryo development medium (ED) (Li *et al.*, 1998). The study presented strong evidence that the secondary embryogenesis process increased the multiplication rate of genotype PSU-Sca 6 by 30-fold compared to primary embryogenesis alone. Similar to primary embryogenesis, the response was variable among the genotypes evaluated. However, after a few primary embryos were regenerated from genotypes with low embryogenic potential, the secondary embryogenesis process assured the further multiplication of these genotypes. The comparison of the sizes of the primary and secondary embryos indicated that secondary embryos have significantly lower variation in size (10-fold). Additionally, the histological analysis of early stages of embryogenesis pointed toward a predominantly unicellular origin of SSEs compared to multicellular origin of PSEs. Considering all advantages of secondary embryogenesis the process was selected as the step in the regeneration system that would be suitable for co-cultivation with *Agrobacterium* and a basis for the development of a genetic transformation system (described in Section 2.3).

An important final step in the regeneration system was the development of protocols for conversion of the somatic embryos to plants and their acclimation to greenhouse conditions (Traore, 2000). The conversion procedure was first optimized for PSU-Sca 6 and later applied for other genotypes. The hormone-free embryo conversion medium (Li *et al.*, 1998) was supplemented with different basal salts formulations. These included MS basal salts, woody plant salts (WPM), and DKW salts. The highest percentages of converted embryos (77%) were recorded after culture on DKW and WPM basal salts. Supplementing DKW with additional 0.3 g

potassium nitrate and $2.5 \mu\text{M}$ the amino acids arginine, glycine, leucine, lysine, and tryptophan increased the conversion rate to 92%. In addition to the media composition, it was observed that the morphological characteristics of the mature embryos are important factors for the success of the conversion. Two different classes of embryos (I and II) were first described by Li *et al.* (1998) based on morphological appearance. Type I embryos displayed yellow translucent color and larger cotyledons compared to type II embryos that had ivory white color and small cotyledons. The conversion of type II embryos resulted in higher conversion rate to plants. Plants produced by this method were transplanted to soil in a greenhouse with 100% humidity and 70% shade (approximately $100 \mu\text{mol m}^{-2} \text{s}^{-1}$). Humidity was maintained by misting for 10 s every 15 min. After 4 weeks the misting was omitted and plants were successfully maintained to maturity at 60–65% humidity with 50% shade ($250\text{--}500 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Maximova *et al.*, 2005). Plants produced by different genotypes are in the process of field evaluation in Saint Lucia, Ecuador, and Puerto Rico.

2.2 SE Research and Genetic Transformation Studies of Cacao at the Malaysian Cocoa Board, COCOA Biotech Center

Various explant sources (leaf, stem, young shoots, seed, flowers, etc.) from different commercial cocoa clones planted in Malaysia were evaluated for their potential for SE using previously published protocols (Aguilar *et al.*, 1992; Figueira and Janick, 1995; Alemanno *et al.*, 1996). From these, only seed, cotyledon tissue, and flower organs regenerated somatic embryos (T.C. Lock, unpublished data). Young cotyledon tissues (white to pale purple color) were more reactive compared to older cotyledon tissues (T.C. Lock, unpublished data). Although somatic embryos were produced from cotyledons, this source was not suitable for clonal propagation due to the fact that cotyledon tissue is a product of fertilization and is genetically different from the source materials. Further experiments were conducted with flower parts—anther filaments, staminodes, and petals (Tan and Furtek, 2003). Similar to the results reported by Li *et al.* (1998), the best regeneration

was recorded for staminode tissues (Figure 1). The genotype of cocoa was also an important factor for the success of embryogenesis as genotype-specific variations in the response were observed. Additionally, the time of the year when flowers were collected from the field and introduced in culture affects the embryo production (T.C. Lock, unpublished data). It was observed that after occurrence of a distinct dry period followed by a wet season, floral tissues performed better during SE. However, there is no actual data recorded that correlate the climatic conditions with embryogenesis rate and future experiments need to be conducted to verify these observations.

For induction of PSE, unopened flower buds (4–6 mm in length) were collected from the field and rinsed with tap water. The flowers were then sterilized in 70% ethanol for 5 min followed by 20 min in 10% commercial bleach (5.25% (v/v) sodium hypochlorite) and 0.10% sodium dodecylsulfate (SDS) solution, and 20 min in 1% bleach and 0.10% SDS solution. Buds were rinsed three times for 5 min in sterile reverse osmosis water. Staminodes were dissected in sterile conditions and cultured on somatic embryo induction medium (MIM41n). MIM41n consisted of DKW basal salt (Duchefa, Biochemie BV, The Netherlands), 20 g l^{-1} glucose, MS vitamins, 2 mg l^{-1} of 2,4-D, 0.1 g l^{-1} N^6 -[2-isopentenyl]adenine (2-iP), 500 mg l^{-1} glutamine, and 2 g l^{-1} Phytigel (Sigma), pH 5.8. Calli were formed after 2–3 weeks of culture on MIM41n media (Figure 1b). After 4–8 weeks, the calli were transferred to embryo differentiation media (MEM22a) consisting of DKW basal salts, MS vitamins, 50 mg l^{-1} and 2 g l^{-1} Phytigel, pH 5.5. Newly formed somatic embryos (Figure 1c) were separated from the calli and subcultured onto differentiation media for another 4–6 weeks. Secondary embryos (Figure 1d) were formed spontaneously on MEM22a medium and after culture on MEM22a supplemented with 0.01 mg l^{-1} 2-iP and 0.01 mg l^{-1} kinetin. The mature somatic embryos produced were approximately 40 mm long with distinct shoot and root apices. These were introduced into conversion media (MGM) consisting of one-fifth strength DKW basal salts, 5 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 2.2 g l^{-1} Phytigel (Sigma), pH 5.8. MGM medium was dispensed onto 250 ml glass jars

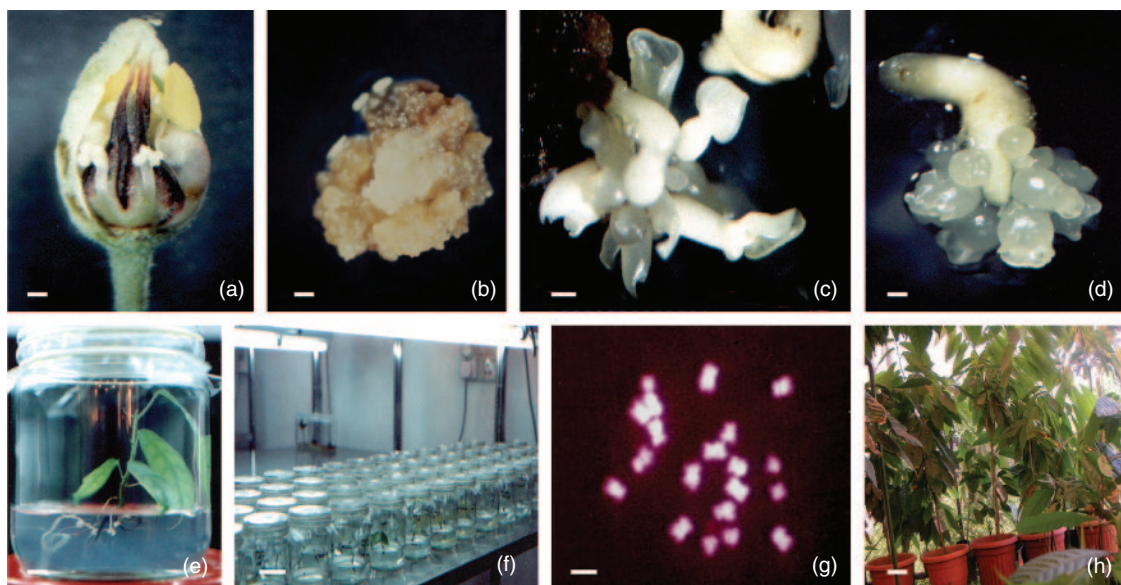


Figure 1 Somatic embryogenesis of *Theobroma cacao*. (a) Unopened flower bud, scale bar = 1.6 mm, (b) callus from staminode, scale bar = 1 mm, (c) primary somatic embryos from callus, scale bar = 2 mm, (d) secondary somatic embryos from primary embryo, scale bar = 0.9 mm, (e) plantlet from somatic embryo, scale bar = 10 mm, (f) plantlets in light culture room, scale bar = 5 cm, (g) cytological analysis of root tips from plantlet, scale bar = 10 mm, (h) somatic embryo derived plants established under *ex vitro* conditions, scale bar = 21 cm

(Biocraft, Singapore) and covered with Suncaps (Sigma) for ventilation. The conversion cultures were kept under light (16 h photoperiod, daylight fluorescent tubes, $25 \text{ mmol m}^{-2} \text{ s}^{-1}$) at $25 \pm 2^\circ \text{C}$ (Tan and Furtek, 2003). Roots usually appear after 3 weeks on conversion media followed by leaf development (approximately 4 to 8 weeks on conversion media). Once a healthy root system and dark green leaves were developed, the plantlets were transferred to *ex vitro* conditions (Figures 1e and f). Embryogenesis was most successful for genotypes QH1560, KKM19, PBC137, and KKM4 (commercial clones in Malaysia). The percentages of embryos producing explants (0% to 50%) varied greatly with the season and age of the source plants, and the genotypes. Between 5% and 15% of the somatic embryos were converted to plantlets.

To test for mutations and somaclonal variation as a result of the SE, SE-derived plants were analyzed and compared to the explant source plants by molecular fingerprinting and morphological characterization. Two- to three-year-old somatic embryo-derived plants and their mother plants were fingerprinted using 39 different microsatellite

markers (T.C. Lock, unpublished data). Genomic DNA was isolated from young, fully expanded leaves from somatic embryo-derived plants and mother plants using DNeasy 96 Plant kit (Qiagen). Polymerase chain reaction (PCR) amplification of the target sequences was performed using Applied Biosystems 96-Well GeneAmp[®] PCR System 9700. Each reaction contained 1U of *Taq* polymerase, 10 ng cocoa DNA, 0.2 mM dNTP mix, 2 mM MgCl_2 , $1 \times$ reaction buffer, and 2 pmol of fluorescently labeled primers in total volume of 20 μl . The PCR conditions applied were: 94°C for 4 min, followed by 30 cycles of 30 s at 94°C , 30 s at 50°C , and 1 min at 72°C . One to 10 dilutions were made from each PCR product and 1 μl from each sample was mixed with 1 μl ROX in an individual tube. The samples were denatured at 95°C for 3–5 min, cooled on ice, and fractionated by capillary electrophoresis using Amersham MegaBACE 500 analysis system. The SSR profiles were analyzed using GENOTYPER software (Applied Biosystems, Foster City, CA). The genotyping analysis demonstrated no differences between the somatic embryo-derived plants and the flower source plants. Additional cytological

analysis of root tips from the somatic embryo-derived plants and the source plants revealed an equal chromosome count of $2n = 20$ (Figure 1g) (T.C. Lock, unpublished data). Preliminary morphological examination of leaves and flowers also showed no differences from the mother plants (Figure 1h).

Agrobacterium-mediated transformation experiments were conducted with untreated staminode tissue, 3-week-old calli from staminodes, zygotic and somatic embryo pieces. The *Agrobacterium* strain used in the transformation was AGL1 carrying vector pGPTV-Kan: contains neomycin Phosphotransferase II (*nptII*) (kanamycin resistance) gene and β -glucuronidase (*GUS*) reporter gene (Becker *et al.*, 1992). The *Agrobacterium* was incubated for 48 h at room temperature (approximately 28 °C) in 523 medium with 50 mg l⁻¹ rifampicin, 50 mg l⁻¹ tetracycline, 50 mg l⁻¹ carbenicillin, and 100 μ M acetosyringone. The calli were inoculated with the *Agrobacterium* suspension (1.0 OD at 600 nm) for 30 s, blotted on sterile paper towels, and were then cultured on MEM22a medium with mg l⁻¹ cefotaxime and 250 mg l⁻¹ augmentin for 7 days. Subsequently, the calli were transferred to MEM22a medium with 250 mg l⁻¹ cefotaxime, 250 mg l⁻¹ augmentin, and 100 mg l⁻¹ paromomycin for selection. After approximately 5 weeks, the calli and newly developed somatic embryos were histochemically stained for GUS expression. The blue color observed indicated the development of putatively transformed calli (Figure 2a). Additionally, the formation of chimeric somatic embryos was observed (Figure 2b) (Tan *et al.*, 2001), which could be due to the possible multicellular origin of the primary embryos (Maximova *et al.*, 2002). Similar results were observed after transformation of callus derived from zygotic and somatic embryo

pieces (data not shown). Additionally, biolistics transformation was carried out using pVDH65 and 3-week-old staminode calli using BioRad PDS-1000/He system. The vector construct used included a triple cauliflower mosaic virus (CaMV) 35S promoter fused to a *GUS* gene (Tan *et al.*, 1999). The GUS histochemical staining resulted in the formation of approximately 40–50 blue spots per 3 mm² of callus as an indicator of transient expression. The number of blue spots observed decreased over time and no differentiation of transgenic somatic embryos was recorded.

2.3 *Agrobacterium*-Mediated Cacao Transformation: Protocol Development and Expression of Genes with Potential Agronomic Importance

2.3.1 Genetic transformation protocol development

Prior to year 2000 only one research publication could be found reporting attempts at cacao transformation. In this study, cacao leaf strips were inoculated with *Agrobacterium tumefaciens* carrying a vector with GUS and *nptII* genes (Sain *et al.*, 1994). The experiment resulted in the formation of transformed callus cells, but no transgenic plants were regenerated. In 1996, the Pennsylvania State University Cacao group began a program dedicated to the development of a reliable genetic transformation protocol based on the recently established SE tissue culture system described above. The development and optimization studies included evaluations of different vectors and reporter genes, various selective antibiotics, different somatic embryo tissues,

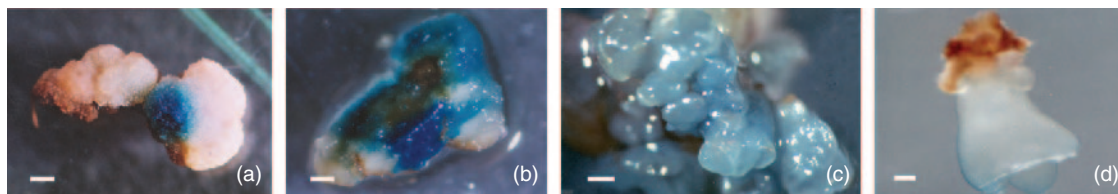


Figure 2 Genetic transformation of *Theobroma cacao* L. (a) Partially transformed callus, scale bar = 0.9 mm, (b) putative transformed callus, scale bar = 0.9 mm, (c) putatively transformed somatic embryos, scale bar = 0.8 mm, (d) partially transformed somatic embryo, scale bar = 0.7 mm

and the potentials of particle bombardment and *Agrobacterium*-mediated methods (Guiltinan and Maximova, 2000; Traore, 2000; Antunez de Mayolo *et al.*, 2003; Maximova *et al.*, 2003). To assess the potential of the biolistic method, cells grown in suspension cultures and somatic embryo cotyledon tissues were bombarded with 1 μm gold particles in a helium-driven PDS-100/He system (Traore, 2000). The gold particles were coated with pBI221 DNA containing *GUS* and *nptII* genes (De Block *et al.*, 1984). Three days after treatment *GUS* expression was evaluated and large numbers of blue spots were observed in the callus and the cotyledon tissue. However, no embryos were ever regenerated from these experiments (Traore, 2000). This method is considered a useful and quick method to check for construct expression in cacao cells prior to use in further attempts at stable transformation, but highlighted the need for improvement of cacao regeneration systems.

Thus, after several years of optimization of the SE system, attempts were made for *Agrobacterium*-mediated transformations using somatic embryos as explant sources. A major contribution to the development of the protocol was the utilization of the green fluorescent protein (GFP) reporter gene (Chalfie *et al.*, 1994). The ability to observe green fluorescence as an indication of transformation events within 48 h after *Agrobacterium* inoculation greatly increased the early screening capabilities (Maximova *et al.*, 2003). Additionally, the incorporation of GFP allowed the nondestructive observation of the explants throughout the entire culture period including the detection of the transgenic somatic embryos that were regenerated in low frequencies. This permitted relatively rapid optimizations of the many variables in the protocol.

Another important variable optimized was the selective antibiotic used in the regeneration medium. To select the best antibiotic for *nptII* resistance of transgenic cacao cells, the effect of different types of aminoglycoside antibiotics including paromomycin, neomycin, kanamycin, and geneticin at concentrations of 125, 150, 175, and 200 mg l^{-1} was evaluated. The number of GFP expressing explants and the total number of GFP expressing areas were recorded at 1, 2, and 3 weeks after bacterial inoculation. The highest percentage of expressing explants (49%) and the highest total number of GFP areas (64)

was observed after application of geneticin at 125 mg l^{-1} (S.M. Maximova and M.J. Guiltinan, unpublished data). Thus, this antibiotic was selected for further experiments subsequently adjusted to a concentration of 50 mg l^{-1} for 2 weeks after inoculation (Maximova *et al.*, 2003). A similar approach was exploited for selection of the *Agrobacterium* counter-selection antibiotic (Antunez de Mayolo *et al.*, 2003). For this study, it was necessary to evaluate the effect of the applied antibiotics on SE and in eliminating *Agrobacterium* in the regeneration culture after the initial co-cultivation period of 48 h. Different antibiotics were supplemented to the regeneration medium in four different concentrations (100, 150, 200, and 300 mg l^{-1}). The antibiotics included: from the cephalosporin class—cefotaxime and moxalactam, and from the penicillin class—amoxicillin and carbenicillin. The percentage of explants lost to *Agrobacterium* overgrowth was evaluated in addition to the effect of the antibiotic type and concentration to the average number of embryos produced in the absence of *Agrobacterium* inoculation. As a result moxalactam at 200 mg l^{-1} was selected as the most promising antibiotic that assured that no explants were lost to bacterial overgrowth and in fact increased the average number of primary and secondary embryos produced per explant by threefold compared to no antibiotic controls (Antunez de Mayolo *et al.*, 2003). This increase of regeneration efficiency could be due to a possible auxin response of the tissue to one of the several breakdown components of moxalactam, phenylacetic acid, which is a naturally occurring auxin (Webber and Yoshida, 1979).

As a result of combining high efficiency SE with *Agrobacterium*-mediated transformation using a GFP reporter gene, transgenic *T. cacao* plants were obtained and grown to maturity (Maximova *et al.*, 2003). With optimization of the protocol, the numbers of transgenic plants recovered from a single experiment increased from one in early experiments to a high of 10 independent transgenic plants. All binary vectors utilized included the Bin19 backbone (Bevan, 1984), *nptII* (De Block *et al.*, 1984) and EGFP (Clontech, Palo Alto, CA) genes both under the control of E12- Ω constitutive promoter, a very highly expressed derivative of CaMV 35S promoter (Mitsuhara *et al.*, 1996). Additionally, pGH00.0131 included

Tobacco Matrix Attachment regions (MARs) (Spiker and Thompson, 1996) flanking the transfer DNA (T-DNA) region. The presence of the MARs contributed to increased GFP fluorescence in primary transgenics and stable and uniform GFP expression among the tertiary somatic embryos propagated from the cotyledons of primary transgenics (Maximova *et al.*, 2003). The physiological and morphological observations of the transgenic cacao plants produced indicated that genetic transformation events and transgene expression did not cause any detectable pleiotropic phenotypic changes. On occasion gene silencing was observed during reiterative embryogenic multiplication (S.M. Maximova and M.J. Guiltinan, unpublished data). Many mature somatic embryos were converted to plants and transferred to greenhouse conditions for further evaluation. Ten transgenic plants from the first established transgenic lines were closely observed during a period of 5 months and their growth parameters were recorded and compared to ten nontransgenic somatic embryo-derived control plants (Maximova *et al.*, 2003). The parameters measured were height, stem diameter, number of leaves, and total leaf area. No significant differences were recorded among the plants for any of the parameters. After approximately 1.5 years of growth in the greenhouse, the transgenic plants developed flowers exhibiting bright GFP expression. The flowers were pollinated with pollen from nontransgenic *T. cacao* genotype Catongo. The pods produced from this crosses exhibited high levels of GFP fluorescence of all maternal pod tissues including pod surface, exocarp, placental tissues, and seed coat. Very high levels of GFP fluorescence were observed in the cotyledons and the embryos of the transgenic seeds. The GFP gene in the T₁ generation from several of the transgenic plants segregated as a single locus insertion (1:1 segregation ratio).

2.3.2 Expression of cacao *TcChi1* gene in transgenic cacao

As discussed in the introduction, cacao pests and diseases are major constraints to sustainable cacao production, and many of these are fungal pathogens. With this in mind, the first gene with potential agronomic importance that was

introduced into cacao was a class I chitinase gene (*TcChi1*), encoding a potential antifungal protein (Maximova *et al.*, 2006). The *TcChi1* gene isolated from *T. cacao* encodes an endochitinase protein (NCBI accession U30324 (Snyder, 1994; Snyder-Leiby and Furtek, 1995)). The gene/protein belongs to the pathogenesis-related (PR) protein family and it was demonstrated to be expressed in cacao fruit in response to fungal elicitor treatment (Snyder-Leiby and Furtek, 1995). As a test of its putative function in pathogen resistance, it was hypothesized that the ectopic overexpression of this gene in cacao would contribute to increased resistance to fungal pathogens. The coding sequence of the chitinase gene was placed under the control of the highly expressed constitutive E12- Ω promoter (Mitsuhara *et al.*, 1996), the same used to drive the EGFP and *nptII* genes as described above. The gene was introduced into cacao via the SE co-cultivation system described above. Transgenic embryos were produced and converted to plants (Maximova *et al.*, 2003). Clonally propagated plants from nine transgenic lines were characterized for transgene integration and expression, and *in vitro* and *in vivo* chitinase activities were measured (Maximova *et al.*, 2006).

Southern genomic DNA blot analysis revealed multiple transgene insertion sites in eight of the lines, and one line with a single insertion. Expression of the *TcChi1* transgene was verified by Northern blot (Maximova *et al.*, 2006) and by quantitative real-time reverse transcription polymerase chain reaction (Q-PCR) analysis. For Q-PCR total leaf RNA was extracted from control PSU-Sca6 plants, three chitinase transgenic lines (T44.1, T56.2, and T61.7), and from one GFP transgenic control line (T-31-GFP) (Maximova *et al.*, 2006). RNA (400 ng) from each sample (three individual samples per transgenic line) was treated with DNase 1 (0.2 units) (#2222, Ambion, Austin, TX) in 20 μ l total reaction volume with 60 ng of yeast tRNA (Invitrogen, Carlsbad, CA) by the manufacturer's instructions. The reactions were incubated for 30 min at 37 °C, then for 5 min at 65 °C, and immediately placed on ice. The DNase treated RNA (50 ng) was added to High Capacity cDNA (complementary DNA) Archive Kit mix (#4322171, Applied Biosystems, Foster City, CA) to a final volume of 20 μ l and the reverse transcription reaction was performed by the manufacturer's instructions.

Gene specific primers were synthesized at the Penn State Nucleic Acid Facility with a MerMade12 automated DNA synthesizer (Bioautomation, Plano TX). Gene specific fluorescent probes were synthesized by Biosearch Technologies (Novato, CA). The fluorescent label used at the 5' end of all cacao gene probes was 6-carboxyfluorescein (6-FAM) and quencher at the 3' end of all gene probes was BHQ1 (Biosearch). The total volume of the PCR reaction was 25 μ l and the mix included; 5 μ l of cDNA (~12.5 ng), 12.5 μ l 2 \times TaqMan[®] Universal Master Mix (#4304437, Applied Biosystems, Foster City, CA), 400 nmoles of each primer, and 200 nmoles of probe. The PCR reactions were run in 96 well thin-walled PCR plates in an Applied Biosystems 7300 Q-PCR system (Foster City, CA) with the following reaction conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was amplified in triplicate and the results were averaged. In addition to the *TcChi1* transgene, the expression of four endogenous pathogenesis related (PR) genes in the control and transgenic lines were also evaluated. These genes were selected based on previous results that indicated their involvement in defense responses in cacao plants induced with chemical elicitors (Verica *et al.*, 2004). The genes included: two additional PR3 chitinases genes—Class 4 and Class 7; PR4-Osmotin; PR6-Type I proteinase inhibitorlike protein; and NPR1—a potential key regulator in the signal transduction pathway that leads to systemic acquired resistance. The mean expression of two cacao house keeping control genes, *actin* and *ubiquitin*, were used to normalize the data. Amplification efficiencies of all target and reference genes were calculated from the slopes of the dilution curves for each sample ($E = 10^{(-1/\text{slope})} - 1$) (Bustin, 2004). Average efficiency for each gene was then calculated and used for efficiency data correction. The data normalization, efficiency correction, statistical randomization test, and relative transgenic/control nontransgenic expression ratios were computed using REST software (Pfaffl *et al.*, 2002). Ratios (fold difference) with *p*-values less than 0.05 were considered significant (Table 1).

The results from the Q-PCR analysis indicated a large increase in *TcChi1* expression in all lines containing the transgene (Table 1). The fold difference compared to the nontransgenic

Table 1 Quantitative real-time reverse transcription polymerase chain reaction analysis (Q-PCR) of gene expression of *TcChi1* transgene and selected pathogenesis related genes. Total RNA was isolated from control PSU-Sca6 plants, transgenic line T31-GFP (expressing *EGFP* and *hptII* only), and three different transgenic lines containing the *TcChi1* gene. Differences in gene expression between the transgenic and nontransgenic tissues are represented as relative expression ratios (fold difference). The ratios were calculated using REST software (Pfaffl *et al.*, 2002) based on Q-PCR efficiencies and the mean crossing point (CP) values of the samples from individual transgenic line versus the mean CP values for PSU-Sca6 control nontransgenic samples. Ratios with *p*-values less than 0.05 were considered significant and listed in the table. When the transgenic samples were not significantly different, they are recorded as not significant (Ns)

Gene name	T31-GFP	T44.1	T56.2	T61.7
<i>TcChi1</i>	Ns	1,555.5	5,053.6	8.8
<i>TcChi4</i>	Ns	Ns	2.2	Ns
<i>TcChi7</i>	Ns	-1.7	-1.5	-1.9
<i>TcNPR1</i>	Ns	Ns	Ns	Ns
<i>TcPR6</i>	Ns	Ns	Ns	Ns

samples varied from 8.8-fold for line T61.7 to 5053.6-fold for the highest expressing line T56.2. Line T56.2 also demonstrated a 2.2-fold increase in *TcChi4*. Interestingly, a down-regulation of between 1.5- to 1.9-fold was observed for the *TcChi7* gene in all lines overexpressing *TcChi1*. The *in vitro* endochitinase protein activity assay also detected significant increases of chitinase activity in all *TcChi1* transgenic lines compared to the control T31-GFP and nontransgenic lines (Maximova *et al.*, 2002). The levels of chitinase activity in the three transgenic lines correlated well with the expression levels of the transgene: T61 had the lowest protein activity, followed by T44 with intermediate activity and T56 with the highest activity (Maximova *et al.*, 2006). The level of chitinase activity also corresponded well with the *in vivo* antifungal activity against *Colletotrichum gloeosporioides*, a pathogen of economic importance for cacao in India and Venezuela. To test the antifungal activity *in vivo*, excised cacao leaves were challenged with spores from *C. gloeosporioides* (Maximova *et al.*, 2006). The lines with the highest mRNA levels and the highest *in vitro* chitinase activity were better able to suppress the development of the fungus and formed smaller necrotic lesions compared to the lines with lower *in vitro* chitinase activity and the control nontransgenic plants. This study

demonstrated the utility of the transformation system as a tool for gene functional analysis and supports a functional role of the cacao *TcChl* gene in pathogen resistance in cacao. While by itself, overexpression of this gene is unlikely to confer high level fungal pathogen resistance, it is possible that combined with other resistance genes via breeding, and pest management strategies, it could contribute to an integrated approach for enhanced pathogen resistance.

2.3.3 Biotechnology for CPB resistance in cocoa

Conopomorpha cramerella, the CPB is one of the most important pests of cacao. Currently CPB affects almost all cocoa producing provinces in Indonesia. By 2000, CPB had infested 60 000 ha, inflicting losses of \$40 million per year (ICCO, 2006). Losses in cocoa yield depend on the age of the infested pods and the degree of infestation. The youngest pods are most susceptible to infection, and with increase in the insect pressure it was estimated that the losses could reach 93% on the individual plantations (Lumsden, 2003). In 2006, an outbreak of CPB was also reported in Papua New Guinea where according to a US State Department report, cacao represents the second largest export product (Merrett, 2006). From January to May 2006, CPB caused a 21% decrease in cacao export of Papua New Guinea compared to 2005. Methods for CPB control include: cultural practices, application of pesticides, biocontrol, use of resistant/tolerant planting materials, and use of pheromones. The effectiveness of any of these methods is hindered due to the fact that the CPB larvae are protected inside the cocoa pod almost half of their life cycle. The selection and breeding efforts for resistance to CPB are mainly directed toward plants with improved physical characteristics of the pod. These include hardness and thickness of sclerotic layer, husk thickness and smoothness of pod surface (Lumsden, 2003; Teh *et al.*, 2006). Although a few clones have been identified as moderately resistant to CPB, in general cacao breeding is a long and slow process and the introduction of new varieties is especially difficult considering the small-holder production systems in the developing countries. Thus, engineering resistance to CPB

in transgenic plants could potentially benefit the cacao producers.

The potential candidate genes are those encoding for the production of *Bacillus thuringiensis* toxins (*Bt*). *Bt*-toxins are natural proteins with insecticidal activity against lepidopteran insects and are also considered harmless to humans and most higher organisms (Schnepf *et al.*, 1998). Different *Bt* toxins have been evaluated for their activity against CPB larvae (Santoso *et al.*, 2004). Twelve different purified CryI class *Bt* toxins were added to CPB artificial diet and fed to CPB larvae. The larvae mortality was observed every day for 3 days. Application of a single dose of 100 ng cm⁻² of four different *Bt* toxins resulted in approximately 20–50% larvae mortality.

With the goal to produce transgenic cacao including *Bt* toxin genes with potential resistance to CPB, a collaborative research project was established between the Plant Research International, Wageningen, The Netherlands and the Indonesian Biotechnology Research Institute of Estate Crops, Bogor, Indonesia (Chaidamsari, 2005). The *Bt* toxin gene selected was SN19 hybrid gene encoding a protein consisting of domains I and III of *CryI_{Ab}* and domain II of *CryII_a* (Naimov *et al.*, 2001). The sequences of domains I and III of the gene were modified by PCR site directed mutagenesis to increase its expression in plants (Chaidamsari, 2005). Binary vectors pSN48, pTC4, pTC9, and pTC12 contained different versions of the gene containing modifications of one, two, or all three domains of the gene. Vector pTC13 included a control nonmodified gene *SN19*. The constructs were first introduced in transgenic *Arabidopsis thaliana* ecotype WS as a model system to rapidly evaluate the expression and effectiveness of the *Bt* transgenes. Larvae of two insect species, *Pieris rapae* and *Plutella xylostella*, were fed with leaves from transgenic plants containing the various constructs and larvae mortality was observed. Depending on the vector, between 25% and 86% of the plants evaluated caused 100% larvae mortality for both the insect species. The largest percentage of plants causing 100% mortality was recorded for pSN48 containing synthetic domains I and III. The analysis of protein content of these plants indicated that SN48 constituted 0.01–0.1% of the total soluble protein.

Transformation vectors pMH58 and pMH59 were constructed for transformation of cacao.

Vector pMH58 contained the *SN19* gene with synthetic domains I and III encoding fragments (comparable with pSN48) and vector pMH59 contained modifications of all the three domains of *SN19*. Both genes were placed under the control of the CaMV 35S promoter. *Agrobacterium* transformations were performed with somatic embryos derived from staminodes of clone Sca6. After recovery of transgenic somatic embryos, secondary embryos were transferred to PCG medium (Li *et al.*, 1998) to allow callus formation. Calli were maintained on the same medium for 5 months and *Bt* protein levels evaluated by immunoassay. Preliminary data demonstrated that calli transformed with pMH58 showed the highest protein content/expression levels, ranging from 0% to 1.4% of total soluble protein, while pMH59 ranged from 0% to 0.2% (Chaidamsari, 2005).

Because CPB primarily affects the pods and specifically enters through the pod wall, it would be advantageous to utilize promoters to drive *Bt* expression only in the pod wall. Thus as part of this study, Chaidamsari (2005) also identified the *TcLea5* gene as primarily expressed in the inner pod wall and somewhat in the outer pod wall but not in pulp, beans, and leaves. The putative *TcLea5* promoter was characterized and the promoter activity was verified in tomato as a model species. Further production and evaluation of transgenic plants producing *Bt* toxins under tissue-specific promoters are necessary to demonstrate the success and potential application of this technology for cacao.

3. FUTURE ROAD MAP

The future of transgenic cacao is currently uncertain. The use of this system for studying gene expression and protein function has been demonstrated but with the current protocols, the efficiency and expense of this method is prohibitive to most laboratories. Heterologous model plant systems such as *Arabidopsis* and tomato may prove to be much better species for the study of cacao gene function. However, precise investigations of molecular interactions may be limited in heterologous expression systems due to subtle differences between species. Therefore in the future, advanced studies of cacao gene function will ultimately require improved and more efficient

cacao transformation methods. Higher transformation efficiencies, faster regeneration methods, and tissue-specific and regulated promoters are all required.

In addition to its utility as a method to study gene function in cacao, transformation holds a huge potential to contribute to the improvement of insect- and disease-resistant lines of cacao or for development of other desirable traits such as drought, cold, and herbicide resistance, etc. However, before commercial lines of cacao with desired traits are even considered, the societal, legal, and ethical issues involved must be carefully thought-out. By far, the largest impediment to the development of these technologies and to the application of genetic engineering for cacao improvement is the public perception of the potential ethical and biosafety issues surrounding this field. The perception of chocolate as a luxury food, and its association with children makes cacao biotechnology especially sensitive to public scrutiny. Because of this, research to develop this technology for cacao has suffered from lack of funding, and thus has lagged well behind other plant species such as corn, rice, and soybean. Furthermore, because cacao is grown in the developing countries, issues surrounding IP rights, farmer rights, and biosafety protocols in these countries are currently highly variable and not clear in many cases.

However, fact remains that cacao germplasm does not contain resistance genes for many of the opportunistic pathogens that inflict huge losses to farmers in many countries where cacao has been introduced. Integrated pest management approaches are being employed to address these problems; however, implementation of these methods requires educating millions of poor farmers who may not have the time or resources to apply them. Clearly, genetically encoded traits such as those made possible with transgenesis would be a powerful tool to help solve the problems facing cacao farmers today. The paradox between public hesitance and farmers' needs will play out in the future as cacao pathogens continue to spread from country to country and new pathogens invade the cacao fields.

In the future, native gene transformation (transformation with gene sequences originating only from the same plant species) and gene targeting methods need to be developed which

will eliminate many of the concerns regarding the precision and safety of transgenic technologies. These breakthroughs will be as important for cacao as for any other plant species. Manipulation of the key transcription factors that regulate plant growth and development as well as responses to biotic and abiotic stress will lead to novel approaches to crop improvement, but a much deeper understanding of the basic mechanisms of plant molecular biology is required before this can be adapted to cacao.

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Tea

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1. INTRODUCTION

Tea (*Camellia sinensis* L.) belongs to the family Theaceae. It is the oldest nonalcoholic caffeine-containing beverage crop of the world. The Chinese were the first to use tea as a medicinal drink, later as a beverage, and they have been doing so for the past 5000 years (Eden, 1958). The cultivated taxa of tea comprise three main natural hybrids: *C. sinensis* (L.) O. Kuntze or China type, *Camellia assamica* (Masters) or Assam type, and *C. assamica* subsp. *lasiocalyx* (Planchon ex Watt.) or Cambod or southern type. Tea is an evergreen, perennial, cross-pollinated plant and grows naturally as tall as 15 m. However, under cultivated condition, the bush height of 60–100 cm is maintained for harvesting the young leaves, which continues even more than 100 years. Flowers are white in color and borne singly or in pairs at the axils. Fruits are green in color with two to three seeds and start bearing within 5–6 years after planting.

1.1 History

Tea plants are believed to have been discovered accidentally by the Chinese legendary emperor Sheng Nong around 2737 BC. As soon as its medicinal value began to be attributed to China, a demand for supplies of tea sprang up, which resulted in cultivation of tea plant in Sichuan province about 3000 years ago. Subsequently, the

knowledge of tea cultivation spread everywhere with fine arts of Buddhism. Though, in India, wild tea plant was discovered by C.A. Bruce in Assam during 1823, seeds were brought by G.J. Gordon from China in 1836 for establishing a commercial garden. Later C.A. Bruce was appointed as the superintendent of the government tea plantation, who took active interest in the indigenous tea plants. Soon commercial interests moved on and the world's first privately owned tea company, the Assam Tea Company, was established on February 12, 1839, with the directives from the British Parliament. This was the beginning of the present-day tea industry in India.

1.2 Origin and Distribution

Southeast Asia is the original home for tea. According to Wight (1962), primary center of origin of tea is considered around the point of intersection of latitude 29° N and longitude 98° E near the source of the river “Irrawaddy”; the point of confluence where lands of Assam, north Burma, southwest China, and Tibet meet. The secondary center of origin is considered to be located in southeast China, Indochina, Mizoram, and Meghalaya (Kingdon-Ward, 1950). The above areas are, therefore, considered to be the zone of origin and dispersion of the genus *Camellia* as a whole (Sealy, 1958). However, presently tea is grown within the latitudinal range of 45° N to 34° S.

Tea was introduced into Japan from China in the early part of the 8th century. From Japan, tea cultivation extended to Indonesia during the 17th century. In Sri Lanka, tea was first planted in 1839 when seeds were brought from Calcutta. In USSR, tea cultivation started when seeds were imported from China toward the end of the last century. Later, from USSR, seeds were exported to Turkey in the year 1939–1940. In Europe, tea was introduced during 1740 by the East India Company's Captain Goff, but the plants that were planted in the Royal Botanical Garden at Kew in England could not survive (Sealy, 1958) and the first successful introduction was achieved by a British merchant and naturalist John Ellis in 1768 (Aiton, 1789; Booth, 1830). From there tea cultivation spread to the African countries at the end of the 19th century. Presently, it is cultivated in more than 52 countries worldwide.

1.3 Botanical Description

A summary of morphological characters of cultivated tea plants is given below (Barua, 1963).

1.3.1 *C. sinensis*

The plant is a 1–3-m-tall shrub with many stems arising from the base of the plant near the ground. The relatively small, thick, and leathery leaves have stomata that appear to be sunken in the lamina. Short and stout petioles give the leaf an erect pose and are usually three to seven in number. The flowers that are borne singly or in pairs in the axils and have 6–10-mm-long pedicels with two to three subopposite scar. Flowers are characterized by seven to eight cup-shaped, 1.5–2.0 cm long, and broad-oval to suborbicular petals with about three to five styles that are generally free for greater part of their length but occasionally free up to the base of the ovary. The capsules have one, two, or three locules containing one to three nearly spherical seeds of about 10–15 mm in diameter.

1.3.2 *C. assamica* (Masters)

It is a 10–15-m-high tree with a trunk and robust branching system. The relatively large, thin,

glossy leaves with more or less acuminate apex have distinct marginal veins and broadly elliptic leaf blades that are usually 8–20 cm long and 3.5–7.5 cm wide. A single or paired pedicellate flower has smooth and green scars of three bracteoles, numerous stamens, five to six persistent sepals, and seven to eight white petals.

1.3.3 *C. assamica* subsp. *lasiocalyx* (Planch. ex Watt.)

The plant is 6–10-m-tall, upright tree with several, almost equally developed, branches and more or less erect, glossy, light green to coppery-yellow or pinkish red leaves, the size of which is intermediate between *C. sinensis* and *C. assamica*. Although flowers are more or less similar to the Assam type yet, they have four or more bracteoles, three to four ovules with five locules, and three to five styles that are free up to half the length.

1.4 Taxonomic Position

In 1689, a Dutch physician and botanist Andre Cley published in Latin a work mentioning the tea plant as “tsumacky”, which means “tsubaki” in Japanese. Later, in 1702 James Petiver and in 1712 Engelbert Kaempfer described the same name. In 1753, Linnaeus used Kaempfer's illustrations to describe the China plant under the name *Camellia tsubaki* and subsequently it appeared in his book *Species Plantarum Volume 1* as *Thea sinensis*. The modern generic name *Camellia* was dedicated to Moravian Jesuit Kamel, a German missionary stationed in the Philippines, who wrote about the plant, found in Asia during the later half of the 17th century. Moreover, tea was known under various botanical names, such as *Camellia thea* Dyer, and also *T. sinensis*, *Thea Bohea*, or *Thea viridis* (Eden, 1958).

Thus in 1935, in order to avoid confusion, the International Botanical Congress held at Amsterdam decided to merge the two genera *Thea* and *Camellia* into a single genus, i.e., *Camellia*. The committee decided *C. sinensis* (L.) to be the correct name of the tea plant. Technically, *C. sinensis* (L.) O. Kuntze is considered to be the full name of tea plant. The family Theaceae, tribe Gordonieae, comprises 16–25 genera with more

than 325 species. The genus *Camellia* of which tea is a member belongs to the family Theaceae, tribe Gordoneae, along with eight other genera, among which *Camellia* is the largest. Sealy (1958) listed 82 species within the genus *Camellia* and 16 other imperfectly known species whose status was not decided.

1.5 Genome Size and Diversity

The genome size in terms of 4C DNA amount for *C. sinensis* is 15.61 ± 1.06 where 1C DNA amount in terms of mega base pair (Mbp) is 3824 (Hanson *et al.*, 2001) and 1 pg (picogram) = 980 Mbp (Cavalier-Smith, 1985). Generally, chromosomes are small in size and tend to clump together due to “stickiness”. Tea is diploid ($2n = 30$; basic chromosome number, $x = 15$) and karyotype of chromosome ranges from 1.28 to $3.44 \mu\text{m}$ (Bezbaruah, 1971). The r value (ratio of long arm to short arm) for all the 15 pairs of chromosomes range from 1.0 to 1.91. This consistency in diploid chromosome number suggests a monophyletic origin of all *Camellia* species. However, few higher ploidy levels, such as triploids, e.g., Tocklai Vegetative (TV)-29, HS-10 A, United Planter Association of South India (UPASI)-3, UPASI-20 ($2n = 45$), tetraploids ($2n = 60$), pentaploids ($2n = 75$), and aneuploids ($2n \pm 1$ to 29) have also been identified among the natural tea populace (Singh, 1980).

Owing to extensive internal hybridization between different *Camellia* taxa, several intergrades, introgressants, and putative hybrids have been formed. These can be arranged in a cline based on morphological characters that extend from China types through intermediates to those of Assam types. Indeed, because of the extreme homogenization, the existence of the archetypes of tea is doubtful (Visser, 1969). To date, the numerous hybrids currently available are still referred to as China, Assam, or Cambod tea depending on their morphological proximity to the main taxa (Banerjee, 1992). Tea breeds well with wild relatives and thus tea taxonomists have always been interested in identifying such hybrids due to their suspected involvement in the genetic pool of tea. Two particularly interesting taxa are *Camellia irrawadiensis* and *Camellia taliensis* whose morphological distribution overlaps with

that of tea (Banerjee, 1992). It has also been postulated that some desirable traits, such as anthocyanin pigmentation or special quality characters of Darjeeling tea, might have been introduced to tea gene pool from wild species (Wood and Barua, 1958). Other *Camellia* species that are suspected to have contributed to the tea genetic pool by hybridization include *Camellia flava* (Pifard) Sealy, *Camellia petelotii* (Merrill) Sealy (Wight, 1962), and possibly *Camellia lutescens* Dyer (Sharma and Venkataramani, 1974). The role of *C. taliensis* is, however, not clear because the species itself is considered to be a hybrid between *C. sinensis* and *C. irrawadiensis* (Wood and Barua, 1958; Visser, 1969). Therefore, it is generally agreed that at least three taxa, i.e., *C. assamica*, *C. sinensis*, *C. assamica* subsp. *Lasiocalyx*, and to an extent *C. irrawadiensis*, have mainly contributed to the genetic pool of tea. The term “tea” should, therefore, cover progenies of these taxa and the hybrids thereof or between them.

1.6 Economic Importance and Health Benefits

The economic importance of the genus *Camellia* is primarily due to tea. Apart from being used as a beverage, leaves of tea are also used as vegetables like the “leppet tea” in Burma and “meing tea” in Thailand. While *Camellia oleracea* produces oil, used in the cosmetic industry, *Camellia sasanqua*, *Camellia Japonica*, and *Camellia reticulata* have great importance due to their ornamental values. Tea seeds yield about 17.3% of oil as compared to that of *C. sasanqua* (58%) and *C. japonica* (66%). The oil is of nondrying class and resembles the oil of *C. sasanqua* by its properties and characteristics. Although the oil is used as a lubricant, extraction of oil from tea seed is not economically viable (Wealth of India, 1950). Moreover, tea seed cakes also contain saponins. Although it has a poor value as a fertilizer and is unfit for animal feed on account of its low nitrogen, phosphorus, and potassium contents, yet at present it is being successfully utilized in the manufacture of a nematocide (Wealth of India, 1950).

Though tea is mainly consumed in the form of “fermented tea” or “black tea”, yet “nonfermented” or “green tea” and lesser known

“semifermented” or “oolong tea” are also available. These types vary in their method of manufacture, chemical constituent, appearance, and organoleptic taste. While black tea is widely used in India and other European countries, green tea is popular in China, Japan, and Taiwan. Oolong tea is mainly consumed in some parts of China as well as Taiwan. Worldwide 80% black tea, 18% green tea, and 2% oolong tea are being produced.

For black tea, the young tender leaves are completely fermented after withering. The fermentation results in oxidation and polymerization of polyphenols, changing the nature of the chemical constituents of tea leaves and forming theaflavin and thearubigin. These polyphenols are responsible for the briskness, strength, color, taste, aroma, and pungency associated with black tea. The infusion of black tea has a bright red or copper color, astringent taste and characteristic aroma. On the other hand, green tea is unfermented and is the least processed among the three types. The plucked leaves are harvested and steamed immediately to inactivate the enzymes to prevent oxidation and polymerization of primary polyphenols that result in retaining of green color in the finished product. Green tea infusion has a leafy taste and a smell of fresh vegetables, and low caffeine content. In oolong tea, the primary polyphenols are allowed to partly oxidize. Oolong tea is not common and is intermediate in characteristic between green and black tea. Immediately after plucking, the tea leaves are partially fermented for about half the time of black tea. It has the color of black tea and flavor of green tea.

Tea was used initially as medicine, later as beverage, and has a proven future potential of becoming an important industrial and pharmaceutical raw material. Scientific reports in the last two decades have validated many beneficial claims for tea. The majority of the beneficial effects have been attributed to the polyphenolic constituents. Several studies suggest that phenolics may be of importance in reducing the incidence of degenerative diseases, such as cancer and arteriosclerosis. The most relevant compounds in dietary regime are cinnamic acid derivatives and flavonoids. As natural polyphenols remain unchanged in green tea, it can be said that green tea is more beneficial than black tea. The strong antioxidant properties that sequester metal

ions and scavenge oxygen species as well as free radicals (Wiseman *et al.*, 1977) are responsible for beneficial effects of tea.

Tea catechins have been found to be better antioxidants than vitamin C, E, and carotene. The polyphenols block free radicals' damage to lipids (found in cell membranes and serum lipids), nucleic acids, and proteins (like those found as cellular enzymes and structural proteins). Damage to these cell components can lead to tumor formation.

Tea polyphenols and flavonoids have been reported to inhibit either enzymatic or nonenzymatic lipid peroxidation, an oxidative process implicated in several pathological conditions including atherosclerosis (Chen *et al.*, 2000). Besides, polyphenols have anticancer activity (Vasisht *et al.*, 2003), antidiabetic effect (Gomes *et al.*, 1995), antiarthritic activity (Tapiero *et al.*, 2002), antiplaque activity (Yu *et al.*, 1995), antiviral activity (Okubo and Juneja, 1997), anti-AIDS activity (Hashimoto *et al.*, 1996), anorectic effect (Kwanashie *et al.*, 2001), antimicrobial activity (Hamilton-Miller, 1995), and numerous other activities such as neuromuscular, antiangiogenic, antihepatotoxic, antiproliferative/apoptotic, and immunomodulatory effects (Sueoka *et al.*, 2001).

1.7 Traditional Breeding

The plant improvement program of tea can be classified into introduction, selection, and hybridization, which resulted in the development of more than 600 cultivated varieties with better yield, quality, and traits, such as resistance to drought, diseases, etc., by different tea research institutes and dedicated planters across the world (Mondal *et al.*, 2004).

1.7.1 Introduction

Barring China and India, most of the tea-growing countries initially undertook the massive introduction programs either to popularize the cultivation or to widen the germplasm base for developing improved cultivars befitting the need of that region, which are discussed below.

Tea propagule, such as seed, was primarily introduced from China to different countries including India, Japan, or Korea, etc. either by the

Buddhist pilgrimage or by colonial soldiers during the 8th century to be used as medicinal beverage.

Secondary introduction always happened after developing the improved varieties from the initial planting materials established through the primary introduction by the commercial tea growers with their personal efforts, and information regarding this is scant as stringent quarantine rules prevailed across the world for carrying the plant propagule. Interestingly, the early records indicate that around 60% world's acreage has received its initial planting material directly or indirectly from tea genetic resources of Tocklai Experimental Station of India. Initially, the commercial tea estate was established and later that was used as breeding stock for developing the new cultivar.

1.7.2 Selection

Selection is the most popular, age-old practice in tea breeding. Since commercial tea gardens were established earlier with seeds, a great variability exists among them. Many instances of elite plants have been identified among the existing bushes, multiplied vegetatively and released as clones. Majority of the tea clones have been developed through selection. TV-1 is a good example of a popular clone that was developed for north India. However, pedigrees of the clone remain unknown.

1.7.3 Hybridization

Hybridization in tea can be either natural or by hand pollination. In natural hybridization, based on known characters, such as previous performance of yield, quality or diseases resistance, two parents are planted side by side in an isolated place and allowed to bear fruits. Subsequently, the seeds (F_1) are harvested, raised, and planted. If average performances of these plants are found to be better than either parent, then seeds are released as hybrid seed or biconal seed. However, some of the outstanding performers among these progenies are marked and verified for multilocation trial and if still found suitable released as clones. These clones are geographically specific and most of the tea research institutes of the world have generated the clone for their own regions. Sometimes in the above process more than two parents are used,

known as polyclonal seeds. The idea is to introduce more variability among the F_1 seeds. Since it is difficult to know about the pedigree of the cultivar (as pollen may come from any male), the chance of reproducibility is low, and the process is thus least preferred presently.

Artificial or hand pollination or controlled crossing, despite being important approach, has met a limited success in tea breeding. The reasons identified could be the following: (i) low success rate; (ii) availability of tea flower for a short period (2–3 months); (iii) long time taken for seed maturation (12–18 months); and (iv) differences in flowering time of different clones. A few clones have been released in Kenya and Malawi recently using this technique.

1.8 Breeding Objectives

Tea breeding programs vary from country to country depending upon the local needs. The various objectives in different regions along with the probable genes are furnished in Table 1. However, by and large they are focused on improving quality and yield.

However, in general, the breeding work at black tea producing countries such as India, Kenya, and Sri Lanka is biased toward the development of clones with high yield and better quality whereas the green tea producing countries near equator such as Japan, China, etc. are focused on the development of cold-resistant and frost-resistant clones.

1.9 Rationale of Development of Transgenic Tea

Production of desired plant types of human choice through conventional breeding is an age-old practice in agriculture. Although, conventional tea breeding is well established and contributed much for tea improvement over the past several decades, the process is slow due to some bottlenecks. Specifically in tea, they are as follows: (1) perennial nature, (2) long gestation period, (3) high inbreeding depression, (4) self-incompatibility, (5) unavailability of distinct mutants to withstand different biotic and abiotic stresses, (6) lack of distinct selection criteria, (7) low success rate of hand pollination, (8) short flowering time, i.e.,

Table 1 Breeding objectives in tea

Objectives	Importance	Region	Transgenic solution
Improving quality	Directly linked to the profitability	Black tea producing countries such as India, East Africa, Sri Lanka, Bangladesh, and Indonesia	Chalcone synthetase, phenyl ammonia lyase, etc.
Increasing yield	Horizontal increase of production by extension planting is limited worldwide	Worldwide	<i>rolb</i> gene
Drought tolerance	Reduces productivity and occurs in all tea-growing regions in the world	Worldwide where tea grows as a rain-fed crop	Absciscic acid induced (<i>abi3</i>) gene
Reduce winter dormancy	No leaf production during winter months and occurs in northeast India, Japan, China, etc.	Tea plantation in near equator	Superoxide dismutase (Cu/Fe/Zn <i>sod</i>) gene
Hail resistance	Causes economic loss as young leaves during rainy season are mostly affected; generally occurs in the hilly region	Hilly region of the tea producing countries	Genes need to be identified
Water-logging tolerance	Reduce productivity during rainy season; generally occurs in northeastern India	Northeast India	Ascorbate peroxidase (<i>apx3</i>) gene
Cold hardiness	Reduced productivity during winter due to snow; generally occurs in Japan and Russia	Mainly Japan, Russia, and China	Glycerol 3-phosphate acyltransferase (<i>gpat</i>) gene
Resistance to diseases such as blister blight, stem canker, etc.	Blister blight causes severe damage as only young leaves are infected; generally occurs in Japan, Sri Lanka, south India, and Darjeeling hills of northeast India	Mainly India, Sri Lanka, Indonesia, and Japan	Chitinase, ribosome inactivating proteins, etc.
Resistance to pests such as red spider mite, tea mosquito bug, leaf sucking pest, etc.	Most important biotic stresses as all cause severe damage to the leaves; generally occur in all the tea-growing regions in the world	Worldwide	<i>Bt</i> , plant lectins, and proteinase inhibitor gene
Suitability to type of manufacturing	For matching the customer's demand as well as better recovery percentage in made tea	Black tea producing countries such as India, East Africa, Sri Lanka, Bangladesh, and Indonesia	Genes need to be identified
Low input responsive clone	Required for organic tea farming	Organic tea	Genes need to be identified

2–3 months, (9) long duration for seed maturation, i.e., 12–18 months, and (10) clonal difference of flowering time and fruit-bearing capability in some clones. Thus transgenic technology is a better alternative as it has several advantages; important among them are as follows: (1) any origin of gene can be transferred; (2) greater speed; (3) expression of transgenic being free from epistatic interaction. Due to the nonavailability of new land for expansion of cultivation along with the objectives of reducing the cost of cultivation, tea breeders need to develop the improved tea cultivars as per the requirement of the industry. Therefore, gene transfer through biotechnological

means appears to be not only a time effective but also an advantageous better alternative.

2. DEVELOPMENT OF TRANSGENIC TEA

2.1 *In Vitro* Culture

Like any other woody plants, *in vitro* culture of tea focuses mainly on micropropagation and somatic embryogenesis both of which are utilized to develop transgenic plants. However, central to any successful transgenic technology is an efficient *in vitro* regeneration protocol. While regeneration

method is essential for the introduction of foreign genes into plant tissues, micropropagation is important for the transfer of a large number of genetically modified (GM) plants to the field within a short span of time.

The micropropagation works in tea have been reviewed from time to time (Vieitez *et al.*, 1992; Dood, 1994; Das, 2001; Mondal, 2002; Mondal *et al.*, 2004). It is clearly evident that the development of micropropagation of tea passed through three different phases. Till 1980s, the emphasis was to standardize the parameters of *in vitro* protocol, such as suitable explant, overcoming the microbial contamination, and optimization of media composition along with growth regulation for better proliferation. It is now widely adopted that nodal segments (0.5–1.0 cm) cultured on a Murashige and Skoog (MS) medium with 6-benzylaminopurine (BAP) ($1\text{--}6\text{ mg l}^{-1}$) is the best for multiplication of shoots along with either a high-dose (500 mg l^{-1}) pulse treatment of auxin, such as indole-3-butyric acid (IBA), or a low dose ($1\text{--}2\text{ mg l}^{-1}$) for long duration is suitable for *in vitro* rooting. Later, till 1990s, efforts were made toward the hardening of microshoots to achieve a higher percentage of survival. Accordingly, several nonconventional approaches, such as CO₂-enriched hardening chamber, biological hardening, and micrografting were developed. Presently, attention is paid to the evaluation of field performance of the micropropagated plants.

One of the prerequisites for genetic transformation of tea is to develop an efficient regeneration system from a single cell to complete plant. Till today, somatic embryogenesis is considered to be the most efficient regeneration system for tea. Several reviews have been documented in the recent past (Akula and Akula, 1999; Mondal *et al.*, 2004) that indicate that though Wu *et al.* (1981) initiated the work of somatic embryogenesis in tea, the technology has been applied for several purposes today, such as clonal propagation (Mondal *et al.*, 2001a), genetic transformation (Mondal *et al.*, 2001b), artificial seed production (Mondal *et al.*, 2000), some interspecific crosses of *Camellia* (Nadamitsu *et al.*, 1986), and androgenic or haploid plant production of tea (Chen and Liao, 1982). Somatic embryogenesis of tea depends upon several factors, such as explants' type (Akula and Akula, 1999), time of culture (Mondal *et al.*, 2000), and genotypes (Kato, 1996).

Type, concentration and time of application of different plant growth regulators (PGRs) have also been extensively worked out. In general, a high cytokinin to low auxin ratio or low cytokinin alone was found to be necessary for the induction of somatic embryos in tea; even reduction or omission of cytokinin in subsequent subculturing is also known. Generally, 6-benzyl adenine ($0\text{--}10\text{ mg l}^{-1}$) has been widely used for tea and related species (Zhuang and Liang, 1985) followed by kinetin ($0.05\text{--}10\text{ mg l}^{-1}$) (Wachira and Ogado, 1995). Among the different auxins, IBA ($0\text{--}2\text{ mg l}^{-1}$) was used mostly for somatic embryo induction in tea. However, different concentrations of α -naphthalene acetic acid (NAA) (Balasubramanian *et al.*, 2000), 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid (Sood *et al.*, 1993) and α -naphthoxyacetic acid, tetraphenylboron, and phenylboronic acid (Ponsamuel *et al.*, 1996) were also used.

Apart from growth regulators, some other factors, such as nitrate salts of potassium and ammonium, together with sulfate salts of aluminum, potassium, magnesium, and ammonium, sucrose concentration as well as maltose and transcinamic acid (Mondal *et al.*, 2002), brassin, a synthetic analog of a naturally occurring brassinoid (Ponsamuel *et al.*, 1996), also have a positive role for tea embryogenesis.

Hitherto, emphasis has been given to manipulate the nutrient composition, growth regulators in the culture medium, physical conditions of incubation, and other stress treatments to induce somatic embryos. However, induction of *in vivo* embryogenesis of tea could be achieved without using the conventional tissue culture media (Mondal *et al.*, 2001c). Though reason for this observation is not clear presently, yet seeds of *Camellia* appear to have a considerable inherent capacity for embryogenesis. Thus, at the right physiological stage, with appropriate levels of internal hormone and appropriate moisture profile of the substrate under sterile conditions, the tea seeds are able to produce embryos without any exogenous nutrient.

2.1.1 Hardening

Information on hardening of tea somatic embryo-derived plants is scant in the literature. Although Wu *et al.* (1981) were the first to transfer the

plantlets to soil, the composition of soil mixture and other conditions used by them were not mentioned clearly. Later, Kato (1996) grew healthy tea somatic seedlings under natural conditions by transferring them into a mixture of vermiculite and soil (1:1) and they were covered under the plastic. On the other hand, Jha *et al.* (1992) hardened tea plantlets for 8 weeks in quarter-strength MS salts before transplanting to pots containing a mixture of peat and soil (1:1) and achieved an acclimatization rate of 70%. Wachira and Ogado (1995) reported that multiple shoots differentiated from the germinated embryos were successfully rooted in mixture of sand:peat (3:1) in small pots. In order to improve rooting, Ponsamuel *et al.* (1996) treated the plantlets with 1 mM indole-3-acetonitrile, 1 mM brassin, and 10 mM phloroglucinol in a liquid MS medium for 15 days, and after profuse root proliferation, the plantlets were acclimatized in pots containing vermiculite. Eventually, the plants were transplanted in the greenhouse.

Akula and Akula (1999) transferred the small plantlets with a strong tap root and four to six leaves into small pots filled with a presterilized potting mixture (sand:peat:vermiculite—1:2:1) and kept in a greenhouse with misting facility, at 80–95% humidity under low light. The new leaves were observed within 5–6 weeks when they were taken into bigger pots. Following this procedure, they achieved a 90–95% survival rate and more than 200 plantlets were transferred to the field in Indonesia. Tea plantlets with young leaves and stout roots, with a height of 4–5 cm, were taken from culture room to Hikko-trays containing presterilized sand and cow dung (1:1). These Hikko-trays were then kept in a polytunnel with intermittent watering for 60 days inside an indigenously developed polyhouse (90% survival rate). Later, they were transferred to polythene sleeves filled with black virgin soil and kept for further 1 year in the same polyhouse (Mondal *et al.*, 2001a). Following these techniques, they could produce 3000 somatic seedlings at the Research and Development Department of Tata Tea Ltd, India, which have been transferred to the field.

Perhaps the most critical phase of *in vitro* culture is the establishment of plantlets to the greenhouse. In tea, a commendable progress has been made that deserves a special merit to elaborate.

Conventionally, tea microshoots are hardened for 6 months on soil mixture containing various additives such as cow dung, soil rite, etc. and then kept inside the indigenously developed polytunnel with various shapes and sizes. Generally, the standard procedure is to transfer the rooted plantlets of tea to potting mixtures containing peat and soil (1:1) under high humidity created by using misting or fogging units (Banerjee and Agarwal, 1990; Agarwal *et al.*, 1992), but use of vermiculite:soil (1:1) has also worked very well (Kato, 1985). Detailed studies with various parameters, such as time of harvesting of microshoots, shoot size, soil pH, PGRs, CO₂ enrichment, and light condition, were carried out by Sharma *et al.* (1999). Among the different parameters, soil pH coupled with CO₂ enrichment is the most critical factor for hardening to achieve a high percentage of survival rate.

Importantly, to overcome the mortality due to microbial contamination at the hardening stage, biological hardening has been developed by Pandey *et al.* (2000) who found that when microbial inoculants of two bacteria, *Bacillus subtilis* and *Pseudomonas corrugate*, are used during hardening of micropropagated shoots, the survival rate increases significantly in all the three seasons: rainy, winter, and summer.

Micrografting is a new alternative strategy for hardening the micropropagated shoots of woody plants. In this strategy, *in vitro* raised scions are directly transplanted either on *in vitro* raised rootstocks under sterile conditions or *in vivo* raised stocks. In tea, this technique has been standardized (Prakash *et al.*, 1999; Mondal *et al.*, 2005), which indicated not only a survival rate more than 95% but also shortening of the hardening time by 1 year due to a faster growth rate of micrografted shoots. This has immense potential in tea where breeding work suffers due to a long gestation period.

2.1.2 Field performance

The ultimate success of any *in vitro* protocol depends upon the performance of the plants in the fields as compared to their vegetative counterparts. The Research and Development Department of Tata Tea Ltd, India, is the pioneer, where more than 45 000 plants of eight different cultivars

of tea were transferred to the field, and leaves from those plants are being harvested regularly to manufacture black tea for last several years.

From a study on 1.7-, 4-, and 8-year-old field-grown micropropagated and vegetatively propagated tea plants in the laboratory of the above department and elsewhere in India, it was observed that overall yield and quality were comparable. Although the different physiological parameters such as photosynthetic rate, chlorophyll content, etc. remained the same, two different morphological variations were noticed. The number of lateral shoots produced after “centering” were significantly more in micropropagated plants as compared to the vegetatively propagated plants. This is perhaps due to the effects of the various growth regulators that the tissue culture raised plants experienced under *in vitro* conditions. The root volumes of tissue culture plants were also higher than those of the vegetatively propagated plants. The reason might be that the micropropagated shoots were treated with IBA to induce rooting, which could be responsible for better root development in the field. Therefore, it can be concluded that the micropropagation protocol should be used only

when there is a need to produce high number of propagules from a limited source.

2.2 Genetic Transformation

The development of transgenics in tea is furnished in Table 2 and explained below.

2.2.1 *Agrobacterium tumefaciens*

The first protocol for production of transgenic tea, *C. sinensis* (L.) O. Kuntze cv. Kangra jat, was developed via *Agrobacterium*-mediated genetic transformation (Figure 1; Mondal *et al.*, 2001b). Two disarmed *A. tumefaciens* strains, EHA 105 and LBA 4404, both carrying the binary plasmid p35SGUS-INT with the *nptII* (neomycin phosphotransferase II) gene and *gus* (β -glucuronidase) intron were evaluated as vector systems. Several parameters were evaluated to maximize the transformation efficiency. While preculture, wounding, and acetosyringone treatment in different concentrations were inhibitory, the bacterial growth phase (optical density: OD₆₀₀ = 0.6), cell

Table 2 A chronological summary of transgenic tea research

Serial number	Techniques used	Remarks	References
1	<i>Agrobacterium tumefaciens</i>	Antibiotic selection for <i>Camellia</i> species was reported	Tosca <i>et al.</i> , 1996
2	<i>Agrobacterium rhizogenes</i>	First attempt of <i>A. rhizogenes</i> transformation	Zehra <i>et al.</i> , 1996
3	<i>Agrobacterium rhizogenes</i>	Transformation technology was exploited to induce the hairy root for hardening the micropropagated tea plants	Konwar <i>et al.</i> , 1998
4	<i>Agrobacterium tumefaciens</i>	Preliminary study for gene transfer to tea plants	Matsumoto and Fukui, 1998
5	<i>Agrobacterium tumefaciens</i>	Standardization of somatic embryogenesis and transient expression of <i>gus</i> gene	Mondal <i>et al.</i> , 1999
6	Particle bombardment	First attempts for standardization of the biolistic-mediated transformation protocol	Akula and Akula, 1999
7	<i>Agrobacterium tumefaciens</i>	Transgenic calli were produced; 100–500 μ M acetosyringone was effective	Matsumoto and Fukui, 1999
8	<i>Agrobacterium tumefaciens</i>	Detailed study on <i>Bt</i> gene transformation was reported	Luo and Liang, 2000
9	<i>Agrobacterium tumefaciens</i>	Development of selection system for tea plant	Mondal <i>et al.</i> , 2001d
10	<i>Agrobacterium tumefaciens</i>	Production of transgenic plants	Mondal <i>et al.</i> , 2001b
11	<i>Agrobacterium tumefaciens</i>	Tea leaves with glabrous surface having lower phenol and wax content are more suitable for infection	Kumar <i>et al.</i> , 2004
12	<i>Agrobacterium tumefaciens</i> and particle bombardment	Green florescence protein gene was transferred with organelle target signals	Kato <i>et al.</i> , 2004
13	<i>Agrobacterium tumefaciens</i> and particle bombardment	Attempt was made for standardization of the protocol	Wu <i>et al.</i> , 2003

Surface disinfection of mature sinker seeds

Germination on half MS + 30 g l⁻¹ sucrose + 0.7%

2 weeks

Induction of somatic embryos from cotyledon slices on half MS + 20 g l⁻¹ sucrose + 2.5 g l⁻¹ NAA + 0.2 g l⁻¹ BAP

6–8 weeks

Multiplication of primary embryos on embryo multiplication medium (EMM) MS + 2 g l⁻¹ BA, 0.2 g l⁻¹ IBA, 1.0 g l⁻¹ L-glutamine

6 weeks

Infection of globular embryos with *Agrobacterium* suspension (OD₆₀₀ = 0.6 × 10⁹ cells/ml)

20 min

Co-cultivation in EMM medium (pH 5.6) in darkness

5 days

Washing two times with sterile water and two times with liquid EMM containing 400 mg l⁻¹ sporidex and blot drying

Transfer of embryos to EMM + sporidex (250 mg l⁻¹) + carbenicillin (250 mg l⁻¹)

2 weeks

Selection on above medium + kanamycin (50 mg l⁻¹)

8–12 weeks

Transfer to MS + 40 g l⁻¹ maltose + 3.0 g l⁻¹ trans-cinamic acid + kanamycin (75 mg l⁻¹)

4 weeks

Transfer to MS + 1.5 mg l⁻¹ GA3 + kanamycin (75 mg l⁻¹)

8 weeks

Multiplication of transgenic shoots on MS + 1 mg l⁻¹ TDZ + 2 mg l⁻¹ NAA

8 weeks

Micrografting on seedling rootstocks and growth in greenhouse

Figure 1 Protocol for *Agrobacterium*-mediated genetic transformation

density (10⁹ ml⁻¹), co-cultivation period (5 days), and pH of the co-cultivation medium (5.6) had positive effects on transformation. Following co-cultivation, globular somatic embryos were placed on a multiplication medium and stressed with kanamycin (50 mg l⁻¹). Further selection occurred in the maturation and germination medium at an elevated kanamycin level (75 mg l⁻¹). An average of 40% transient expression was registered based on the GUS histological assay (Figure 2). Then the kanamycin-resistant, GUS-positive embryos were germinated and the resulting microshoots were

multiplied *in vitro* on a MS medium fortified with 5 µM thidiazuron and 10 µM NAA. Later, they were micrografted onto seed-grown rootstocks (Mondal *et al.*, 2005) of cv. Kangra jat and eventually hardened in a walk-in polyhouse. Integration of the transgenes into the tea nuclear genome was confirmed by polymerase chain reaction (PCR) analysis using *nptII*- and *gus*-specific primers and by southern hybridization using an *nptII*-specific probe.

However, use of phenolic inducer, acetosyringone, did not enhance the efficiency of

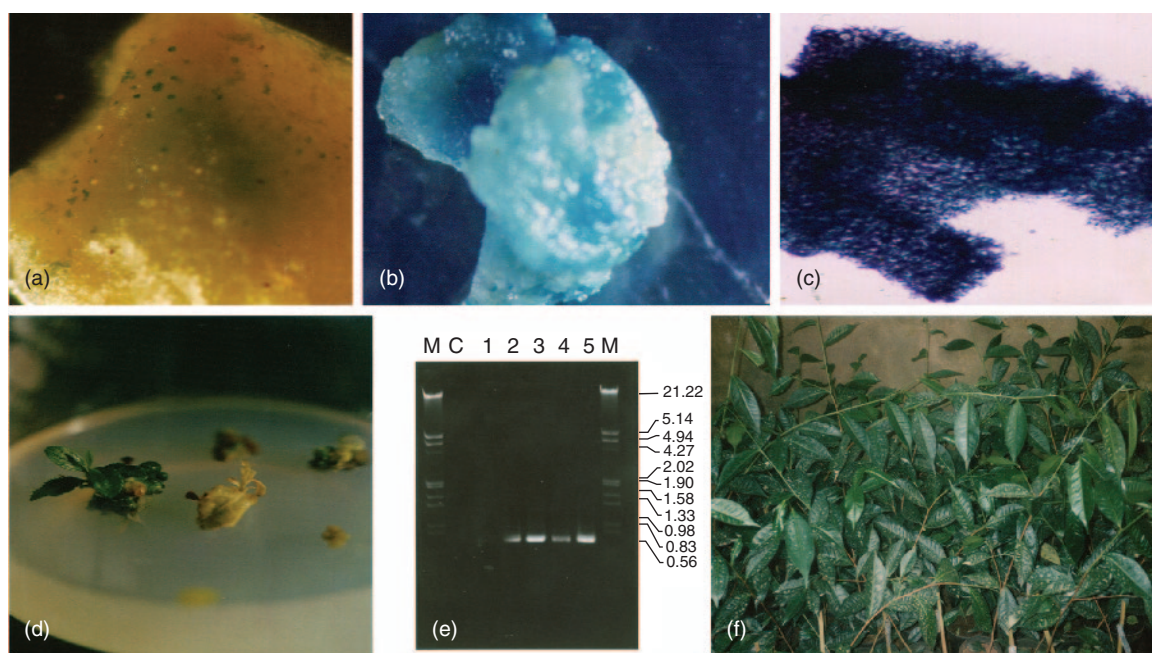


Figure 2 *Agrobacterium*-mediated genetic transformation of tea somatic embryos: (a) blue spot indicating gus activity after 48 h of infection; (b) a gus-positive secondary somatic embryo of 4 months old; (c) a hand section of leaf tissue of transformed shoot of 12 months old; (d) kanamycin selection; (e) PCR amplification of a 693 bp fragment of *nptII* gene (lane M: DNA double digested with *Eco*R1 and *Hind*III; lane C: DNA from untransformed tea plant (control); lanes 1–5: DNA from independently transformed plants; (f) the greenhouse-grown transgenic tea plants (Mondal *et al.*, 2002)

transformation (Mondal *et al.*, 2005). In several other experiments on woody plants, acetosyringone did not help in increasing transformation efficiency (Confalonieri *et al.*, 1997). The inability of acetosyringone to improve the transformation efficiency could be due to the inherent prevalence of high amounts of phenolics in tea. Contrary to this, Matsumoto and Fukui (1999) found that acetosyringone has a positive role in tea transformation as this plant is considered to be one of the recalcitrant plants for *Agrobacterium* infection. Naturally occurring crown galls are hardly observed on tea plant and this bacterium is not cited as an economically important pathogen in Japan. Therefore, they concluded that the low efficiency of the *Agrobacterium* infection was overcome by acetosyringone. Resistant calli emerged only on the explants treated with a higher concentration of acetosyringone. Thus, they concluded that application of 100 μ M acetosyringone to a co-culture medium was effective for tea transformation.

Another important factor that affects transformation efficiency is the selection of transgenic tissue on an antibiotic medium. In order to select transformed explants, selective agents are added into the medium to produce selective pressure. In general for tea, among the different antibiotics, hygromycin was more effectively used at a low concentration (20 mg l^{-1}) for somatic embryos followed by kanamycin with a range from 50 to 200 mg l^{-1} . In a wild species of tea, Tosca *et al.* (1996) found that 75 mg l^{-1} kanamycin was lethal to the tissues. Similarly, Mondal *et al.* (2001b) reported that 50 mg l^{-1} kanamycin followed by an elevation of kanamycin to 75 mg l^{-1} were the levels to be used for the effective selection of tea somatic embryos. On the other hand, Matsumoto and Fukui (1998, 1999) found that 200 mg l^{-1} kanamycin was effective for tea when leaves were used as explants.

Another prerequisite for genetic transformation is the molecular characterization of putative transformants. While Matsumoto and Fukui

(1998, 1999) have reported stable transformations in callus on the basis of PCR analysis and southern hybridization, it were Mondal *et al.* (2001b) who reported on stable transformed transgenic tea plants after molecular characterization. They found that 5 out of 12 independent kanamycin-resistant and GUS-positive lines yielded positive PCR amplification. This indicated the presence of the marker kanamycin (*nptII*) linked to the *gus* gene as a single “transfer-DNA (T-DNA) strand” in the genomic DNA of the transformed plant. The failure of the rest of the lines in yielding PCR-amplification products may be attributed to the presence of “false positives” during the antibiotics selection. Further, when the leaves of these five independent transgenic plants were subjected to Southern blot analysis, *Pst*I digests of genomic DNA from each of four putative transgenic lines generated an internal transgene fragment of 1.6 kb that hybridized to the *nptII* probe. Additional shorter fragments produced in some transgenic lines further indicated a deletion of a part of the T-DNA containing *nptII*. The deletion might perhaps occur during transformation or regeneration. Different banding patterns observed in the southern hybridization could be due to multiple insertion, rearrangement, and/or deletions of the integrated transgenes in the regenerated plants as is common to *A. tumefaciens*-mediated transformation (Mercuri *et al.*, 2000). Although 80–90% survival of transgenic plants was achieved under greenhouse conditions, the stability of the transgene remains to be elucidated as tea plants take years to flower and set seeds.

The host range specificity between the bacterium and five different tea cultivars as well as an unrelated plant, *Artemisia parviflora*, having extreme surface characteristics was evaluated (Kumar *et al.*, 2004). The degree of *Agrobacterium* infection in the five cultivars of tea was affected by leaf wetness, micromorphology, and surface chemistry. Wettable leaf surfaces of TV-1, UPASI-9, and Kangra jat showed a higher rate (75%) of infection. This indicated that the leaves with a glabrous surface having lower Ø (large surface area covered by water droplet) and higher phenol and wax content were suitable for *Agrobacterium* infection. Caffeine fraction of tea promoted *Agrobacterium* infection even in leaves containing less wax (UPASI-10), whereas caffeine-free wax inhibited both *Agrobacterium*

growth and infection. Thus, this study suggests the importance of leaf surface features in influencing the *Agrobacterium* infection in tea leaf explants.

Considering the fact that lepidopteran and coleopteran insects account for 31.5% and 18.8% crop loss, respectively, in China, Luo and Liang (2000) pioneered for constructing a vector containing *Bt* (*Bacillus thuringiensis*) gene and transformed tea. The vector pGA471 containing *Bt* gene was digested with *Hind*III and *Bgl*II and inserted into the vector pCambia2301. The constructed plasmid with *Bt* gene (*cryIac*), *GUS* intron gene, and *NPTII* was transformed into *Escherichia coli* and introduced into *Agrobacterium* strains LBA 4404, EHA 105, and pRi15834 through a triparental cross. They detected the transient expression of *GUS* gene in calli and leaves of putative transgenic tea plants. However, no transgenic plants were recovered.

Three genes including *rolb*, *Bt*, and *Chitinase* have been transferred to tea cv. TTL-1 at the Research and Development Department, Tata Tea Ltd, Kerala for developing a tea plant with better yield, pest resistance, and resistance to blister bight disease. Preliminary study indicates that there is no difference in quality of Centro de Tecnologia Canavieira (CTC)-made tea in the transgenic plant but *rolb* produces more root and shoot biomass in 2-year-old young transgenic plants (data not shown).

Later, Lopez *et al.* (2004) also produced transgenic tea. Cotyledon-derived embryogenic callus cultures were co-cultivated with *A. tumefaciens* harboring a binary vector carrying the hygromycin phosphotransferase (*hptII*), glucuronidase (*uidA*), and green fluorescent protein (*GFP*) genes. Following co-cultivation, embryogenic calli were cultured in a medium containing 500 mg l⁻¹ carbenicillin for a week and then transferred on an antibiotic selection medium containing 75 mg l⁻¹ hygromycin for 8–10 weeks. Hygromycin-resistant somatic embryos were selected. The highest production efficiency of hygromycin-resistant calli occurred with co-cultivation for 6–7 days in the presence of 400 µM acetosyringone (AS). Hygromycin-resistant somatic embryos developed into complete plantlets in a regeneration medium containing half-strength MS (1962) salts with 1 mg l⁻¹ BAP and 9 mg l⁻¹ gibberellic acid A3. Transformants were subjected to GFP expression analysis, β-glucuronidase (GUS) histochemical

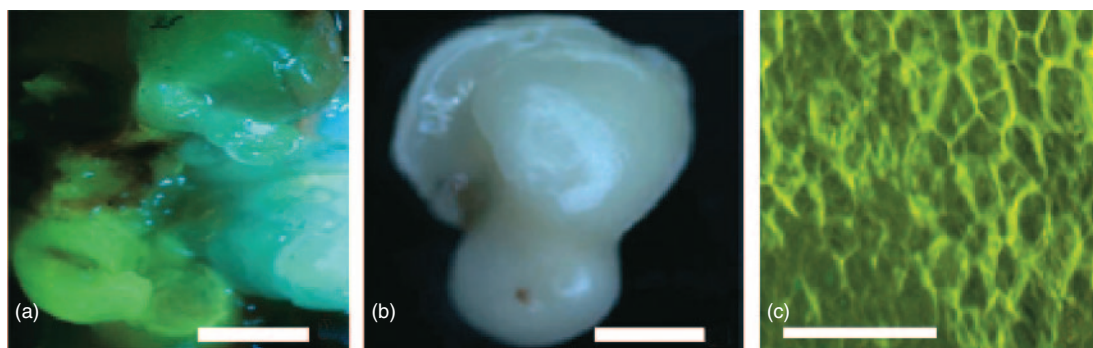


Figure 3 (a) Stable GFP expression following *Agrobacterium*-mediated genetic transformation of embryogenic calli (bar: 3 mm); (b) nontransformed control callus without GFP expression (bar: 3 mm); (c) GFP expression in the cross-section of the transformed callus [Reproduced from Lopez *et al.* (2004) with permission]

assay, PCR analysis, and southern hybridization to confirm gene integration. The GFP is a stable, cell-autonomous fluorescent protein derived from jellyfish (*Aequorea victoria*). It has been extensively used as a nondestructive reporter gene for plant transformation (Chiu *et al.*, 1996). After 30 days of co-cultivation, GFP expression was evident in calli. Nontransformed tissue from original explants did not express green fluorescence, and explant growth was suppressed (Figures 3a–c). Leaves of transformed plantlets also expressed GFP, and the green fluorescence was easily detectable with blue light illumination of calli.

Production of transgenic tea tissue was also reported by Jayaraman and Nithya (2005). Embryogenic tissues of tea were co-cultivated with *A. tumefaciens* strain LBA4404. The plasmid pBi121, which contains the *nptII* gene providing kanamycin resistance as a selectable marker and the β -glucuronidase (*uidA*) reporter gene, was used as binary vector. The highest transformation frequency (12 transformants per gram fresh weight of treated embryogenic tissue) was obtained with 5-day-old tissues grown in a liquid medium and co-cultivated with *Agrobacterium* for 2 days in the same medium but containing 50 μ M AS. There was improvement in the recovery of kanamycin-resistant tissues when tissues were first grown for 10 days on a medium containing 350 mg l⁻¹ Timentin to prevent bacterial overgrowth, before application of the selection pressure. Resistant tissues obtained after 6 weeks on the kanamycin selection medium showed a stable *uidA* expression. PCR analysis demonstrated

the presence of the transgenes, while southern hybridization confirmed their integration into the genome. Following this protocol, they could produce transgenic plants within 4 months after co-culture. As reported for other species (Bergmann and Stomp, 1992; Sangwan *et al.*, 1992), the physiological status of the tissue was considered to be an important factor for successful transformation of tea. They found that rapidly dividing embryogenic suspension culture, obtained 5 days after a 7 days subculture, provides suitable material to achieve a high frequency of transformation. Additionally, co-cultivation in a liquid medium and addition of 50 μ M AS were essential for successful transformation. AS is a low-molecular-weight phenolic compound naturally released by wounded plant cells and acts as an inducer of the virulence genes. For tea, the use of AS probably increases tDNA transfer, as reported in a wide range of plant species (Godwin *et al.*, 1992). It was also shown that Timentin, when used at a high concentration (350 mg l⁻¹), provided better protection against bacterial overgrowth than did cefotaxime and carbenicillin. By using Timentin instead of cefotaxime or carbenicillin, the costs associated with antibiotic use were reduced. Finally, a two-step procedure for screening transformants such as first selection of transformed tissues and a second selection during the germination of putatively transformed embryos on a kanamycin-containing medium (25 and 50 mg l⁻¹, respectively) improved the rapidity and efficiency of the transformation procedure, allowing recovery of transgenic plants within 3–4 months after co-culture.

2.2.2 *Agrobacterium rhizogenes*

Several groups have also transformed the tea plant with *A. rhizogenes*. Zehra *et al.* (1996) infected *in vitro* tea leaves with *A. rhizogenes* A₄. A cell density of 10^8 ml^{-1} for 2 min infection followed by blotting on sterile filter papers and co-cultivation in dark for 2 days induced hairy roots after 35 days. The isolated hairy roots grew healthy in the liquid medium. Mannopine from these roots were analyzed through paper electrophoresis, which confirmed stable integration of the gene. Later, Konwar *et al.* (1998) also transformed 4–6-month-old *in vitro* grown tea shoots by infecting at the basal portion followed by co-cultivation in a liquid basal MS medium supplemented with IBA (5 mg l^{-1}) and rifampicin (100 mg l^{-1}). Root initiation from the basal portion of 66% explants after 32–45 days culture enabled convenient hardening of the shoots in pots or nursery beds.

2.2.3 Biolistic-mediated transformation

Although no transgenic tea plants have produced via particle gun, a preliminary study on transient expression was reported by Akula and Akula (1999). Somatic embryos were bombarded with DNA (p2k7) coated gold particles ($1.5\text{--}3 \mu\text{m}$ diameter). The transformation vector p2k7, which was used in their study, was originally derived from binary vector pBI 221 with *NPTII* gene and *gus* gene. Both genes were driven individually by the cauliflower mosaic virus 35S promoter. The gold particles were coated with DNA ($1 \mu\text{g} \mu\text{l}^{-1}$) by precipitation with CaCl_2 (1 M) and spermidine (0.1 M). Optimization of various factors such as the distance between the site of delivery of the microprojectile and the target tissue, helium pressure, and the state of target tissue to obtain transient expression were evaluated on the basis of β -glucuronidase (GUS) assay after 30–40 h of bombardment. Following bombardment, the highest transient expression levels (up to 1085 blue spots/shoot) were obtained in the somatic embryos using a helium pressure of 550 kPa with target tissue at a distance of 9.5 cm from the site of delivery of DNA. Mannitol pretreatment did not influence the transient expression as

both control and treated cultures gave the same level of expression. However, further details of regeneration of transgenic somatic embryo were not mentioned.

3. FUTURE ROAD MAP

Despite the fact the transgenic technology has tremendous scope for tea, no transgenic plants have been cultivated commercially so far. Among the many reasons, a few could be as follows: (1) tea plant is not easily amenable for *Agrobacterium* infection; (2) nonavailability of generic protocol, which can be applied for a wide range of varieties of tea plant; (3) being woody perennial, production of transgenic tea is time consuming and hence private funding is less. However, it is evident now that among the different techniques *A. tumefaciens*-mediated transformation has been attempted with different groups but transgenic tea is yet to be commercialized. On the other hand, though *A. rhizogenes* transformation in tea has been demonstrated, the technique has not been exploited commercially to produce the secondary metabolites so far, which will be immensely useful for a crop like tea. Contrary to the above techniques, work on particle bombardment is at very initial stages. It is noteworthy to mention here that perhaps transgenic tea has a unique advantage as, during processing, green tea leaves are exposed to a temperature of 120°C at the drying step during which toxins will be destroyed, which is critical for GM foods globally for some agricultural crops. A detail study through international collaboration by a common consortium will be the right approach for production of transgenic tea in the long term.

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Rubber Tree

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1. INTRODUCTION

Natural rubber, cis-1,4-polyisoprene, produced in the milky cytoplasm (latex) of specialized cells, called laticifers, of certain plants, is one of the most important biological macromolecule, used as industrial raw material for the manufacture of a variety of products. Natural rubber has been found in the latex of over 2000 plant species belonging to 311 genera of 79 families. However, *Hevea brasiliensis* (Para rubber tree) remains as the only cultivated species as a commercial source of natural rubber because of its abundance in the latex, high quality, and convenience of harvesting. Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. Rubber has a number of applications and there is hardly any segment of life that does not make use of rubber-based material. All along this chapter, *H. brasiliensis* will be mentioned as rubber or rubber tree, unless otherwise a specific mention is required.

1.1 History, Origin, and Distribution

H. brasiliensis is a native of Amazon River basin of South America. Rubber is one of the recently domesticated crops in the world. The successful transfer of *H. brasiliensis* to Asia and the subsequent establishment of commercial

rubber plantations were in response to the growing demand for this raw material. *H. brasiliensis* was introduced to Tropical Asia in 1876 through Kew Gardens from the seeds brought from the Rio Tapajós region of the upper Amazon region of Brazil by Sir Henry Wickam (Dijkman, 1951). Kew Gardens, UK, played a special role in the domestication of wild plants. It was in the Kew Gardens where the planting materials were assembled from the native land, propagated, and then distributed to other botanical gardens around the world (Baulkwil, 1989). *H. brasiliensis* is now commercially cultivated in the tropical regions of Asia, Africa, and South America in countries like Indonesia, Thailand, Malaysia, India, China, Sri Lanka, Philippines, Vietnam, Nigeria, Cameroon, Ivory Coast, Liberia, Brazil, Mexico, etc. However, the major share comes from Tropical Asia.

1.2 Botanical Descriptions

1.2.1 Taxonomy

H. brasiliensis belongs to the family Euphorbiaceae. Ten species of *Hevea* have been identified in the genus, namely, *H. benthamiana*, *H. brasiliensis*, *H. camargoana*, *H. camporum*, *H. guianensis*, *H. microphylla*, *H. nitida*, *H. pauciflora*, *H. rigidifolia*, and *H. spruceana* (Schultz, 1990).

1.2.2 Botany

Rubber tree (*H. brasiliensis*) is a sturdy, quick-growing tree, which attains a height of 25–30 m. The tree has a straight trunk with light gray bark. Branches are usually developed to form an open leafy crown. The bark of the trunk is the part from where rubber is exploited (Figure 1). The young plants show a characteristic growth pattern of alternating periods of rapid elongation and consolidation. The tree is deciduous with annual leaf fall. Refoliation and flowering follow wintering. The leaves are arranged in groups or storeys. From each storey, a cluster of spirally arranged trifoliate glabrous leaves is produced. The petioles are long, usually about 15 cm, with extra floral nectaries present in the region of insertion of the leaflets (Premakumari and Saraswathyamma, 2000).

Rubber tree is a monoecious tree with diclinous flowers arranged in a pyramid-shaped panicle. The flowers are short stalked and fragrant. The flower has one whorl of bell-shaped yellow-colored perianth with five lobes. Male flowers are smaller in size but more in number than the female flowers. In the male flower, there are 10 sessile anthers arranged on a slender staminal

column in two whorls of five each. Each anther contains two pollen sacs that split longitudinally on dehiscence. Pollen grains are tricolpate, smooth, and sticky. Female flowers are seen at the tip of the panicle and its branchlets. When fully developed, they are recognizable by their relatively bigger size and the green torus basal disc. The gynoecium is tricarpellary and syncarpous with an ovule in each locule. The stigma is short styled and three lobed. Pollination is mediated by insects. Sticky pollen and stigmatic surfaces indicate the typical entomophilous nature of the flower. After fertilization the ovary will develop into a three-lobed dehiscent capsule, regma, with three large mottled seeds. Fruits ripen 5–6 months after fertilization (Kavitha and Saraswathyamma, 2005).

1.2.3 Habitat

Rubber tree could be predominantly grown in the tropics where an equatorial monsoon climate prevails. The optimum climatic conditions for the successful growth of rubber are as follows: (1) rainfall of 2000 mm or more, evenly distributed without any marked dry season, and with 125 to 150 rainy days per annum; (2) maximum temperature of about 29–34 °C; (3) high atmospheric humidity in the order of 80% with moderate wind; and (4) bright sunshine amounting to about 2000 h per annum at the rate of 6 h per day throughout all the months (Watson, 1989; Rao and Vijayakumar, 1992).

1.2.4 Cytology

The chromosome counts made by various investigators showed variations and reported as $2n = 16, 34$, and 36 . However, detailed cytological investigations have confirmed the chromosome complement of rubber tree in the somatic cells as $2n = 2x = 36$ (Ramaer, 1935; Saraswathyamma *et al.*, 1984). The chromosomes are small and vary in length and the total chromosome length of the species is 89.7 μm . Meiotic division is regular and pollen fertility is over 80%. Critical analysis of karyomorphology revealed significant differences between clones with reference to centromeric position and total chromosome



Figure 1 A rubber plantation (RRII 105 clone) at RRII with an enlarged view of the tapping region at the inset (arrow indicates latex flow into a cup from rubber tree after tapping)

length (Saraswathyamma, 1990). There are no chromosomal or genetic barriers between the 10 *Hevea* species. Triploid plants with $2n = 3x = 54$ (Nazeer and Saraswathyamma, 1987) and induced tetraploids with $2n = 4x = 72$ by the application of colchicine (Saraswathyamma, 1990) were also reported. Wide range of meiotic abnormalities was noticed in the triploids and tetraploids (Saraswathyamma, 1997). The cytophotometric determination of DNA content of various cytotypes revealed 44.2 pg (picogram) in the diploids, 62.4 pg in the triploid, and 89.37 pg in the tetraploids (Saraswathyamma, 1990).

1.2.5 Harvesting and processing

In rubber tree, natural rubber is synthesized in highly specialized cells called latex vessels, present in all parts of the tree except the heartwood. These latex vessels originate from the cells produced by the cambium and they are articulated and anastomosing (Hebant and de Fay, 1980). The milky cytoplasmic content (latex) is collected by the controlled wounding of the bark, called tapping. The rubber is separated from latex upon coagulation and further processing.

1.3 Economic Importance

The global area under rubber cultivation is about 9.6 million hectares producing 9.2 million tons annually valuing about US\$18 billion as raw material alone (IRSG, 2007). The major rubber producing countries are Thailand, Indonesia, India, Malaysia, China, Vietnam, etc. Chemically, natural rubber is *cis*-1,4-polyisoprene, having molecular weight of 200 000 to 8 000 000 and with viscoelastic properties. The flexibility of natural rubber to undergo vulcanization with sulfur under high temperatures is an important attribute. The higher strength, low heat build up, and better resistance to wear and flex cracking made natural rubber a suitable raw material for the manufacture of automobile tires. A major quantity of natural rubber produced is consumed by the automobile tire industry. Natural rubber is a good insulator and can be easily manipulated. Being water resistant, it finds use in the manufacture of water proofing materials. More than 35 000 rubber-based

products such as hand gloves, toys, balloons, hoses, footwear, etc. are manufactured. Besides, rubber is also useful in soil stabilization, in vibration absorption, road surfacing, etc.

1.4 Traditional Breeding

The global consumption of natural rubber is steadily increasing and the production has also to be increased so as to meet the demand. The major objective of rubber tree breeding is to develop potential clones with high rubber yield combined with desirable secondary characters such as high initial vigor, smooth and thick bark with good latex vessel system, good bark renewal, high growth rate after initiation of latex harvest, tolerance to major diseases and wind, etc. (Annamma *et al.*, 1990; Varghese *et al.*, 1992). Recently, importance has also been given to develop clones with tolerance to abiotic stresses such as drought, high temperature, cold, etc. (Thulaseedharan *et al.*, 2000). Clones attaining early tapping girth and high initial yields are preferred to clones with higher yields in a later stage (Lim *et al.*, 1973). In countries where labor is cheap and the small-holding sector is predominant, clones capable of withstanding high tapping intensities are preferred. Besides high rubber yield, superior technological properties of rubber, timber and its quality (latex–timber clones), and low incidence of tapping panel dryness (TPD) are also major breeding objectives.

The conventional methods of genetic improvement are introduction, selection, and hybridization. The elite clones developed in one country are introduced to other countries. Popular clones introduced from other countries are evaluated under local agroclimatic conditions and the promising ones are recommended for large-scale planting. Introductions are effected under bilateral and multilateral clone exchange programs. Considerable yield variability in seedling populations was first observed by the Dutch workers in Java and Sumatra in the second decade of the last century (Whitby, 1919). Simultaneously, the technique of bud grafting was perfected, which facilitated the fixation of desired characters and the development of early primary clones through ortet selection. Ortet selection, mother tree selection or plus tree selection, is the oldest selection method

adopted in rubber trees. Ortet is, therefore, the original tree from which members of clone have descended. Hybridization programs are aimed at combining genes for desirable characters found dispersed in different clones. Hand-pollination between selected parent clones, evaluation of F_1 hybrids, selection of promising recombinants from the progeny, and multiplication by bud grafting are still the most important methods of conventional breeding (Varghese and Maydin, 2000).

Rapid progress made in the ortet selection between 1919 and 1926 in Indonesia and Malaysia resulted in the development of many classical primary clones commercially very promising (Marattukalam *et al.*, 1980). Controlled pollination was started in 1918 in Malaysia, 1920 in Sumatra, 1937 in Brazil, 1939 in Sri Lanka, 1948 in China, and 1954 in India, using the primary clones developed through ortet selection. Progress in yield improvement in rubber tree resulted in gradual increment, from 650 kg ha^{-1} in unselected seedlings during 1920s to 1600 kg ha^{-1} in the best clones during the 1950s. The yielding potential was further enhanced to 2500 kg ha^{-1} in PB, RRIM, RRII, RRIC, IRCA, BPM, and RRIV clones during 1990s. During these 70 years of rigorous breeding and selection, notable clones including RRIM 501, RRIM 600, RRIM 712, PB 217, PB235, PB 260, RRII 105, RRIC 100, IRCA 18, IRCA 230, IRCA 331, and BPM 24 were derived (Tan, 1987; Simmonds, 1989; Clement-Demangne *et al.*, 2001; Priyadarshan, 2003). Some of the primary clones, like PB 86, Tjir 1, Pil D65, Gl 1, PB 6/9, and PB 86, selected during the aforesaid period, became parents of improved clones. It must also be acknowledged that primary clones like GT 1 and PR 107 are still widely used, although their identification traces back to the 1920s.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

As a perennial tree species, genetic improvement through conventional breeding in rubber tree is rather a slow and difficult process. The major constraints in rubber tree breeding are long juvenile period, seasonal flowering, lack of any early selection parameters especially for estimating traits such as latex yield, susceptibility to TPD and wind damage, low fruit set, very long period of field

experimentation, and pronounced interaction of genotype and environment. The major advantages are monoecious nature of the tree, which makes hybridization easy and the amenability to vegetative method of propagation (Saraswathyamma, 2002). The recent developments in recombinant-DNA and *in vitro* plant regeneration techniques could provide a direct route for the introduction of specific genes controlling agronomic traits into crop plants. The transfer of selected genes in a single generation by genetic transformation is especially interesting for this species, since its improvement is limited by long breeding cycles and high levels of heterozygosity. The major objectives of genetic transformation of rubber tree in different laboratories are (a) improvement of agronomic traits of elite rubber clones and (b) production of pharmaceuticals and other valuable recombinant proteins in the transgenic rubber tree. In the improvement of agronomic traits, the underlying direction is toward development of transgenic rubber tree with increased rubber biosynthesis, improved quality and volume of timber, tolerance to various diseases as well as abiotic stresses, etc. (Thulaseedharan, 2002; Yeang, 2004). A unique feature of rubber tree is the presence of latex vessels with the continuous production and harvesting of latex, which contains about 30–40% rubber and remaining with a serum containing all the machinery for protein synthesis, provided the gene is integrated through transgenic technology. Hence, the rubber tree could be exploited as a biological factory for the production of desired recombinant proteins by the integration of the desired genes.

2. PROGRESS IN THE GENETIC TRANSFORMATION OF RUBBER TREES

2.1 Donor Genes Employed and Design of Transgenics

2.1.1 Marker and antibiotic selection genes

In spite of the economic importance of the rubber trees, transgenic approaches for crop improvement have started only a little more than a decade ago. Two major factors that helped to lay the foundation for modern plant biotechnology are the capacity to regenerate whole plants from single

Table 1 Different genes, promoters, and antibiotic-resistant genes used for genetic transformation of rubber trees

Genes tried	<i>Agrobacterium</i> strain	Antibiotic used	Promoter	Selectable marker gene	References
<i>GUS</i>	Microprojectile	Kanamycin	CaMV 35S	<i>nptII</i>	Arokiaraj <i>et al.</i> , 1994
<i>GUS</i>	LBA 4404, GV2260	Kanamycin	CaMV 35S	<i>nptII</i>	Arokiaraj <i>et al.</i> , 1996
<i>GUS</i>	GV2260	Kanamycin	CaMV 35S	<i>nptII</i>	Arokiaraj <i>et al.</i> , 1998
<i>GUS</i>	C58p MP 90, C58p GV2260, AGL1, LBA 4404, EHA 105	Kanamycin	CaMV 35S	<i>nptII</i>	Montoro <i>et al.</i> , 2000
<i>HAS</i>	GV2260	Kanamycin	CaMV 35S	<i>nptII</i>	Arokiaraj <i>et al.</i> , 2002a
<i>GUS</i>	C58p MP 90, C58p GV2260, AGL1, LBA 4404, EHA 105	Paramomycin	CaMV 35S	<i>nptII</i>	Montoro <i>et al.</i> , 2003
<i>SOD</i>	EHA 101	Kanamycin	CaMV 35S	<i>nptII</i>	Jayashree <i>et al.</i> , 2003
<i>SOD</i>	EHA101	Kanamycin	FMV 34S	<i>nptII</i>	Sobha <i>et al.</i> , 2003b
<i>GUS</i>	EHA 105	Paramomycin	CaMV 35S	<i>nptII</i>	Blanc <i>et al.</i> , 2006
<i>SOD</i>	EHA101	Kanamycin	CaMV 35S	<i>nptII</i>	Rekha <i>et al.</i> , 2006
<i>IPT</i>	EHA101	Kanamycin	mas'	<i>nptII</i>	Kala <i>et al.</i> , 2003
TB antigen	LBA4404	Kanamycin	CaMV 35S	<i>nptII</i>	Kala <i>et al.</i> , 2006
ScFv	GV2850	Kanamycin	CaMV 35S	<i>nptII</i>	Yeang <i>et al.</i> , 2002

cells without changing the original genetic features of the cell (Birch, 1997) and successful gene transfer into plant genomes with stable expression of an introduced foreign gene (Zambryski *et al.*, 1983) through the natural genetic transformation mechanism by *Agrobacterium tumefaciens*. The first transgenic rubber tree was developed in 1994 incorporated with *GUS* (β -glucuronidase) gene (Arokiaraj *et al.*, 1994). Rubber plants being very recalcitrant and rubber cultivation being restricted to specific locations around the world, only few laboratories are actively involved in rubber research. A list of genes successfully tried for rubber tree transformation is presented in Table 1. In order to optimize the *Agrobacterium*- and biolistic-mediated genetic transformation systems in rubber trees, different workers employed *GUS* as the marker gene (Arokiaraj *et al.*, 1994, 1996, 1998; Montoro *et al.*, 2000, 2003; Blanc *et al.*, 2006). The *GUS* gene used is from *Escherichia coli* and the expression is controlled by the cauliflower mosaic virus (CaMV) 35S promoter and the selectable marker gene used was the sequence coding for neomycin phosphotransferase II (*nptII*).

2.1.2 Genes for recombinant protein production

The advent of genetic engineering in the early 1970s and its application to plant biotechnology

have revolutionized agriculture. Recent years have seen a dramatic increase in application of biotechnology for the production of a variety of recombinant proteins in plants. Transgenic rubber trees that secrete human serum albumin (HSA) in the serum fraction of rubber latex were generated by Arokiaraj and coworkers in 2002. They constructed a binary vector pLGMR, which contained a 1.8-kb polymerase chain reaction (PCR)-amplified sequence coding for HSA fused with the CaMV 35S promoter and polyA tail to control the expression. *nptII* gene complementary DNA (cDNA) under the control of *nos* (nopaline synthase) 5' promoter and *nos* 3' polyA tail were used as the antibiotic selection. Further, Yeang *et al.* (2002) reported the expression of a functional recombinant antibody fragment in the latex of transgenic rubber. The binary vector used for the genetic transformation contained the gene for a mature immunoglobulin single chain variable fragment (ScFv) with specificity for the dental bacterium *Streptococcus gordonii*, together with the coding region of the tobacco pathogenesis-related protein signal sequence for targeting the recombinant fragment antibody. The CaMV 35S promoter and the *nos* terminator sequence were used for the expression of the gene. *nptII* gene was used as the selectable marker gene. Kala *et al.* (2006) used a cDNA sequence coding for a 10.8-kDa (kilodalton) TB antigen protein isolated from *Mycobacterium tuberculosis*.

2.1.3 Genes for tolerance to stress and TPD

Transgenic breeding for tolerance against environmental stress as well as TPD were initiated at the Rubber Research Institute of India (RRII). Transgenic rubber plants were developed using the functional gene coding for superoxide dismutase (Jayashree *et al.*, 2003). The 702 nucleotide rubber (*H. brasiliensis*) superoxide dismutase (Hb.SOD) cDNA was obtained by the reverse transcription PCR from the mRNA (messenger-RNA) isolated from the bark tissues of the trunk region, using forward and reverse sequences corresponding to a previously reported sequence of Hb.SOD (Mia and Gaynor, 1993). The Hb.SOD coding sequence was inserted into the binary vector pDU92.3103 (Tao *et al.*, 1995) at the unique *Bam*HI site between the CaMV 35S promoter and 3' polyadenylation sequences, thereby creating the binary vector pDU96.2144 (Jayashree *et al.*, 2003). Similarly, *SOD* gene containing binary vector were developed under the control of figwort mosaic virus (FMV) 34S promoter and transgenic plants were obtained (Sobha *et al.*, 2003a). Rekha *et al.* (2006) reported genetic transformation of rubber tree with *SOD* gene in the antisense orientation in order to understand the role of *SOD* in the normal growth and development as well as environmental stress tolerance, including TPD in rubber. In order to enhance the cytokinin level in the bark to confer tolerance against TPD, attempts were also made to develop transgenic plants integrated with gene coding for isopentenyl transferase (*ipt*). The plasmid vector used was pDU.970612, which contained the sequence coding for *ipt* from *Agrobacterium* under the control of its own 5' promoter and 3' polyadenylation signal (Kala *et al.*, 2003).

2.2 Status of Genetic Transformation in Rubber Trees

2.2.1 Optimization of transformation techniques

The possibility of genetic transformation in rubber trees was first explored by Arokiaraj and Rahaman (1991). They employed co-cultivation of *in vitro*- and *in vivo*-propagated plantlets with *A. tumefaciens* (Strain 541) and cultured

in the Murashige and Skoog (MS) medium without growth regulators. Co-cultivated explants developed tumors and produced octopine indicating effective transformation. Subsequently transformations were developed for direct DNA delivery through microprojectile bombardment as well as through *A. tumefaciens*-mediated methods (Arokiaraj *et al.*, 1994, 1996). In their studies, anther-derived calli were used as explants and binary vectors harboring *GUS* as reporter gene and either *nptII* or chloramphenicol acetyl transferase (*CAT*) as the selection gene. Genetic transformation was confirmed by histochemical staining and fluorometric assay for GUS activity, ELISA (enzyme-linked immunosorbent assay) for detecting expression of the *nptII* gene, and direct enzyme assay for detection of *CAT* gene expression were carried out. The presence of foreign gene in the transformed calli, embryoids and transgenic plants was further confirmed by PCR analysis. Arokiaraj *et al.* (1998) studied the constitutive promoter (CaMV 35S) directed β -glucuronidase expression in the laticifer system of transgenic rubber and the stability of expression of the transgene in successive vegetative generation raised through bud grafting of the original transformants. Anther calli were genetically transformed using *Agrobacterium* strain GV2260 harboring the β -glucuronidase (*uidA*) and *nptII* genes. β -glucuronidase protein was expressed in the leaves of transformed plants. GUS was also expressed in the serum fraction of latex. Transverse sections of the leaf petiole from a transformed plant revealed GUS expression, especially an enhanced expression in the phloem and laticifers. GUS expression was detected in three successive vegetative cycles propagated from the original transformants.

Studies were also undertaken to enhance the efficiency of *Agrobacterium*-mediated genetic transformation of rubber callus. It is reported that the virulence capacity of the *Agrobacterium* strain and the combination of the *Agrobacterium* strain and the type of binary vector used significantly influenced the transient expression of the GUS (Montoro *et al.*, 2000). Out of the five *A. tumefaciens* strains, C58pMP90, C58 pGV2260, AGL1, LB4404, and EHA 105 and the two binary vectors, pGIN and pCAMBIA 2301 tested the combination of EHA 105 and the binary plasmid vector pCAMBIA 2301 showed the highest transient expression. It is also reported

that transfer of friable callus from a maintenance medium containing 9.0 mM CaCl_2 to a calcium-free medium before *Agrobacterium* infection as well as use of a calcium-free *Agrobacterium* resuspension medium to inoculate friable calli significantly enhanced the transformation efficiency. Paramomycin was proved more effective than kanamycin for the selection of transformed cells (Montoro *et al.*, 2003). The influence of cryopreservation of explants, co-cultivation temperature, and duration of co-cultivation of the *Agrobacterium*-mediated genetic transformation of rubber callus was studied by the above group. It is reported that, the transformation efficiency and competence of the embryogenic calli were improved after two cycles of cryopreservation. When the co-cultivation temperature was reduced from 27 °C to 20 °C and the duration of this phase was increased up to 7 days, the GUS activity was increased (Blanc *et al.*, 2006).

2.2.2 Development of transgenic plants for environmental stress tolerance

After developing efficient plant regeneration protocols through somatic embryogenesis, RRII has initiated active research for the development of transgenic plants integrated with genes for desirable agronomic traits. An efficient *Agrobacterium*-mediated genetic transformation system for transgenic plant regeneration has been developed in the recent past (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003b). Initial focus was to develop transgenic plants tolerant to abiotic stresses like elevated light and temperature, drought, and TPD. In nature, plants encounter a wide range of environmental stresses that detrimentally affect their growth and development. Plants exposed to environmental stress generate excess reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Asada and Takahashi, 1987). Superoxide dismutase (SOD) is the first enzyme involved in the detoxifying process of reactive oxygen species (Fridovich, 1986). Significant yield loss occurred in plantations across the rubber-growing countries due to TPD, a physiological disorder, characterized by the browning of bark followed by the cessation of latex flow. Since, no pathogens are unequivocally proved as the causative organism yet, this is considered

as a physiological disorder. It is reported that the TPD-affected bark shows an increased free radical accumulation and a reduced level of SOD enzyme as well as cytokinin (Das *et al.*, 1998). Therefore, attempts were made at RRII to develop transgenic rubber plants tolerant to a variety of environmental stresses as well as TPD, by incorporation of the genes coding for SOD and *ipt*.

Agrobacterium-mediated genetic transformation was carried out with *Hb.SOD* gene under the control of CaMV 35S promoter. Two-month-old immature anther calli were used as the initial explants in the transformation experiments. The plasmid used was pDU96.2144, which contains *uidA* as the reporter gene and *nptII* as the selectable marker gene. Transgenic plants integrated with the SOD gene under the control of different promoters such as CaMV 35S and FMV 34S were developed separately from different cell lines obtained through independent transformation events. The stable integration and copy number of the transgene were confirmed through molecular analysis (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003a). The preliminary biochemical studies conducted with SOD-transformed callus cultures showed enhanced activity of SOD and related enzymes such as catalase and peroxidase (Sobha *et al.*, 2003b).

Genetic transformation with *ipt* gene for the overproduction of cytokinin was also attempted. Rubber (Clone RRII 105) anther calli were transformed through *A. tumefaciens* (strain EHA 105) harboring the binary vector carrying the gene coding for *nptII* as the selectable marker gene, *GUS* as the reporter gene, and the sequence coding for *ipt*. The putatively transformed calli were able to grow hormone autotrophically and showed increased cytokinin levels compared with the controls. The embryos showed developmental abnormalities and most of the transformants were severely deformed. The constitutive expression of *ipt* gene by its promoter elevated the endogenous cytokinin levels, which might have enhanced ethylene production leading to phenotypic abnormalities hampering the plant development (Kala *et al.*, 2003).

2.2.3 Transgenic plants for enhanced rubber biosynthesis

Natural rubber is cis-1,4-polyisoprene. The biosynthesis of rubber from sucrose is through a

series of reactions catalyzed by various enzymes. Few enzymes involved in the conversion of acetate into rubber in rubber tree latex have been analyzed, which indicates that the activity of 3-hydroxy-3-methyl glutaryl Coenzyme A reductase (*hmgr1*) is low compared to the other enzymes (Lynen, 1969) suggesting that the constitutive level of this enzyme may be a limiting factor in rubber biosynthesis. Arokiaraj *et al.* (1995) overexpressed *hmgr1* in transgenic rubber, where HMGR activities of transformed anther derived callus ranged from 70 to 410% of the value of wild-type control and the activity in the transformed embryos ranged from 250 to 300%.

In order to enhance the rubber biosynthesis, experiments were initiated at RRII to develop transgenic plants integrated with the genes coding for important enzymes involved in this pathway. Initially, the genes coding for *hmgr1*, farnesyl-diphosphate synthase (FDP) and rubber elongation factor (REF) were selected. The *hmgr1* and *REF* genes were cloned into the binary vector pBIB 121 for the genetic transformation which contains the antibiotic genes *nptII* and *hpt* (hygromycin phosphotransferase). The *FDP* gene was cloned into the binary vector pCAMBIA under the control of CaMV 35S promoter. Three *Agrobacterium* strains such as EHA 105, LBA 4404, and pGV 3101 were used for infecting the tissue for the transformation of *hmgr1* gene while EHA 105 alone is used for *FDP* and *REF* genes. Two-month-old calli were infected with different *Agrobacterium* strains carrying the above genes and the transgenic lines were selected. Transgenic embryos and few plantlets integrated with the *FDP* gene were regenerated. Further work in this direction is in progress at RRII.

2.2.4 Transgenic plants for recombinant protein production

For commercial production of diagnostic and therapeutic products, the pharmaceutical industry essentially depends upon microorganisms involving sophisticated bioreactors. Since plants are easy to maintain, require only sunlight, water, and agricultural inputs, they are cheaper compared to microorganisms involving bioreactors. As protein-manufacturing factories, they are also ecologically friendly. Production of diagnostic and therapeutic

products using plant biotechnology has become well recognized in the field of pharmaceutical industry. Recently, various recombinant proteins have been expressed in crop plants making them living factories for the production of commercially valuable proteins (Nilesh *et al.*, 2004).

Natural rubber from rubber tree is continuously extracted through tapping. The rubber tree has many unique advantages for biopharming. Rubber tapping is a systematic and controlled wounding of the bark. The bark of the rubber tree contains a complex network of articulated laticifers or latex vessels, notably in the soft bark of the trunk from which rubber is collected. The latex is a cytoplasm that contains rubber particles, microvacuoles known as lutoids, and double-membraned organelles rich in carotenoids assimilated to plastids, the Frey-Wysling particles (Paradkooper, 1989). All the organelles of nonphotosynthetic plant cells: vacuoles (the lutoids), plastids, mitochondria, nuclei, and endoplasmic reticulum are also present (de Faiy *et al.*, 1989). It also contains various types of RNAs (Priyadarshan and Clemant-Demange, 2004), polysomes, and ribosomes as well as other ingredients required for the protein synthesis. It means latex contains all the machinery for the protein synthesis, if the desired gene is inserted into rubber plants.

The disadvantage of plants in general as biological factories for recombinant protein production is that the proteins may be accumulated in certain plant portions and the harvesting is mainly by destructive methods. After each harvest, it takes time for new growth to take place before the next harvest, making the harvest batchwise, rather than continuous processes. The ideal plant for recombinant protein production would be one that is cheaper to maintain and easy to multiply clonally, while allowing for continual harvesting of the protein (Arokiaraj, 2000). In rubber tree, latex harvesting is by nondestructive method and a continuous process (every alternate days or once in 3 days) throughout the year and the latex replenishment after each tapping is rapid. Moreover, once the tree is genetically transformed, the trait could be fixed in the T₁ generation itself with large-scale clonal propagation. Therefore, among the plants, rubber tree is having the unique advantage as the most suitable candidate for biopharming.

The expression of a recombinant protein, the HSA in the transgenic rubber tree was reported

for the first time by Arokiaraj *et al.* (2002b). An attempt was made to express the 68-kDa HSA protein in transgenic rubber. Transgenic rubber plants that secrete HSA in the serum fraction of rubber latex were generated using a binary vector pLGMR.HSA (13.8 kb) containing HSA cDNA (1.8 kb) with its native leader sequence in the *A. tumefaciens* (GV2260) containing a supervirulent plasmid (pToK 47). In this vector (pLGMR.HSA), the inserted HSA cDNA with its leader sequence is placed under the control of a CaMV 35S promoter. Rubber anther callus (clone GL1) was used as the explant for the infection using *Agrobacterium* GV2260. The presence of the inserted gene was confirmed by PCR using the primers of *nptII* and HSA. Detection of HSA in the leaf and latex extracts was carried out using an antibody coupled to a protein chip array. Western blot analysis was also done for the immuno detection of the HSA. An expression level in the latex serum of about 24 µg of HSA per milliliter of latex extract in young plants was also detected.

Yeang (2004) reported the expression of a functional recombinant antibody fragment in the latex of transgenic rubber. The plasmid for the genetic transformation contained the gene for a mouse immunoglobulin ScFv with specificity for the dental bacterium *Streptococcus gordonii*. The CaMV 35S promoter and the *nos* terminator regulatory sequences and the selectable marker *nptII*, together with the coding region of the tobacco pathogenesis-related protein pathogenesis related protein 1a (PR-1a) signal sequence regulated the expression of the recombinant antibody fragment. The gene construct was transformed into *A. tumefaciens* (GV 2850). The presence of the inserted genes in the putative transgenic plants was analyzed by PCR. The ScFv protein concentration detected in the latex was about 6 µg ml⁻¹ latex serum for the most productive plant.

Recently, attempts were initiated at RRII for the production of a recombinant TB antigen protein in rubber. The embryogenic calli derived from the leaf explants were transformed with the *A. tumefaciens* strain LB 4404 carrying the binary plasmid vector having the gene coding for a 10-kDa TB antigen and the *nptII* as the selectable marker gene under the control of CaMV 35S promoter. Co-cultivated tissues were transferred onto a selection medium containing kanamycin (350 mg l⁻¹) and cefotaxime (500 mg l⁻¹). High-

frequency transformation was obtained and embryos were developed from the transgenic tissues on the modified MS medium with benzylamino purine (BA) (2.0 mg l⁻¹), gibberellic acid A3 (GA3) (1.2 mg l⁻¹), α-naphthalene acetic acid (NAA) (0.2 mg l⁻¹) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.1 mg l⁻¹). The gene integration was confirmed by PCR analysis. Work is in progress to develop plantlets (Kala *et al.*, 2006).

2.3 Methods of Genetic Transformation

Genetic transformation is a potential tool in different areas such as manipulation and understanding of the biochemical processes, knowledge of genome regulation, and integration of the genes, which is not feasible through conventional breeding. Different techniques for gene transfer into plant systems have been developed, which includes *A. tumefaciens*- and *A. rhizogenes*-mediated transformation, microprojectile bombardment, tissue and protoplast electroporation, polyethylene glycol (PEG)-mediated direct gene transfer to protoplasts, microinjection, and fiber-mediated transformation. The possibility of genetic transformation in rubber tree was first explored in 1991 by *A. tumefaciens*-mediated transformation of callus derived from *in vitro* and *in vivo* seedling cultures (Arokiaraj and Rahaman, 1991). The first transgenic rubber plant was produced by the integration of *GUS* and *nptII* genes into callus cultures by particle gun method. However, because of the efficiency and convenience, *A. tumefaciens*-mediated genetic transformation system remains as the most widely used method for rubber genetic transformation.

2.3.1 *Agrobacterium*-mediated gene transfer in rubber trees

2.3.1.1 *A. tumefaciens*—a natural vector

The natural capacity of the gram-negative soil bacterium, *A. tumefaciens* to introduce a segment of oncogenic DNA present in the tumor-inducing (Ti) plasmid makes it an efficient vector system in genetic transformation. In rubber, *Agrobacterium*-mediated genetic transformation has been used widely and effectively for genetic transformation

(Arokiaraj *et al.*, 1996, 1998; Jayashree *et al.*, 2003; Sobha *et al.*, 2003a; Kala *et al.*, 2003). Strains of *Agrobacterium* used commonly for transformation include C58, EHA101, EHA 105, LBA 4404, pGV2260, and pGV3850. These have been reported to show high efficiency in rubber genetic transformation. Presence of phenolic compounds in rubber cells might have contributed to the success of *Agrobacterium*-mediated transformation since this act as *vir* gene inducers aiding in transfer-DNA (T-DNA) transfer. In most of the reports on rubber genetic transformation using *Agrobacterium*, *nptII* has been used as the selection marker and qualitative marker is the commonly used GUS reporter. In recent reports on transformation, most vectors were found to avoid the qualitative markers since these were found to hamper plant regeneration.

2.3.1.2 *Agrobacterium* protocols

A detailed protocol of *Agrobacterium*-mediated genetic transformation developed at the RRII is described here (Figure 2). *Agrobacterium* strain EHA 101 was transformed with the binary vector pDU 96.2144, which contained *uidA* as the reporter gene, *nptII* as the selectable marker gene, and the 702-nucleotide *Hb.SOD* cDNA under the control of CaMV 35S promoter (Jayashree *et al.*, 2003). *Hb.SOD* gene under the control of FMV 34S promoter were also tried separately (Sobha *et al.*, 2003a). The bacteria harboring the vector was grown in the AELB medium overnight at 28°C in the presence of 50 mg l⁻¹ kanamycin and 20 mg l⁻¹ gentamycin until an A₆₀₀ of 0.5. The bacterial cell density was adjusted to 5 × 10⁸ cells ml⁻¹ in this medium and used for tissue infection.

Approximately 2.0 g of calli were precultured on a callus proliferation medium prior to infection with *Agrobacterium*. The explants were immersed in the *Agrobacterium* cultures for 10 min and were blotted dry in a sterile filter paper and transferred to a co-cultivation medium (MS + acetosyringone 20 mg l⁻¹ + glycine betaine-HCl 153.6 mg l⁻¹ + proline 113 mg l⁻¹). After 3 days of co-culture the explants were subcultured on a selection medium containing 500 mg l⁻¹ cefotaxime and 300 mg l⁻¹ kanamycin for 50 days and maintained at 25 ± 2°C in the dark. After 8 weeks of

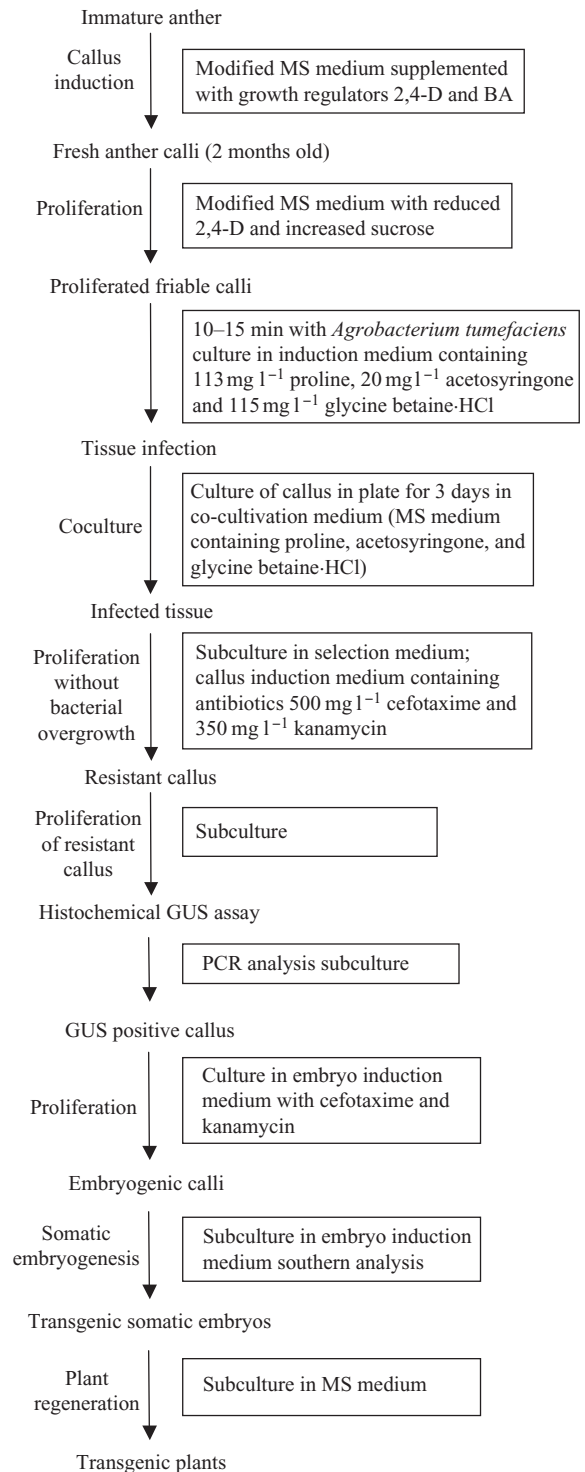


Figure 2 Flow chart of *Agrobacterium*-mediated genetic transformation and plant regeneration protocol in rubber

culture, kanamycin-resistant callus lines were subjected to histochemical GUS assay according to Jefferson (1987). The transformed cells were incubated overnight at 37°C in 2 mm X-gluc (5-bromo-4-chloro-3-indolyl β -D glucuronide) in phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100, and were observed under microscope.

The transformed callus lines were subcultured into a fresh medium for proliferation. After 2 months of culture, the embryogenic calli obtained were subcultured to an embryo-induction medium. The gene integration in the transgenic plants was confirmed by PCR analysis and Southern hybridization. The different hybridization patterns obtained for the transgenic plants indicate random integration and multiple insertions of the T-DNA in the genome of the plants.

In order to understand the effect of the physiological state and sources of explants on transformation efficiency, Rekha *et al.* (2006) examined six different explants, i.e., intact explants (immature anther, ovule), 2-month-old callus and embryogenic callus of the anther and ovule for *Agrobacterium* infection. Results suggested that the target tissue is one of the important factors that determine the transformation efficiency. Among the different explants used, maximum transformation frequency (62%) was obtained with the embryogenic callus of the anther. Similar results were also observed when embryogenic callus derived from leaf explants was used for *Agrobacterium* infection (Kala *et al.*, 2006). A detailed investigation has been carried out by Montoro *et al.* (2000) on the effect of exogenous calcium on *Agrobacterium*-mediated gene transfer in rubber friable callus. It is demonstrated that the exogenous CaCl_2 reduces the efficiency of *Agrobacterium*-mediated gene transfer. The use of Ca-free media, both in bacteria and plant tissue preparations, dramatically increased the number of transformation events and consequently the positive effect of acetosyringone on transformation efficiency. Further, this group has done extensive studies to improve the transformation efficiency by manipulating the competence of the explants and the co-cultivation temperature (Blanc *et al.*, 2006). They observed a drop in transformation efficiency as the age of callus increases. Reducing the temperature and lengthening

the co-cultivation period with the *Agrobacterium* suspension increased the transformation frequency significantly. Rattana *et al.* (2000) reported that co-cultivation at 20°C slowed down the *Agrobacterium* proliferation and callus browning by which duration of co-cultivation could be extended. In that way, contact between the plant cells and the bacteria was favored, making gene transfer more efficient (Blanc *et al.*, 2006).

2.3.2 Biolistic transformation system

Gene delivery into intact tissues by DNA-coated microprojectiles allows genetic transformation of several recalcitrant species. Usually, this technique results in transient gene expression and chimera formation. Effective gene transfer using this system is found to be influenced by several factors. Nature and size of particles and target tissues usually influence gene expression. Biological factors such as growth stage and media supplements that help cell survival are also detrimental. Acceleration of a microcarrier was achieved by a shock wave generated from gunpowder charge or sudden release of compressed air. In rubber, this technique was used by Arokiaraj *et al.* (1994) for transforming anther-derived callus with vectors harboring the *gus* gene, *nptII*, and *cat* gene. Plasmid DNA was precipitated on to tungsten particles, loaded on to a microprojectile, and accelerated toward the target placed 5 cm below the stopping plate with a biolistic particle gun. The calli were then dark incubated for 24 h after which they were transferred to the incubation medium containing antibiotics from which kanamycin-resistant transformants were obtained. Optimization of parameters that would influence DNA delivery such as microprojectile velocity, coating mixture, and particle dispersal were carried out, which proved that microprojectiles can deliver DNA into rubber cells and helped recovery of kanamycin-resistant transformants.

2.4 Regeneration of Whole Plant

An efficient plant regeneration pathway through somatic embryogenesis is an essential prerequisite for crop improvement through transgenic approaches besides using this as a micropropagation

system (Thulaseedaran *et al.*, 2004). Wang *et al.* (1980) and Wan *et al.* (1982) successfully regenerated rubber plants through somatic embryogenesis from anther walls. Carron (1981) used inner integument tissue of seeds for somatic embryogenesis and was successful in plantlet development. Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration. Studies were also conducted to optimize cultural conditions, nutritional and hormonal requirements during somatic embryogenesis. The parameters include the effect of polyamines (El Hadrami *et al.*, 1989), hormone balance (Michaux-Ferriere and Carron, 1989), water status of the medium and explant (Etienne *et al.*, 1991a), mineral and carbohydrate nutrition (Etienne *et al.*, 1991b), interaction of growth regulators, sucrose, and calcium on callus friability (Montoro *et al.*, 1993, 1995), role of sucrose and ABA on embryo induction (Veisseire *et al.*, 1994a, b; Cailloux *et al.*, 1996; Linossier *et al.*, 1997), and carbohydrate types (Blanc *et al.*, 2002). In spite of all these studies, the plant regeneration frequency remains very low. In most of the above studies inner integument tissue was used as the explant. It is reported that the calli obtained from the integuments of immature seeds frequently display browning leading to tissue degeneration and a loss of embryogenic competence (Housti *et al.*, 1991).

There has been a renewed interest in rubber for development of techniques for plant regeneration through somatic embryogenesis especially for use in genetic transformation (Kumarijayasree *et al.*, 1999). In order to identify the suitable explant source, a variety of explants such as leaf, tender shoots, integumental tissues of immature fruit, immature anther, immature inflorescence, etc., were tried at the RRII. Extensive optimization experiments were carried out to improve the plant regeneration efficiency through somatic embryogenesis for the Indian clones of rubber. Attempts were also made to standardize the optimum growth regulator concentration and the balance between different growth regulators, the nutritional requirements, and the physical factors such as light and temperature for maximum callus proliferation, embryo induction, and subsequent regeneration of normal and healthy plantlets. As a result, initially immature anthers (before microsporogenesis) (Kumarijayasree *et al.*, 1999),

immature inflorescence (Sushamakumari *et al.*, 2000) and leaf (Kala *et al.*, 2005, 2006) were identified as suitable explant source and protocols were developed for high-frequency somatic embryo induction and plant regeneration for RRII-105, the most popular Indian rubber clone. Kumarijayasree *et al.* (1999) reported a standardized protocol for the induction of friable embryogenic callus, somatic embryogenesis, and further plant regeneration from the immature anthers. Optimum callus induction was obtained in the modified MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin. Somatic embryo induction was found to be better with 0.7 mg l^{-1} kinetin and 0.2 mg l^{-1} NAA. Further development of the embryos into plantlets was achieved on a hormone-free medium (Figure 3).

Culture conditions and other nutritional requirements for improving the efficiency of somatic embryo induction and germination were investigated by Kumarijayasree *et al.* (2001). Immature anthers precultured in a liquid medium for 10 days followed by 25-day culture in a solid medium were found to promote callus induction. The embryo induction efficiency was promoted by supplementing 200 mg l^{-1} glutamine and 400 mg l^{-1} casein hydrolysate in the embryo-induction medium. Dark incubation favored callus induction and proliferation as well as induction of embryogenesis, whereas plantlet regeneration was found to be light dependent. A study on the gibberellic acid requirement on embryo induction and maturation revealed that incorporation of GA3 up to 2.0 mg l^{-1} increased the embryo-induction frequency. Germination percentage was also significantly enhanced by higher concentrations; however, further plant development was affected by increasing GA3 levels (Kumarijayasree and Thulaseedharan, 2004). A detailed investigation was also done on the response of various cytokinins such as BA, zeatin (ZEA), kinetin and thidiazuron (TDZ) on germination of somatic embryos derived from immature anther explants. TDZ was proved to be superior to BA and ZEA while kinetin showed the least response. Maximum percentage of embryo germination and plantlet regeneration was 80% and 82%, respectively, when the medium was supplemented with TDZ (Kumarijayasree and Thulaseedharan, 2005).

Sushamakumari *et al.* (2000) studied the role of sucrose and abscisic acid (ABA) on embryogenesis

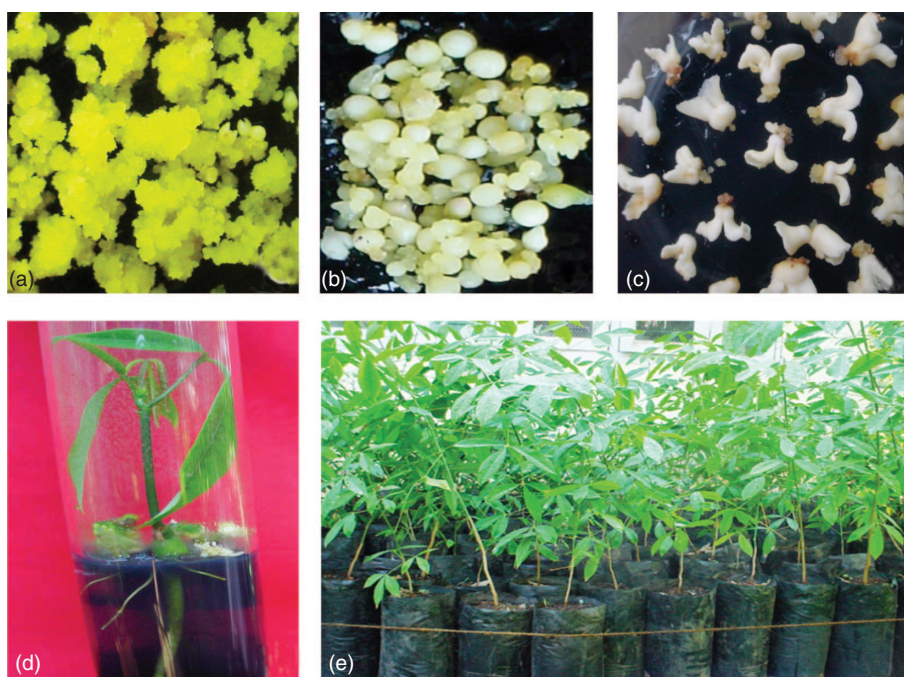


Figure 3 Somatic embryogenesis and whole plant regeneration from immature anther explants of *H. brasiliensis* (RRII 105 clone): (a) embryogenic callus; (b) globular embryos; (c) bipolar embryos; (d) full-plantlet development; and (e) somatic embryo-derived plants were established in poly bags and ready for field planting

from immature inflorescence-derived explants. A higher sucrose level was found to be essential for effective embryo induction as well as maturation. However, lower level was found to be beneficial for plant regeneration. Further, efforts have been made to enhance the embryo induction and plant regeneration frequency by the manipulation of the nutrients and hormonal combinations. Recently a system was also standardized for plant regeneration from leaf tissues of rubber where the explants are available throughout the year. Callus induction could be obtained in the MS medium with enhanced calcium nitrate (850 mg l^{-1}) and supplemented with casein hydrolysate (1.0 mg l^{-1}), B₅ vitamins, sucrose 20 g l^{-1} , and phytohormones BA (1.0 mg l^{-1}), 2,4-D, (1.5 mg l^{-1}), and NAA (0.2 mg l^{-1}). Embryo induction was obtained in the modified MS medium by the addition of amino acids, glutamine (300 mg l^{-1}), proline (100 mg l^{-1}), and arginine (37 mg l^{-1}), and organic supplements like casein hydrolysate and coconut water with phytohormones, 2.0 mg l^{-1} BA, 1.0 mg l^{-1} GA₃, 0.2 mg l^{-1} NAA and 0.1 mg l^{-1} 2,4-D. Maturation and apex induction of embryos could be obtained

in woody plant medium containing coconut water 10%, malt extract 100 mg l^{-1} , casein hydrolysate (400 mg l^{-1}) and hormones BA (0.3 mg l^{-1}) and GA₃ (1.2 mg l^{-1}). The hormone-free 1/2 MS medium helped plant regeneration (Figure 4). All media except callus induction needed the presence of activated charcoal (Kala *et al.*, 2005, 2006).

With minor modifications, the transgenic rubber callus integrated with different genes could be regenerated. At RRII protocol for the regeneration of transgenic plants integrated with the *SOD* gene under the control of different promoters were developed (Jayashree *et al.*, 2003; Sobha *et al.*, 2003a). The proliferation of the transgenic callus and embryo induction could be obtained as reported earlier (Kumarijayasree *et al.*, 1999). A combination of ABA (0.1 mg l^{-1}) and phytigel (0.4%) promoted the frequency of embryo induction. A high sucrose level was beneficial for both embryo induction as well as maturation in rubber. Addition of organic supplements and polyamines played a significant role in the induction and maturation of the embryos. Inclusion of spermine (2.0 mg l^{-1}) in the embryo-induction medium had

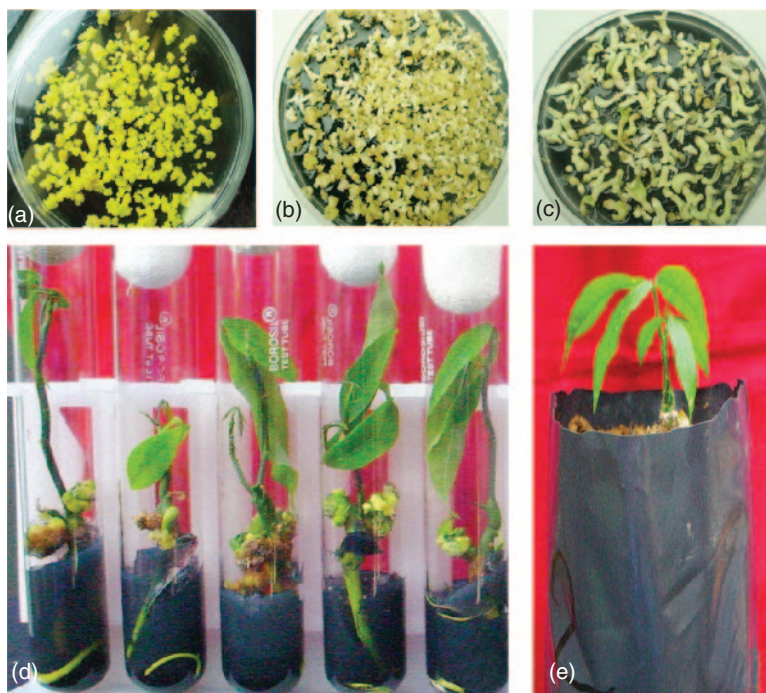


Figure 4 Somatic embryo induction and plant regeneration from leaf explants of RR11 105 clone of *H. brasiliensis*: (a) callus initiated from leaf explants; (b) embryogenic callus with embryos; (c) bipolar embryos; (d) development of full plants; and (e) a plantlet established in poly bag

a positive effect on embryogenesis. Although, casein hydrolysate (200 mg l^{-1}) was good for the embryo induction, maturation was favored by the addition of 150 mg l^{-1} banana powder. Addition of amino acids like glutamine and proline influenced the maturation frequency dramatically. Plant regeneration was promoted in the medium with reduced levels of sucrose (20 g l^{-1}) and phytagel (0.2%) (Figure 5).

3. FUTURE ROAD MAP

3.1 Expected Improved Products through Genetic Engineering

Biotechnology would play an important role in the future of the rubber industry. Conventional rubber breeding is a long process involving various methods such as selection of population followed by hybridization, progeny evaluation, and backcrossing. It takes more than 25 years to

release a new clone by using any of these methods. Rubber tree is a good candidate for transgenic manipulation due to the long breeding cycle and heterozygous nature. *In vitro* techniques including plant regeneration via somatic embryogenesis have been established in this tree species. Thus, the basic technology for genetic manipulation of rubber plant at the cellular and molecular levels is available, making rubber a suitable crop for genetic engineering. Many agronomic traits could be considered for a rubber biotechnology program, namely, high yield potential, tolerance to TPD, tolerance to environmental stresses and diseases, production of recombinant proteins, improvement in wood quality, etc.

3.1.1 Enhancement of latex yield in transgenic rubber trees

As in the case with most other crops, rubber biotechnology has focused much effort on

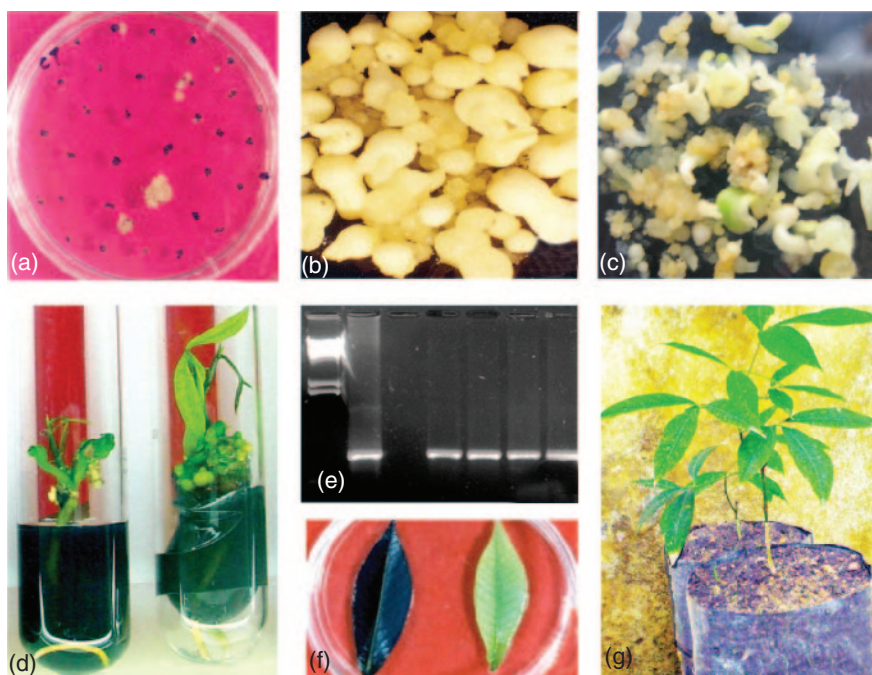


Figure 5 Development of *Hevea* transgenic plants for the overexpression of *Hb.SOD* gene using immature anther-derived callus as initial explants: (a) emergence of new transgenic callus lines on selection medium containing kanamycin (350 mg l^{-1}) and cefotaxime (500 mg l^{-1}); (b) globular embryos from transgenic callus; (c) putatively transformed bipolar embryos; (d) germination of transgenic plants; (e) PCR confirmation of transgenic plants using *nptII* gene-specific primers; (f) histochemical GUS expression analysis; and (g) transgenic plants growing in poly bags

increased latex yield. In any crop improvement program, the overall objective is to increase yield and adaptation to environmental conditions. To meet this objective, the genetic variation must be exploited by conventional breeding methods. The major limitation to crop improvement is likely to be the availability of germplasm from which desirable genes can be readily introgressed into the plant of interest. However, major improvements have been made over the last century in the productivity of rubber, as the yield of dry rubber per acre have been increased significantly by releasing better clones. There is no doubt that the conventional breeding will continue to increase the latex production in rubber trees, but a stage would eventually come when the rate of rubber biosynthesis with the tree becomes the limiting factor (Arokiaraj, 2000). At this stage, latex yield will be enhanced only by treatments designed to affect the rate of rubber biosynthesis. It was reported that the constitutive level of *hmgr1* enzyme may be a limiting factor

in rubber biosynthesis. On the basis of this hypothesis, Arokiaraj *et al.* (1995) initiated genetic transformation experiment to overexpress *hmgr1* in rubber, where *hmgr1* activity of transformed callus ranged from 70 to 410% more value of wild-type control and the activity in transformed embryos obtained ranged from 250 to 300%. However, they failed to produce transgenic plants. Among the latex associated proteins, the amount of REF in the whole latex is proportional to the rubber content (Priya *et al.*, 2006). As such, if REF protein amount is shown to be correlated simultaneously with rubber yield, there is a possibility that this relationship could be used to select for clones that are able to sustain high rubber biosynthetic efficiency. It could be possible to enhance rubber yield by overexpression of *REF* gene involved in rubber biosynthesis. At present, the authors' laboratory had undertaken a research project in which rubber biosynthesis genes such as *hmgr1*, *FDP*, *REF*, and *cis-prenyl transferase* were cloned into binary vectors and

transformation works are in progress to develop transgenic plants.

3.1.2 Transgenic plants for TPD tolerance

Plant stress is one of the major problems for crop production. In rubber tree, TPD syndrome is considered to be a physiological disorder, which greatly affects latex yield. Once the TPD occurs, the tapping incision is partly or entirely blocked and the amount of latex production is significantly decreased or stops completely. The incidence of TPD occurs in 12–50% of rubber trees in almost every rubber-planting country. Under normal conditions, active oxygen species (AOS) are efficiently scavenged by detoxifying enzymes such as SOD. Nevertheless, during TPD stress conditions this defense system becomes saturated and cellular damage is inevitable (Chrestin, 1989). The presence of excessive AOS results in severe latex yield losses. It has been reported that SOD activity protected plants from oxidative and other stresses. In order to overcome this TPD problem, an attempt was made to overexpress *SOD* gene in transgenic rubber plants. Transgenic rubber plants have been successfully developed and established in soil at the RRII (Jayashree *et al.*, 2003). Further evaluation is in progress.

3.1.3 Transgenic trees with improved wood quality

Until now, the most important product of the rubber tree was its latex and considerable efforts have been made to improve the latex yield. With the depletion of tropical forests leading to a shortage of timber for many industrial and engineering uses, attention has moved on rubber wood as an alternative source of timber for markets (Arokiaraj *et al.*, 2002b). As the composition of wood is important for the pulp industry, genetic engineering to modify lignin content in rubber is a very active area of research that has been stimulated in recent years by the characterization of important genes controlling lignification. The export of rubber wood from Malaysia rose from RM 900 million in 1993 to RM 3.7 billion in 1998 and subsequently to

RM 5.2 billion in 2001 (Arokiaraj *et al.*, 2002b). These figures clearly indicate how rubber wood industry is growing. For tree species, only limited information is available about the process of differentiation and development that are involved in wood formation. Knowledge of what determines the pathway of differentiation that cambium cells undergo is essential to any attempt to design better wood characteristics and improve latex yield. In rubber tree, the homeobox (*HB*) gene has been isolated and it is presumed that *HB* genes may be involved in differentiation of cambium cells to form latex vessels (Arokiaraj *et al.*, 2002b). Research in this direction is important because it clearly demonstrates that modifying specific genes in the wood-forming process can also potentially influence important tree characteristics and hence improve timber production.

3.1.4 Production of recombinant proteins in transgenic rubber plants

Currently, numerous immunotherapeutic proteins, antibodies, and vaccines have been produced. Recent work suggests that plants will be a facile and economic bioreactor for large-scale production of industrial and pharmaceutical recombinant proteins (Kusnadi *et al.*, 1997). Genetically engineered (transgenic) plants have several advantages as sources of proteins compared with human or animal fluids or tissues, recombinant microbes, transfected animal cell lines, or transgenic animals. These include the following: (1) efficiency of the transformation events at large scale; (2) correct assembly of multimeric antibodies (unlike bacteria); (3) increased safety, as plants do not act as hosts for human pathogens, such as human deficiency virus (HIV), prions, and hepatitis viruses; (4) production of raw materials on an agricultural scale at low cost; and (5) reduced capitalization costs relative to fermentation technology. Other than latex and timber, useful products such as serum proteins, sugars, lipids, carotenoids, inositols, organic acids, and minerals in the latex can be exploited profitably with a new research strategy. Commercial-scale production of recombinant proteins from rubber plants will also benefit from the technology and equipment

commonly used in the food and beverage industry. Already, HSA proteins were expressed in a transgenic rubber plant (Arokiaraj *et al.*, 2002b). Rubber trees synthesize enormous volume of latex upon tapping and the trees could be exploited without any destruction for large-scale production of the foreign protein throughout the year. Depending upon the promoters used, transgenic proteins will be sequestered throughout the plant or in specific parts of the plants or specific organelles within a given plant cell. A laticifer cell-specific hevein promoter has been reported earlier (Pujade-Renaud *et al.*, 2005). In this respect, the RR II has been working on isolation of laticiferous specific promoters, which will eventually enhance the recombinant protein production in transgenic rubber trees. Recently, laticiferous-specific promoter sequences for rubber elongation factor (Priya *et al.*, 2006), hevein (Saleena *et al.*, 2006), β -1,3-glucanase (AY325498), and hmgr1 (DQ785798) have been isolated and characterized. Further work is in progress to develop transgenic plants for recombinant protein production as well as for enhanced rubber production by inserting the appropriate genes under the control of laticifer cell-specific promoters.

3.2 Risks and Concerns

The introduction of transgenic crops into the existing natural system has generated a number of questions about possible negative consequences. The issues on transgenic plants can be of various groups of concerns as delineated below.

3.2.1 Damage to human health and natural environment

The possibility that we might see an increase in the number of allergenic reactions to food as a result of genetic engineering has a powerful emotional appeal. Since rubber is not used as food crop, there is no such food safety issue with transgenic rubber plants. Gene flow from transgenic crops to others requires the following: (1) the presence of sexually compatible wild relatives close to the crop; (2) an overlap of flowering times between

the crop and wild relatives and the presence of a pollinating agent such as a bird or an insect unless the likelihood that transgenes spread can be different for each crop and wild combination in different area of the world. If pollen grains from transgenic rubber plants are released in these areas they do not encounter any compatible plants to pollinate and therefore the risk of gene flow is remote. Also rubber-planting materials are developed via bud grafting, which further reduces the risk.

3.2.2 Concerns about damage to current farming practices

Hybridization of transgenic crops with nearby conventional crops raises concerns on several fronts. Movement of pollen from a transgenic field to a conventional field involves farmers in discussions about the distance required between fields to ensure purity of a crop, and about who must pay if unwanted genes move into a neighbor's crop. It will be important to ensure that hybridization is not occurring in the field. Many agencies publish recommended minimum isolation distances for a variety of crops. These distances have been decided to maintain a level of purity that has been acceptable to the agricultural community in the past. When there is a danger of gene flow to nearby fields, it is possible to prevent contamination of nearby crops by planting tall barrier plants to physically block the flow of pollen. If genetically modified (GM) pollen pollinates plants in neighboring field, then the issue of genetic trespass may arise. These issues have already prompted several lawsuits and they will continue to be a factor in the development and use of transgenic plants for years to come. As far as transgenic rubber plants are concerned, it takes about 6 years for first flowering, so the crop suitability can be assessed before pollen production. The main objective of rubber transformation is to produce plants for enhanced latex yield, TPD tolerance, disease resistance, and recombinant protein production. Also, the ultimate product from transgenic rubber plant is latex with protein and it is to be purified from latex, so neither the inserted gene nor the selection marker gene is thus available to the consumer.

3.3 Intellectual Property Rights for Transgenic Rubber Plants

The historical context of overincreasing protection for plant innovations, coupled with ongoing efforts to achieve international harmonization, provides an optimistic backdrop to some of the uncertainties that surround present efforts to obtain strong intellectual property protection for the full scope of innovations in the area of transgenic plants. An important objective of intellectual property law is to provide a reliable framework of rules, so that important commercial decisions about investigations in research may be made with a degree of certainty about how the products of the research will be protected. On the basis of this requirement for predictability, intellectual property laws are typically drafted in general terms and applied to new technology on the basis of established principles.

The requirements for patentability are generally judged with respect to the claimed invention. In addition, an invention must be within the categories of subject matter deemed patentable in a particular jurisdiction. There is also a generally recognized requirement that the description of an invention in a patent must be provided to allow a skilled technical person to make and use the invention, without the need for further innovation. Also a patentee is free to define the invention broadly in the claims and to cover various aspects of the invention, provided the requirements for patentability can be met with respect to each claimed aspect of the invention. To be new, an invention generally must not previously have been made available to the public, either through human activities or by virtue of its occurrence in nature. Many inventions are combinations of pre-existing knowledge in a way that yields an unexpected and hence patentable result. Typically, genetic inventions must be carefully characterized in claims in ways that distinguish them from a naturally occurring product. Genomic innovation in the field of transgenic plants may include genes that are novel in the sense that they have not previously been identified, genes that are novel only in the sense that their function has not previously been identified, new combinations of genetic material, such as a recombinant gene made up of a tissue-specific promoter, and a coding sequence with which it would not normally be associated

with expressed sequence tags. The exclusive right conferred by a patent in most jurisdictions includes the right to exclude others from making, using, and selling the invention defined by the claims for the term of the patent. Generally, patents have a term of 20 years from the filing date of the patent application. The innovation protectable under a plant patent can be seen as the combination of a three-step process: (a) cultivation or discovery of the plant, (b) identification of the new and distinct characteristics of the plant, and (c) asexual reproduction of the plant. Patents clearly offer the most flexible form of intellectual property protection for transgenic plants. Currently, RRII has undertaken various projects for development of transgenic plants and expected to produce soon. Once the transgenic plants are available then efforts will be taken to protect those transgenic plants intellectual property rights (IPR) by filing patent.

3.4 Public Perceptions of Transgenic Rubber Plants

Transgenic plants will continue to be developed and grown in the future, provided that a number of social and political constraints can be overcome. To understand public perceptions about transgenic plants, it is helpful to understand public perception of technology and educators have long been interested in how people perceive and understand risks associated with health and environmental issues (Wilson and Crouch, 1987). Risk perception must be considered in its social and cultural context. Many problems associated with risk management and communication result from the differences between scientists and the public (Freudenburg, 1988). The technical concept of risk is too narrow and ambiguous to serve as the crucial yardstick for policy making. Public perceptions, however, are the product of intuitive biases, economic interests, emotions, and cultural values. Technical risk must, therefore, be viewed in combination with psychological, social, and cultural processes that can heighten or reduce public perceptions of risk.

GM ingredients in food are perceived as more risky because they are considered to pose an involuntary risk. Many people believe they have very little control over food production

and processing. Furthermore, people are often concerned with secondary effects that the experts are unable to assess. Because political leaders have many of the same perceptions as other citizens, they are likely to base policy decisions on subjective factors as well. Public perception of risk is also influenced by public attitudes toward science and technology in general (Freudenburg, 1988). These problems are particularly serious as related to agriculture, because many people are no longer personally familiar with farming. Most have little understanding of how food is produced. If people are genuinely interested in a subject, such as biotechnology, they talk about it. The surveys in Europe, Canada, and the United States asked consumers to evaluate different applications of biotechnology (Hoban and Katic, 1998). The results are actually quite encouraging. The opponents of biotechnology in Europe have had the chance to tell their side of the story for several years without much balance. Educational efforts are starting to take hold among European leaders and consumers. Overall, we need to increase consumer understanding of food production and processing. However, the products from transgenic rubber plants are not consumed directly as food so there is no risk factor involved. Therefore, it could be easily explained to public how their understanding about transgenic rubber products.

3.5 Industry Perspectives

The success of GM organisms (GMOs) in the fresh-produce market in the world hinges on several factors: (1) the existence of a benefit from any GMO that is evident or can be made evident to the buyer; (2) the education of the buyer (whether a trade buyer or a consumer) about the development (process) and benefits of GMOs (this included the labeling debate); and (3) successful interaction with opponents of GMOs. The ultimate buyer is the consumer, but there are many buyers along every distribution chain. In the food business, seed companies buy materials they need to produce their seeds. Growers buy seeds and plants from the seed companies. Genetic engineering offers many potential benefits to the producer including the ability to grow a crop in hostile conditions, time savings, economic savings, economic gain, and environmental benefits. Where the growers see that

the benefit of a GMO is worth it, he will pay more for it: (1) genetically engineered drought resistance would allow production in hostile climates as well; (2) genetically engineered disease resistance can permit production in areas infested with a given disease and can reduce crop protection inputs, savings the grower money, time, and environmental impact; and (3) genetically engineered rubber plant for recombinant protein production would give additional income to the growers along with rubber.

Consumers may see some of the on-farm benefits as beneficial to society at large and a certain percentage of them might pay more for a product that can be grown with fewer crop protection chemicals. The produce industry, as any other industry, reacts to its customer's actual or potential desires. The desire for greater availability of produce led to breeding new clones that can be cultivated for high yield potential. Growers and researchers have worked to bring value-added products to the market place. Genetic engineering allows breeding to take a huge step toward specificity in trait exchange: whether the genetic enhancement benefited the industry or consumers, labeling was an issue. Labeling on the marketing side was something else entirely. It became a major consumer issue and any major consumer issue usually surfaces as a major trade issue. However, rubber is not consumed as food, so there is no such issue to the industry. When genetically engineered rubber trees are in the small-scale trial, it is quite possible that consumer issues may be discussed before entering into market.

3.6 Political and Economic Consequences

Biotechnology is increasingly affecting the competitive base for much of that industry. As with most revolutionary technological changes, biotechnology has generated both economic and political responses. The new technologies have fundamentally altered the innovation process itself. The advent of biotechnology did two things: first, it created the potential to target the research more finely to specific market needs; second, it made the research process far more complex, with no one individual or small group of individuals able to undertake the entire process. The fragmentation of the innovation system into different structures of knowledge development and

use has fundamentally affected the economics of scale and scope in the industry. Most of the fundamental patentable technologies developed have come from desperate research programs around the world and have then been assembled and commercialized by private companies. The introduction of biotechnology has also participated in a major industrial restructuring in the agrifood sector. The opportunity presented by biotechnology to manage the research process to deliver custom products has created investment potential for some private companies while creating a threat or risk to many existing business. However, rubber is produced mainly from a few Asian countries and then traded globally. As a result, there is increasing potential that traditional net exporters may both export and import. All these trends in economics of scale and scope, product attributes, and consumer demand increase the dependence of the industry on international trade. The fundamental economic changes underway in the rubber sector are creating both winners and losers. Economic studies done over the years show that research in rubber has yielded relatively high private returns and even higher public returns but that farmers tend to get a smaller share of the returns on innovations that improve yield rather than quality and their share is depressed when the related processing sector is imperfectly competitive.

Economic change often is precipitated by or compels political responses. This is especially true for changes in the rubber sector, which is traditionally viewed as a politically important constituency that produces a strategic rubber of value as a geopolitical tool. The most significant government response to the introduction of biotechnology was the extension of intellectual property rights to processes and products of biotechnology. Governments around the world have been urged to regulate biotechnology-based research and production. However, rubber biotechnology research is in the infant stage and it will take more time for practical reality to trigger political responses.

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Medicinal Plants

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1. INTRODUCTION

Plants produce a wide variety of secondary metabolites that are used as pharmaceuticals, food additives, and industrial raw materials. Chemical synthesis has been successful for certain metabolites of plant origin, such as menthol. However, as most of these compounds have complex chemical structures and their bioactivities are critically defined by their chirality, chemical synthesis can require many complicated reaction steps, and the recovery rates are not always high, leading to high costs for the synthesis of these natural products. For these reasons, the extraction of useful secondary metabolites from medicinal plants is in great demand. Transgenic medicinal plants offer new opportunities to enhance both the quantity and quality of these compounds. Transgenic studies have been conducted on numerous medicinal plant species. *Agrobacterium tumefaciens*, widely applied in transgenic studies, is also used to produce transgenic medicinal plants.

A unique feature of transgenic studies on medicinal plants compared to those on field crops, vegetables, and ornamental plants is the routine use of hairy root technology. Like *A. tumefaciens*, *Agrobacterium rhizogenes* transfers

its transfer DNA (T-DNA) from a plasmid, in this case the root-inducing (Ri) plasmid, to the host genome, inducing the formation of hairy roots (Chilton *et al.*, 1982; Nilsson and Olsson, 1997). *A. rhizogenes* can also harbor a binary vector carrying foreign genes, and the T-DNA of the binary vector can be transferred to the plant genome accompanied by the T-DNA of the Ri plasmid, enabling the production of transgenic hairy roots (Simpson *et al.*, 1986). *A. rhizogenes*-mediated transformation has several desirable features for the production of secondary metabolites. First, hairy root cultures, unlike undifferentiated cell cultures, can usually synthesize the same compounds as the roots of the intact plant. In 1986, three study groups independently demonstrated the production of secondary metabolites by hairy root cultures, including the production of tropane alkaloids by *Atropa belladonna* (Kamada *et al.*, 1986) and *Scopolia japonica* (Mano *et al.*, 1986), and nicotine by *Nicotiana rustica* (Rhodes *et al.*, 1986). Second, the rapid and efficient induction of hairy roots from explant tissues in a wide variety of plant species, including medicinal plants, has been reported. Third, plant regeneration via hairy roots has been described for important medicinal plants such as *Nicotiana tabacum* (for nicotine production), *Panax ginseng* (ginsenoside) (Yang

Table 1 Metabolic engineering of alkaloid and terpenoid biosynthesis in medicinal plants (after 2001)

Plant species	Enhanced metabolite	Introduced gene	Transformation plant/tissue	Transgenic	Reference
Tropane and nicotine alkaloids					
<i>Atropa belladonna</i>	Hyoscyamine, scopolamine	Tropinone reductase I	<i>A. rhizogenes</i>	Hairy root	Richter <i>et al.</i> , 2005
<i>Datura metal</i>	Hyoscyamine, scopolamine	Putrescine-N-methyltransferase (PMT)	<i>A. rhizogenes</i>	Hairy root	Moyano <i>et al.</i> , 2003
<i>Hyoscyamus muticus</i>	Hyoscyamine, scopolamine	PMT	<i>A. rhizogenes</i>	Hairy root	Moyano <i>et al.</i> , 2003
<i>Hyoscyamus muticus</i>	Hyoscyamine, scopolamine	PMT, hyoscyamine-6 β -hydroxylase	<i>A. rhizogenes</i>	Hairy root	Zhang <i>et al.</i> , 2004
<i>Nicotiana sylvestris</i>	Nicotine	PMT	<i>A. tumefaciens</i>	Plant	Sato <i>et al.</i> , 2001
<i>Nicotiana sylvestris</i>	Putrescine, Spermidine	PMT	<i>A. tumefaciens</i>	Plant	Sato <i>et al.</i> , 2001
Benzylisoquinoline alkaloids					
<i>Coptis japonica</i>	Berberine	(S)-scoulerine 9-O-methyltransferase	<i>A. tumefaciens</i>	Cell culture	Sato <i>et al.</i> , 2001
<i>Eschscholzia californica</i>	Columbamine Sanguinarine (decreased)	(S)-scoulerine 9-O-methyltransferase	<i>A. tumefaciens</i>	Cell culture	Sato <i>et al.</i> , 2001
<i>Eschscholzia californica</i>	Benzophenanthridine alkaloids	Norcoclaurine 6-O-methyltransferase	<i>A. tumefaciens</i>	Cell culture	Inui <i>et al.</i> , 2007
<i>Papaver somniferum</i>	Reticuline	Codeinone reductase (RNAi)	<i>A. tumefaciens</i>	Plant	Allen <i>et al.</i> , 2004
<i>Papaver somniferum</i>	Morphinan alkaloid	Codeinone reductase	<i>A. tumefaciens</i>	Plant	Larkin <i>et al.</i> , 2007
Terpenoids					
<i>Artemisia annua</i>	Artemisinin	Farnesyl diphosphate synthase	<i>A. tumefaciens</i>	Plant	Han <i>et al.</i> , 2006a
<i>Eleutherococcus senticosus</i>	Triterpene saponins, phytosterols	Squalne synthase	<i>A. tumefaciens</i>	Plant	Seo <i>et al.</i> , 2005
<i>Panax ginseng</i>	Triterpene saponins, phytosterols	Squalne synthase	<i>A. tumefaciens</i>	Plant	Lee <i>et al.</i> , 2004
<i>Panax ginseng</i>	Ginsenosides	Dammarenediol synthase (RNAi)	<i>A. tumefaciens</i>	Root, plant	Han <i>et al.</i> , 2006b

and Choi, 2000), *Papaver somniferum* (morphinan alkaloid) (Park and Facchini, 2000), *Artemisia annua* (artemisinin) (Banerjee *et al.*, 1997), *Scoparia dulcis* (scopadulcic acid) (Yamazaki *et al.*, 1996), and *Ophiorrhiza pumila* (camptothecin) (Watase *et al.*, 2004).

Bajaj (1999) compiled a review of the genetic transformation of more than 25 medicinal plants. Table 1 summarizes recent progress transgenic studies in medicinal plants. In this chapter, recently advanced topics are summarized first, and then transgenic studies on two important medicinal

plants, *Glycyrrhiza* species and *O. pumila*, are reviewed.

2. RECENT PROGRESS IN TRANSGENIC STUDIES ON MEDICINAL PLANTS

2.1 Tropane- and Nicotine Alkaloid-Producing Plants

The members of the family Solanaceae produce a range of biologically active alkaloids, including

nicotine and tropane alkaloids. Tropane alkaloids, such as hyoscyamine and scopolamine, which are mainly found in *Atropa*, *Duboisia*, *Hyoscyamus*, and *Scopolia* species, together with their semi-synthetic derivatives, are useful as parasympatholytics that competitively antagonize acetylcholine (Yamada and Tabata, 1997). The ease of a tissue culture procedure depends on the plant species. The tissue culture of both *A. belladonna* and *Nicotiana* species is straightforward. Because these two plant species produce important alkaloids, tropane and nicotine alkaloids, respectively, various *in vitro* methods for these plants have been developed (Bajaj, 1999). In the early 1990s, the overproduction of hyoscyamine-6 β -hydroxylase (H6H) in *A. belladonna* was found to efficiently convert hyoscyamine to scopolamine in both transgenic plants (Yun *et al.*, 1992) and transgenic hairy roots (Hashimoto *et al.*, 1993). In tobacco, increasing the activity of ornithine decarboxylase in *N. rustica* can result in increased nicotine content (Hamill *et al.*, 1990). The N-methylation of putrescine catalyzed by putrescine N-methyltransferase (PMT) is the first committed step in the biosynthesis of both tropane and nicotine alkaloids. The overexpression of the tobacco PMT cDNA (complementary DNA) in *Nicotiana sylvestris* increased its nicotine content, whereas the suppression of endogenous PMT activity severely decreased the nicotine content (Sato *et al.*, 2001). To effectively control metabolic flow, it is important to regulate the genes that encode the enzymes in a given pathway.

Oksman-Caldentey's group also investigated intensively the metabolic engineering of tropane and nicotine alkaloids. The H6H gene alone or in combination with other genes has been further transferred to different tropane-alkaloid producing plant species. Transgenic root cultures of *Hyoscyamus muticus* with H6H gene were able to produce over 100 times more scopolamine than the control culture (Jouhikainen *et al.*, 1999). While overexpression of only PMT gene in *H. muticus* and *Datura metel* resulted in only slight changes on tropane alkaloid levels (Moyano *et al.*, 2003), when both PMT and H6H genes were simultaneously overexpressed in the hairy roots of *Hyoscyamus niger*, a significantly high scopolamine production was achieved (Zhang *et al.*, 2004).

2.2 Useful Terpenoid-Producing Plants

Terpenoids (isoprenoids) belong to a large chemical family of more than 20 000 compounds that have been isolated from various plants. Plant terpenoids are produced in the cytosol, plastids, and mitochondria. All isoprenoids are biosynthesized from common C5 isoprene units: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Unlike animals, plants synthesize IPP and DMAPP via two pathways, the cytosolic mevalonate (MVA) pathway and the plastidal 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. Sesquiterpenes, triterpenes, and sterols are biosynthesized via the MVA pathway, whereas monoterpenes, diterpenes, carotenoids, and chlorophyll side chains are biosynthesized via the MEP pathway.

A. annua (Asteraceae) has a long history of use in traditional Chinese medicine. Artemisinin, a sesquiterpene lactone, is a new and highly effective antimalarial drug (Klayman, 1985). Because *A. annua* is currently the only source of artemisinin, extensive chemical and molecular genetic studies focused on sesquiterpenoid biosynthesis in this plant have been performed. Several transformation protocols for *A. annua* using *A. tumefaciens* (Han *et al.*, 2005) and *A. rhizogenes* (Banerjee *et al.*, 1997) have been reported. Farnesyl diphosphate is a general precursor of sesquiterpenoids. When farnesyl diphosphate synthase was overexpressed in *A. annua*, the plants synthesized 30% more artemisinin than nontransgenic *A. annua* (Han *et al.*, 2006a). Recently, two enzymes, amorpho-4,11-diene synthase (ADS), one of the sesquiterpene synthases (Mercke *et al.*, 2000), and CYP71AV1, a P450 monooxygenase that oxidizes amorpho-4,11-diene to artemisinic acid (Ro *et al.*, 2006; Teoh *et al.*, 2006), have been shown to have key roles in artemisinin biosynthesis. A study of transgenic plants overexpressing both ADS and CYP71AV1 would be of interest.

Triterpenes exhibit a wide range of structural diversity and biological activities, and these saponin glycosides are economically important as natural medicines. Squalene synthase (SS) catalyzes the first enzymatic step toward both triterpenoid and sterol biosynthesis (Abe *et al.*, 1993). Increased synthesis of SS in both *P. ginseng* (Lee *et al.*, 2004) and *Eleutherococcus senticosus*

(Seo *et al.*, 2005) enhanced the triterpenoid and sterol levels in transgenic plants of both species. Triterpenoids and sterols are formed by the cyclization of 2,3-oxidosqualene. This cyclization, which is catalyzed by oxidosqualene cyclases (OSCs), is one of the most complicated and fascinating reactions found in nature. Whereas OSCs in vertebrates and fungi only convert 2,3-oxidosqualene into lanosterol, a sterol precursor, OSCs in plants convert 2,3-oxidosqualene into cycloartenol and some other cyclic triterpenes (Suzuki *et al.*, 2006; Suzuki and Muranaka, 2007). Plant triterpenes have diverse structures with more than 100 different carbon skeletons, and plants correspondingly have several genes that encode OSCs. Han *et al.* (2006b) isolated dammarediol synthase (DDS), one of the OSCs of *P. ginseng*. This study demonstrated that the treatment of transgenic *P. ginseng* with DDS RNAi results in the silencing of DDS expression, which leads to reduced ginsenoside production in roots. These results suggest the possibility of changing triterpenoid profiles by manipulating a specific OSC. The following section details tissue culture and transgenic studies of *Glycyrrhiza* plants, important triterpene-saponin producing plants that could be used as practical model plants.

2.3 Glycyrrhiza Species

2.3.1 *In vitro* studies of *Glycyrrhiza* plants

The dried root and subterranean stem (stolon) of some species of *Glycyrrhiza* plants (licorice, Leguminosae; Figure 1) are some of the most important drugs used in oriental traditional medicine as anti-inflammatory, antiallergenic, and antiulcer agents. Licorice is also an important commercial product used as a sweetener and a flavor in many foods, tobacco, and confections. The root and stolon contain several compounds of interest, the most important of which is glycyrrhizin (Figure 2), a triterpene saponin. Licorice can grow in only limited regions, including semi-arid areas of southern Europe and Asia. Recently, however, the exhaustion of natural resources and desertification has occurred in these regions. Most licorice is harvested from wild plants or cultivated under intensive farming. Because the quantity and the quality of these products are



Figure 1 Plants of *Glycyrrhiza uralensis* (In the research field in Division of Tsukuba, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Japan)

not sufficiently stable for use in drugs, demand for breeding modern licorice cultivars has recently increased. The development of a rapid *in vitro* propagation method is necessary to breed licorice, and research is also needed to obtain large biomass and secondary metabolite (e.g., glycyrrhizin) yields in a *Glycyrrhiza* tissue culture system.

2.3.2 Micropropagation

A few reports on the *in vitro* propagation of *Glycyrrhiza* plants have been published (Table 2). *In vitro* propagation methods using *Glycyrrhiza* nodal stem segments or other explants are summarized in Henry *et al.* (1991). Multiple shoots produced from *Glycyrrhiza glabra* axillary buds excised from growing plants have been reported (Kohjyouma *et al.*, 1995; Figure 3), and Kakutani *et al.*

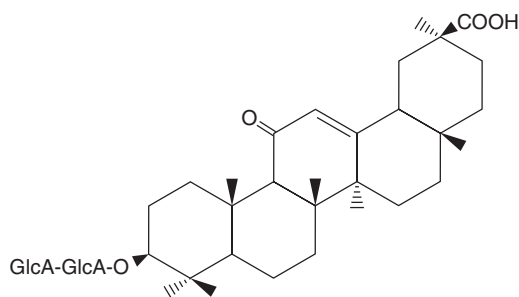


Figure 2 Chemical structure of glycyrrhizin

Table 2 *In vitro* studies of *Glycyrrhiza* plants^(a)

Plant species	Purpose	Explants	Medium ^(b)	State	Secondary metabolites	Reference
<i>G. glabra</i>	Micropropagation	Nodal stem	Modified MS+ sucrose+NAA+BA	Single shoot, multiple shoots		Kohjyouma <i>et al.</i> , 1995
<i>G. echinata</i>	Micropropagation	Leaves	MS+sucrose+NAA+BA+2,4-D	Somatic embryogenesis		Kakutani <i>et al.</i> , 1999
<i>G. uralensis</i>	Micropropagation	Nodal stem of seedling	MS+sucrose+NAA	Cultured stolon		Kojoma <i>et al.</i> , 2005
<i>G. glabra</i>	Growth of suspension cultures	Roots, shoots, leaves of seedlings	B5+sucrose+2,4-D+kinetin	Callus, suspension cultures		Aris-Castro <i>et al.</i> , 1993a
<i>G. glabra</i>	Biotransformation of 18 β -glycyrrhetic acid	Young roots	LS+sucrose+NAA+BA	Suspension cultures	Triterpene	Hayashi <i>et al.</i> , 1990a
<i>G. glabra</i>	Production of secondary metabolites		LS+sucrose+NAA+BA	Suspension cultures	Triterpene (soyasaponin I and II)	Hayashi <i>et al.</i> , 1990b
<i>G. glabra</i>	Production of secondary metabolites	Roots, shoots, leaves of seedlings	B5+sucrose+2,4-D+kinetin	Suspension cultures	Isoflavonoid (formononetin)	Aris-Castro <i>et al.</i> , 1993b
<i>G. glabra</i>	Detection of triterpene glucuronosyl-transferase activities		LS+sucrose+NAA+BA	Suspension cultures	Triterpene (soyasapogenol B)	Hayashi <i>et al.</i> , 1996
<i>G. echinata</i>	Production of secondary metabolites	Leaves and petioles	MS+sucrose+ 2,4-D+kinetin+Yeast extract	Callus	Isoflavonoid (medicarpin)	Nakamura <i>et al.</i> , 1999
<i>G. glabra</i>	Production of secondary metabolites	Roots, hypocotyl stems and leaves	LS+sucrose + 2,4-D+kinetin+cork tissue	Suspension cultures	Lipophilic and hydrophilic flavonoids	Yamamoto <i>et al.</i> , 2001
<i>G. echinata</i>	cDNA cloning isoflavonoid biosynthesis		1/2MS+sucrose+NAA+BA	Suspension cultures	Isoflavonoid	Akashi <i>et al.</i> , 1999
<i>G. glabra</i>	cDNA cloning of β -amyrin biosynthesis		LS+sucrose+NAA+BA	Suspension cultures	Triterpene saponin	Hayashi <i>et al.</i> , 2001
<i>G. glabra</i>	Gene expression of soyasaponin biosynthesis		LS+sucrose+NAA+BA+MeJA	Suspension cultures	Triterpene saponin	Hayashi <i>et al.</i> , 2003
<i>G. echinata</i>	cDNA cloning hormononetin biosynthesis		1/2MS+sucrose+NAA+BA	Suspension cultures	Isoflavonoid	Akashi <i>et al.</i> , 2003
<i>G. echinata</i>	Gene expression of glucosyltransferase		MS+sucrose+IAA+kinetin+Yeast extract	Suspension cultures	Isoflavonoid	Nagashima <i>et al.</i> , 2004
<i>G. glabra</i>	Gene expression of triterpene biosynthesis		LS+sucrose+NAA+BA+MeJA	Suspension cultures	Triterpene saponin	Hayashi <i>et al.</i> , 2004

^(a)Studies before 1990 were summarized by Henry *et al.* (1991)

^(b)MS, Murashige and Skoog (1962); LS, Linsmaier and Skoog (1965); B5, Gamborg *et al.* (1968); NAA, naphthaleneacetic acid; IAA, indole-acetic acid; BA, benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MeJ, methyl jasmonate

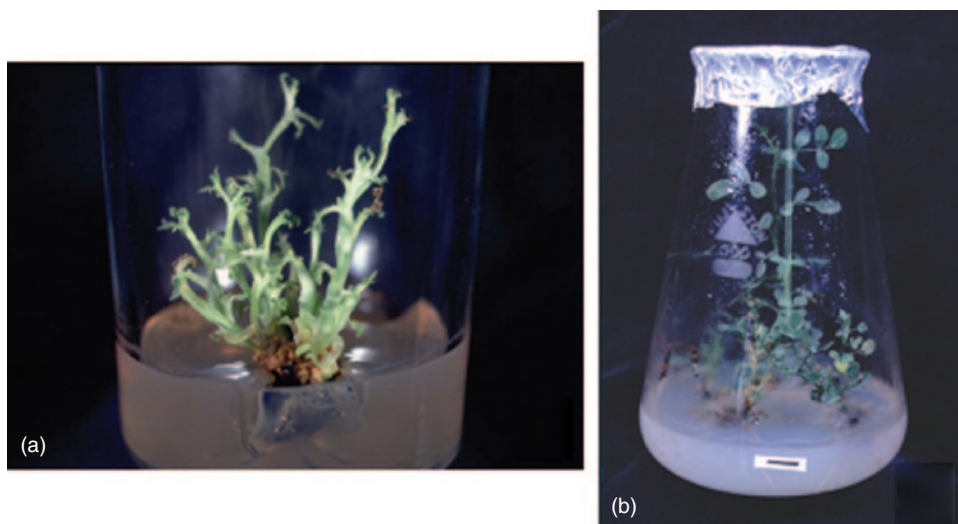


Figure 3 Multiple shoots developed from an axillary bud of *Glycyrrhiza glabra* (a). Regenerated plantlets with adventitious roots (b)

(1999) described the somatic embryogenesis of *Glycyrrhiza echinata* and *Glycyrrhiza squamulosa*.

In vitro stolon cultures of *Glycyrrhiza uralensis* have been established (Figures 4 and 5; Kojoma *et al.*, 2005). The formation of a stolon was induced from a single stem node in Murashige and Skoog (MS) liquid medium supplemented with $0.01 \mu\text{M}$ α -naphthaleneacetic acid (NAA) and grown in darkness. This concentration of NAA and 6% sucrose comprised the best conditions for proliferating the stolon. Adventitious root and shoot regeneration from cultured stolons were easily achieved on MS medium containing $0.01 \mu\text{M}$ NAA and 0.2% Gelrite and culture in light.

2.3.3 Secondary metabolites

Several chemical studies have been conducted on callus and suspension cultures of *Glycyrrhiza* plants (Table 3; the reports published before 1990 are summarized in Henry *et al.*, 1991). Hayashi *et al.* (1988, 1990a, b) reported the biosynthesis of triterpene, betulinic acid, and soyasaponins I and II in *G. glabra* cell suspension cultures, and Aris-Castro *et al.* (1993a, b) described the production of the isoflavonoid formononetin. Chalcones and isoflavones were isolated from callus cultures of *G. uralensis* (Kobayashi *et al.*,

1985), and flavonoid production in cell suspension cultures of *G. echinata* was investigated (Ayabe *et al.*, 1980, 1986; Nakamura *et al.*, 1999).

A few reports have described glycyrrhizin production in cultured tissues (Tamaki *et al.*, 1973, 1975 in, e.g., *G. glabra*, *G. uralensis*, and *G. echinata*; Fujita *et al.*, 1978 in various *Glycyrrhiza* species; Ko *et al.*, 1989 in *G. uralensis*). However, no efforts have been made to produce glycyrrhizin in cultured tissues since that of Ko *et al.* (1989).

2.3.4 Transgenic studies

Although no study has examined the establishment of transgenic *Glycyrrhiza* plantlets, the development of *Glycyrrhiza* hairy root cultures by transformation with *A. rhizogenes* containing Ri plasmids has been reported (Table 3). Many flavonoids have been isolated from *G. glabra* hairy roots by Asada *et al.* (1998, 1999, 2000) and Li *et al.* (2000). Toivonen and Rosenqvist (1995) reported the production of phenolic substances.

Saito *et al.* (1990) showed β -glucuronidase (GUS) reporter gene expression in *G. uralensis*. A hairy root culture was also established by infecting cultured *G. uralensis* stolons (Kojoma *et al.*, 2005). In these hairy root cultures, green fluorescent protein (GFP) reporter gene expression was detectable (Figure 6; Kojoma *et al.*, 2003).

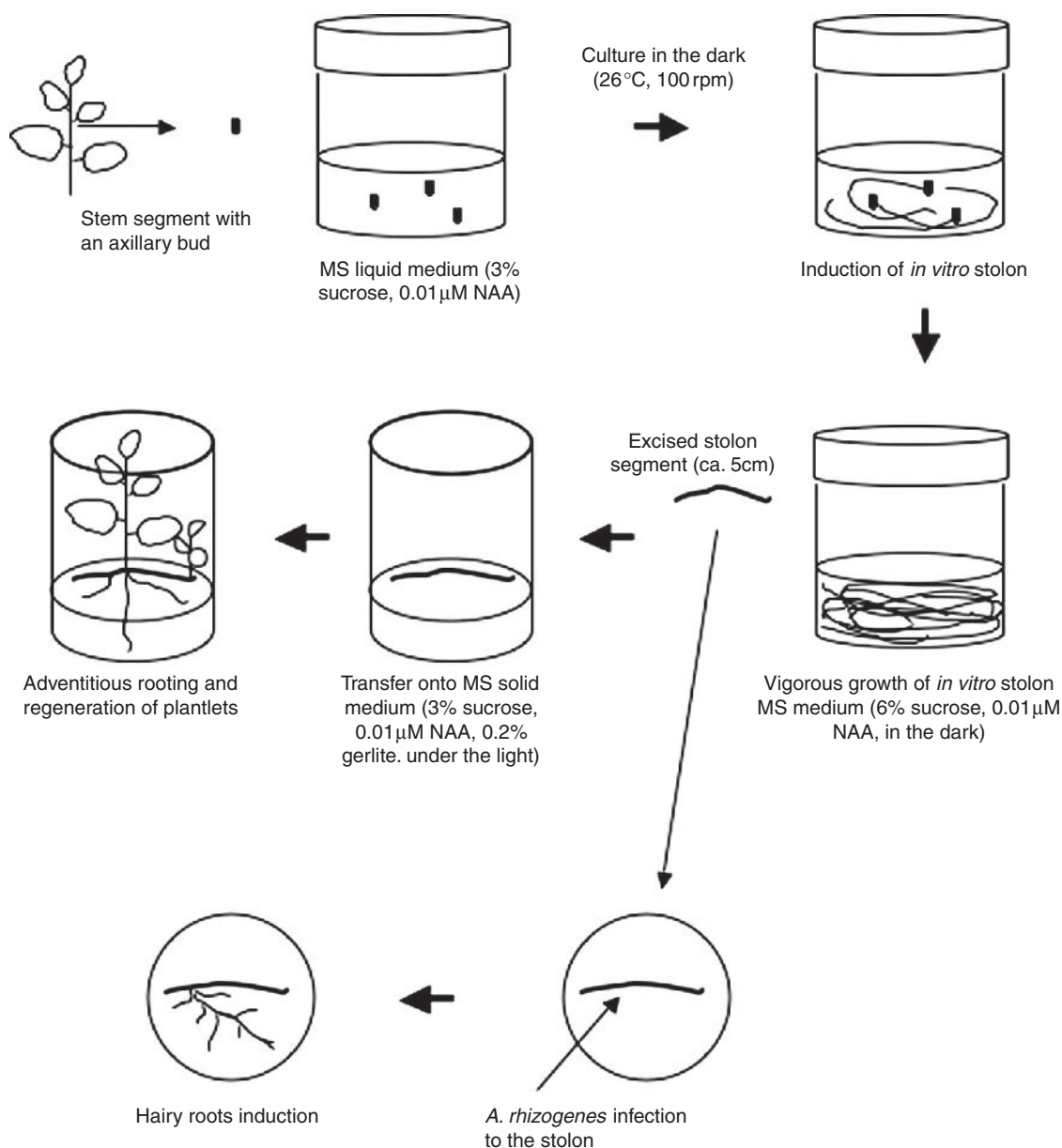


Figure 4 Schematic of the *in vitro* stolon formation and hairy roots induction

2.4 Ophiorrhiza pumila

2.4.1 Camptothecin-producing plants

Camptothecin (CPT) (Figure 7: 1) is one of most important antineoplastic alkaloids of plant origin, and its semi-synthetic water-soluble analogs topotecan (Figure 7: 2) and irinotecan (Figure 7: 3)

are used as clinical antitumor agents throughout the world. The worldwide market of irinotecan/topotecan is currently 1 billion US dollars. In spite of the rapid growth of the market, CPT is still extracted from the seeds of *Camptotheca acuminata* and the bark of *Nothapodytes foetida*.

CPT was first isolated from *C. acuminata* during a screening for antitumor agents by the



Figure 5 *In vitro* cultured stolon from a nodal stem segment of *Glycyrrhiza uralensis* in MS liquid medium with 0.01 μ M NAA

National Cancer Institute in the United States (Wall *et al.*, 1966). CPT has been found in not only *C. acuminata* (Nyssaceae) (Figure 8a) and *N. foetida* (Icacinaeae) (Figure 8b) but also in several other plant species belonging to various plant families, including the Rubiaceae, Apocynaceae, and Gelsemiaceae. The genus *Ophiorrhiza* (Rubiaceae) is widely distributed throughout tropical and temperate Asia, and 150 species belong to this genus. CPT has been detected in four species: *O. pumila* (Figure 8c), *Ophiorrhiza liukuensis* (Figure 8d), *Ophiorrhiza kuroiwa*, and *Ophiorrhiza mungos*.

2.4.2 CPT production by *O. pumila* hairy root cultures

Although Sakato *et al.* (1974) first reported on the establishment of *C. acuminata* cell suspension cultures, however, the levels of CPT production in

these cultures were insufficient. Hairy root cultures induced by *A. rhizogenes* are an attractive method for producing CPT because of their potential for stable, high yields. Saito *et al.* (2001) developed the first feasible CPT production system in *O. pumila* hairy root cultures. In these hairy roots, CPT accumulated at levels similar to those in the roots of the original plants and was excreted into the culture medium in large quantities. These hairy root cultures have been grown in 3-L bioreactors for the production of CPT (Sudo *et al.*, 2002). In addition, *C. acuminata* hairy roots that produce an equal level of CPT have been reported (Lorence *et al.*, 2004), but no studies have reported on hairy root cultures of *N. foetida*, the main species used for the production of CPT in Japan.

2.4.3 *O. pumila* genetic transformation and plant regeneration

For better production of CPT and further genetic engineering, regeneration protocols for transformed plants of the above CPT-producing species are needed. Watase *et al.* (2004) reported a method for plant regeneration from *O. pumila* hairy roots. This group infected sections of *O. pumila* shoot cultures with *A. rhizogenes* strain 15835. The established hairy root cultures were subcultured in fresh B5 (Gamborg *et al.*, 1968) gellan gum-solidified medium once per month and grown under dark conditions. Hairy roots of about 1 month of age spontaneously developed several shoot buds in darkness. The hairy root cultures with shoot buds were exposed to light (Figure 9a), and the shoot buds that turned green were transferred to half-strength MS (Murashige and Skoog, 1962) solid medium containing 1% sucrose for rooting. The regeneration frequency was over 83%: 33 lines from 40 randomly selected hairy roots regenerated. The regenerated plants showed typical features of hairy root-derived plants, including short internodes and malformed leaves (Figure 9b). The transformed plants accumulated CPT in amounts 66–111% of those in wild-type plants. To date, this is the only report describing the generation of transgenic CPT-producing plants.

2.4.4 The future of CPT research

Although the skeleton of CPT belongs to the quinoline alkaloid group, it is synthesized

Table 3 Summary of transgenic studies of *Glycyrrhiza* plants with *Agrobacterium rhizogenes*

Plant species	Strain of <i>Agrobacterium</i>	Vector	Part of infection	Chemical compound	Reference
<i>G. uralensis</i>	ATCC 15834		Aseptic plantlet and stem	Glycyrrhizin	Ko <i>et al.</i> , 1989
<i>G. uralensis</i>		pRi 15834, pGSGlucI	Hypocotyl of seedling (4 days old)	Not detected glycyrrhizin	Saito <i>et al.</i> , 1990
<i>G. uralensis</i>	MAFF 03-01724		Stem of aseptic plantlet	Polysaccharide	Nose <i>et al.</i> , 1998
<i>G. glabra</i>	A4, LBA/9402, C58C1 (pRT GUS 104)		Seedling (three weeks old)	Liquiritigenin, isoliquiritigenin	Toivonen and Rosenqvist, 1995
<i>G. glabra</i>	A4 (agropine type)	pRi 15834, pBI 121 (GUS)	Callus from root	Flavonoid (licoagrodine, etc.)	Li <i>et al.</i> , 1998
<i>G. glabra</i>	A4 (agropine type)	pRi 15834, pBI 121 (GUS)	Callus from root	Flavonoid (Licoagrochalcone A, licoagrocarpin, etc.)	Asada <i>et al.</i> , 1998
<i>G. glabra</i>	A4 (agropine type)	pRi 15834, pBI 121 (GUS)	Callus from root	Flavonoid (licoagrone, etc.)	Asada <i>et al.</i> , 1999
<i>G. glabra</i>	A4 (agropine type)	pRi 15834, pBI 121 (GUS)	Callus from root	Flavanone (glabrol)	Asada <i>et al.</i> , 2000
<i>G. glabra</i>	A4 (agropine type)	pRi 15834, pBI 121 (GUS)	Callus from root	Licoagrochalcone B, etc.	Li <i>et al.</i> , 2000
<i>G. pallidiflora</i>		pRi 15834, pGSGlucI (GUS)	Aseptic young plant	Isoflavone (licoagroisoflavone, etc.)	Li <i>et al.</i> , 2001
<i>G. pallidiflora</i>		pRi 15834, pGSGlucI (GUS)	Aseptic young plant	Licoagroside D, etc.	Li <i>et al.</i> , 2002

via strictosidine, a common intermediate of monoterpenoid indole alkaloids (Hutchinson *et al.*, 1979). Some of the genes that encode the key enzymes involved in the biosynthesis of these alkaloids have been identified. The cDNAs encoding strictosidine synthase (STR) and tryptophan decarboxylase (TDC) have been isolated from *Rauvolfia serpentina* (Kutchan *et al.*, 1988), *Catharanthus roseus* (De Luca *et al.*, 1989; McKnight *et al.*, 1990), and *O. pumila* (Yamazaki *et al.*, 2003a). However, the biosynthetic pathway after strictosidine to CPT and the regulatory steps of the biosynthetic pathway are still unclear (Figure 10). Three plausible precursors of CPT, 3(*S*)-pumiloside and 3(*S*)- and 3(*R*)-deoxypumiloside, have been isolated from *O. pumila* (Aimi *et al.*, 1989; Kitajima *et al.*, 1997). A feeding study with *O. pumila* hairy roots revealed that the secologanin moiety of CPT is synthesized via the MEP pathway (Yamazaki *et al.*, 2004). The enzymatic activity of STR and the expression

of the corresponding mRNA were measured in several *O. pumila* tissues, callus, and hairy roots, and compared to the metabolic profiles of those tissues. The patterns of enzymatic activity of STR and the expression of its mRNA were roughly correlated with the accumulation of CPT (Yamazaki *et al.*, 2003b). *O. pumila* will be a suitable species for metabolic studies of CPT-producing plants because it is straightforward to obtain transformed tissue and genetically transformed plants of this species.

3. FUTURE PROSPECTS OF TRANSGENIC MEDICINAL PLANT DEVELOPMENT

In this chapter, recent advances in transgenic studies on medicinal plants were summarized, and detailed studies on the tissue culture and transgenic plant generation of two important medicinal plants with potential use as practical models

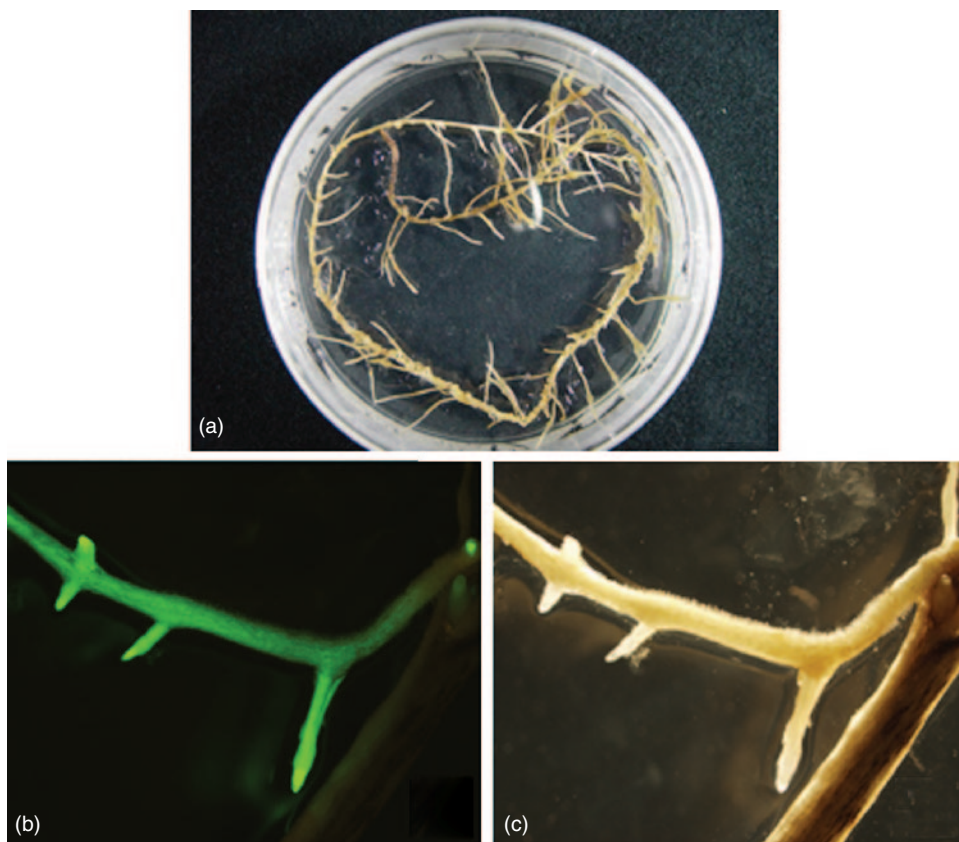


Figure 6 Hairy roots from the cultured stolon of *Glycyrrhiza uralensis* infected with *Agrobacterium rhizogenes*. (a) Light (b) and fluorescent micrographs (c) of GFP-expressing hairy roots

were described. Although routine transgenic plant generation protocols are available for some medicinal plants, efforts are continuing to establish efficient protocols for *Glycyrrhiza* species and *O. pumila*. Research on each step in a tissue culture

protocol is still important for success in this field. Recently, a high-throughput system for activation tagging in transformed hairy roots was established (Seki *et al.*, 2005). This approach will identify genes involved in root-specific secondary metabolites or

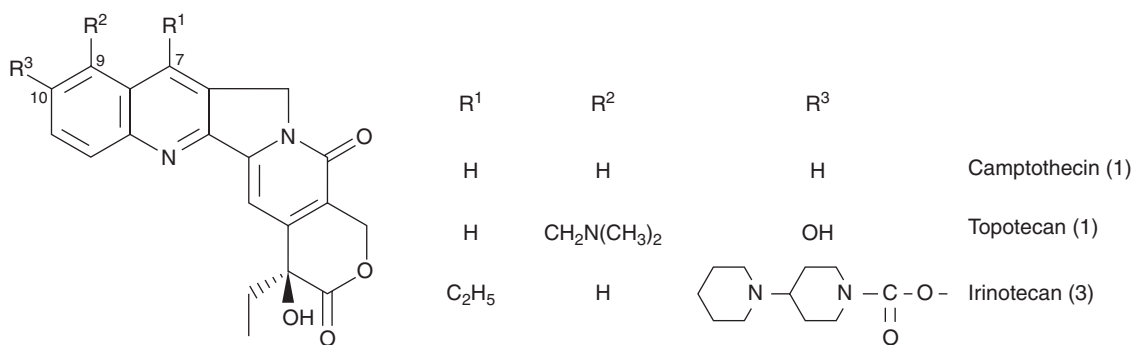


Figure 7 Camptothecin and derivatives



Figure 8 Camptothecin producing plants. (a) *Camptotheca acuminata*, (b) *Nothapodytes foetida*, (c) *Ophiorrhiza pumila*, (d) *Ophiorrhiza liukiensis*

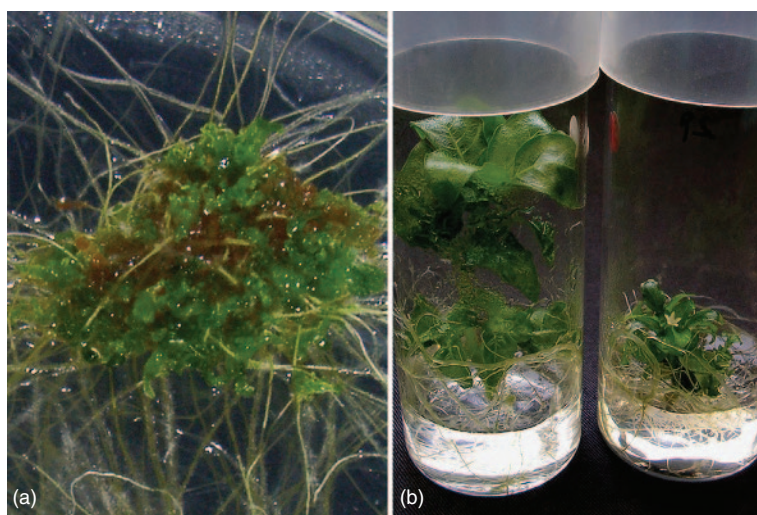


Figure 9 Regeneration of transgenic shoots from hairy roots of *O. pumila*. (a) Shoots primordia emerged from hairy roots after 5 weeks of culture under light conditions. (b) *In vitro* cultures of the transgenic plant derived from hairy root (right) and wild-type plant (left)

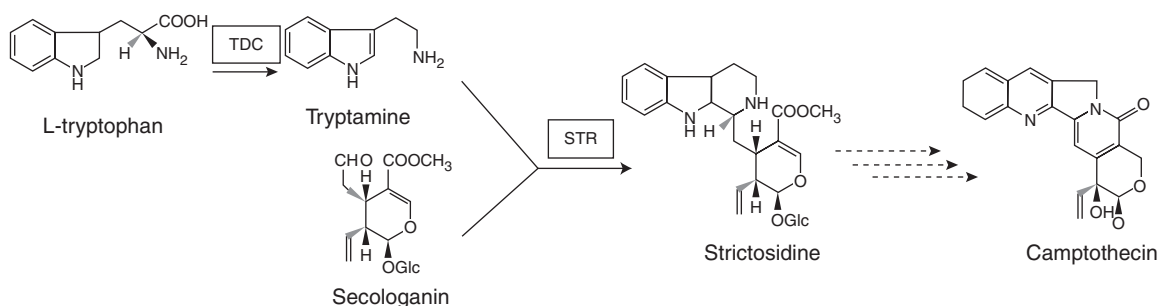


Figure 10 The predicted biosynthesis pathway of camptothecin in *O. pumila*. The enzymes are: TDC, tryptophan decarboxylase; STR, strictosidine synthase

secretion in various medicinal plants. Although a large amount of genome and gene expression information is available for several plant species, such information is still limited for many medicinal plants. As described here, transgenic medicinal plants offer new opportunities to enhance both the quantity and quality of useful compounds. For this purpose, it is important to determine the general concepts and methodology to apply this abundant information to medicinal plants of interest.

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Ornamentals

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1. INTRODUCTION

Substantial economic returns are generated by the sales of cut flowers, potted, and bare-rooted ornamental plants. The revenue generated for cut flowers alone exceeds US\$27 billion per annum, representing about one-third of the total market income (Chandler, 2003; Tanaka *et al.*, 2005). Consumer demand for greater novelty, particularly in terms of flower form and color, drives the search for new genetic material. Conventional breeding, through sexual hybridization and selection, has maintained the generation of this genetic novelty and it is unlikely that plant breeders will lose their momentum in this area. More recently, biotechnological approaches have been developed that are an important adjunct to conventional approaches. Such procedures, based on the robust culture of totipotent cells and tissues, include somatic hybridization through protoplast fusion and transformation exploiting recombinant-DNA technology. Exposure of genetic variation through the culture of somatic cells may be superimposed on these gene transfer procedures. Whilst there has been less input of effort and finance into the application of biotechnology to ornamental plants compared to the massive investment into crops for human nutrition and animal feed, it is certain that biotechnological procedures also have considerable potential for improving ornamental plants. Such approaches will be exploited increasingly in the immediate future.

Several excellent reviews have been published recently that summarize the extensive literature relating to plant cell culture and transformation of ornamental plants, to which the reader is referred in the present text. For example, Li and Pei (2006a, b) have edited two volumes devoted to aspects of transformation technologies as applied to ornamental plants, while Teixeira da Silva (2006a) has edited an excellent series of four volumes devoted to all aspects of the biotechnology of ornamental plants. Additionally, the journal “*Global Plant Letters*”, also edited by Teixeira da Silva (2006b) and initiated in 2006 to coincide with the formation of the International Society for Floricultural and Ornamental Plants (ISFOP), is intended to raise the profile of ornamental plants and related research. Consequently, the present article directs the reader to some of the most recent and relevant publications.

2. DEVELOPMENT OF TRANSGENIC ORNAMENTALS

2.1 Targets for Genetic Manipulation of Ornamental Plants

Harriman *et al.* (2006) discussed the ways that the horticultural industry may benefit from current state-of-the-art biotechnological procedures. Micropropagation has facilitated the mass

propagation of elite plants, with more sophisticated recombinant-DNA procedures being superimposed on this technology in recent years. The targets for transformation of ornamental plants by somatic cell techniques parallel, in many ways, those of conventional breeding. Additionally, the targets for ornamentals are similar to those for other crops with the need to improve tolerance to abiotic stresses such as drought, cold, heat, salinity, and light, and to increase disease resistance. Additional traits for modification that are specific to ornamentals, include flower color, scent, longevity following cutting and alteration of plant morphology. Modification of physiology through endogenous growth regulators has the potential to generate changes beneficial to the horticultural industry.

Molecular fingerprinting procedures have become important tools in clarifying the parentage and genetic relationships of existing species and cultivars, and in identifying plants most suitable as donors for introgressing horticulturally important traits into existing species and cultivars through both sexual hybridization and cell culture-based technologies (Handa *et al.*, 2006). For example, Aros *et al.* (2006) used random amplified polymorphic DNA (RAPD) analysis to discriminate nine wild *Alstroemeria* accessions and 10 commercial varieties, while Chen *et al.* (2006a) employed amplified fragment length polymorphism (AFLP) markers to detect genetic differences in 19 cultivars of tissue culture-derived somaclonal variants of *Syngonium podophyllum*. Similarly, Chen *et al.* (2006c) used sequence tag site (STS) markers developed through AFLP fingerprinting to distinguish new cultivars of orchids of the genus *Oncidium*. This technology has application in distinguishing existing and new cultivars, especially in relation to Plant Breeders' Rights. Scariot *et al.* (2006) exploited polymorphic sequence-tagged microsatellite sites (STMs) to characterize 65 accessions of old garden roses. Importantly, the seven major clusters developed from these analyses were consistent with earlier grouping of the roses into botanical sections and horticultural groups. RAPD analyses based on morphology, physiological and ecological characteristics were used to substantiate the classification of oriental *Cymbidium* species (Choi *et al.*, 2006).

2.2 Somatic Cell Techniques for Genetic Manipulation of Ornamentals

2.2.1 Micropropagation, organogenesis, and somatic embryogenesis

Robust culture procedures that enable cells and tissues to express their totipotency through organogenesis and/or somatic embryogenesis are fundamental to the generation of novel plants by somatic cell approaches. Rout *et al.* (2006) reviewed the literature until October 2005 and concluded that approximately 156 ornamental genera are micropropagated through the culture of meristems, axillary buds, and shoot tips. These authors provided a comprehensive table listing the micropropagation of major ornamental species. An added advantage of meristem culture is that it is a procedure for virus elimination. Other explants used routinely for ornamental species include those from tubers, leaf laminae, and petioles. Rout *et al.* (2006) also summarized the effects of physical factors, including illumination and semisolid and liquid culture media, on shoot regeneration by organogenesis and/or somatic embryogenesis, with a summary of the major ornamentals in which somatic embryogenesis is the primary pathway of plant regeneration. Detailed case studies were discussed for *Ficus* and *Yucca* spp., African violet, begonia, chrysanthemum, cyclamen, and rose. Other topics of this excellent review included aspects of germplasm conservation, *in vitro* mutagenesis, cryopreservation, and somaclonal variation. Other authors have focused attention upon high-value crops, particularly chrysanthemum, since it is one of the most important global cut flowers and pot plants. Thus, Teixeira da Silva (2003) presented an outstanding and comprehensive review of the advances achieved in the culture, cryopreservation, postharvest technology, synthetic seed production, genetics, and transformation approaches of ornamental plants.

Hazarika (2006) discussed some of the morphological and physiological disorders associated with *in vitro*-grown plants and the influence of these abnormalities on the acclimation of regenerated plants to *ex vitro* conditions. More recent papers on the tissue culture of ornamentals emphasize the diversity of target species. For example,

investigations have focused on plant regeneration by somatic embryogenesis in *Catharanthus roseus* (Junaid *et al.*, 2006), *Lilium* × *formolongi* (Ho *et al.*, 2006), *Oncidium* (Jheng *et al.*, 2006), *Euphorbia pulcherrima* (Castellanos *et al.*, 2006), and *Syngonium podophyllum* (Zhang *et al.*, 2006). Polyamines and L-ornithine influence the development of embryogenic tissues in *Cryptomeria japonica* (Nakagawa *et al.*, 2006). Other reports describing plant regeneration by organogenesis, relate to the genera *Acacia* (Yang *et al.*, 2006), *Kniphofia* (Dalia *et al.*, 2006), *Bambusa* (Kapoor and Rao, 2006), the effects of growth regulators, particularly thidiazuron (TDZ) on shoot regeneration in *Kalanchoë* (Sanikhani *et al.*, 2006), and 6-benzylaminopurine in stimulating organogenesis in *Eucalyptus erythronema*, *E. stricklandii*, and their interspecific hybrids (Glocke *et al.*, 2006a, b). Chen *et al.* (2006b) micropropagated *Lychnis senno*, a rare plant with potential as an ornamental, while Ma and Wu (2006) regenerated shoots from cotyledons of *Ochna integerrima*, a medicinal and ornamental plant from southeastern Asia. Dewir *et al.* (2006) scaled up the micropropagation of *Spathiphyllum* using an airlift bioreactor, while Teixeira da Silva *et al.* (2006a) developed a photoautotrophic micropropagation system for this ornamental and novel culture vessels for shoot multiplication under carbon dioxide enrichment (3000 ppm) at low photon flux density. *Lilium* has also been targeted in liquid medium in bioreactors (Thakur *et al.*, 2006). Wang and Bhalla (2006) initiated cell suspensions from leaf and root callus cultures of the Australian ornamental plant, *Scaevola aemula*.

Micropropagation of orchids has received considerable attention because of the commercial requirement for large numbers of cut flowers and potted plants. Additionally, multiplication of shoots in culture is important for those orchids that are endangered. Indeed, the technique has been applied to several genera, including *Cymbidium* and *Phalaenopsis* (Chen and Chang, 2006), *Cypripedium* (Yan *et al.*, 2006), *Dendrobium* hybrids (Martin and Madassey, 2006), *Laelia* (Santos-Hernandez *et al.*, 2005), and intergeneric sexual hybrids between *Ascocentrum* and *Vanda* (Kishor *et al.*, 2006). A robust micropropagation protocol was developed and reported for *Cymbidium* (Teixeira da Silva *et al.*, 2006b). Other

ornamental plants that have been targeted include bromeliads, such as the Brazilian *Vriesea reitzii*, with 90% survival *ex vitro* of the regenerated plants (Alves *et al.*, 2006). Similar success has been achieved with *Tillandsia eizii*, a large colorful plant with persistent flowers up to 1 m in length but which, like *V. reitzii*, is endangered through collecting in the wild (Pickens *et al.*, 2006). Woody plants, such as Japanese photinia (*Photinia glabra*) have received attention (Li *et al.*, 2006a), as have succulents such as *Notocactus magnificus* (de Medeiros *et al.*, 2006). Hatzilazarou *et al.* (2006) correlated the rooting of micropropagated shoots of *Gardenia jasminoides* with peroxidase activity. Literature relating to other recent micropropagation reports and tissue culture-based procedures for ornamental plants have been summarized by Teixeira da Silva (2006c), together with short synopses of the scientific relevance of the contents of the publications. Jain *et al.* (2006) discussed the commercial application of *in vitro* techniques, especially of micropropagation, to satisfy the demand for ornamental potted plants such as *Begonia*, *Cyclamen*, *Ficus*, *Philodendron*, *Rhododendron*, *Saintpaulia*, and *Spathiphyllum*.

2.2.2 Protoplast-based technologies

Isolated protoplasts are essential for gene transfer by somatic hybridization, cybridization, and some aspects of transformation. Details of parameters such as source material, plant genotype, and aspects of protoplast isolation have been reviewed (Davey *et al.*, 2005). The composition of culture media, growth regulators, and gelling agents has also been considered. Innovative approaches to protoplast culture include electrostimulation and the influence of nonionic surfactants, antibiotics, and polyamines on growth, with the use of perfluorochemicals and haemoglobin to modify the gaseous environment within culture vessels. Winkelmann *et al.* (2006) developed a protocol for isolating protoplasts from cell suspensions of *Cyclamen persicum*, with subsequent culture of the protoplasts in alginate films. Protoplast-derived plants of four genotypes were transferred to compost. Komai *et al.* (2006) reported that sustained mitotic division of protoplasts of *Lilium japonicum* necessitated the use of nurse cell culture.

2.3 Genetic Manipulation of Ornamental Plants through Protoplast-to-Plant Technology

2.3.1 Somatic hybridization to combine genetic information from genera and species

Protoplast fusion circumvents pre- and postzygotic sexual incompatibility barriers, permitting nuclear and/or cytoplasmic genomes to be combined from different donors to generate somatic hybrid and cybrid plants. The fusion of protoplasts presents few difficulties. Treatments of isolated protoplasts with polyethylene glycol (PEG), electrofusion, or a combination of these techniques, are the procedures generally employed to fuse isolated protoplasts. The cells and tissues resulting from the heterokaryons generated by fusion may regenerate to plants with balanced or asymmetric nuclear genomes (Davey *et al.*, 2000). Usually, chloroplasts segregate or, more rarely, remain as a mixed population in heterokaryon-derived cells and regenerated plants. Frequently, the DNAs of mitochondria recombine; elimination of the nuclear genome of one parent may result in the production of cytoplasmic hybrids, with transfer of cytoplasmically encoded traits, such as mitochondrially encoded cytoplasmic male sterility (CMS). Liu *et al.* (2005) discussed the relevance of somatic hybridization to the genetic improvement of cultivated plants, and emphasized the importance of both related and distant species as a pool of desirable genes for introgression into crop plants.

The selection of hybrid/cybid cells prior to plant regeneration remains the most difficult aspect of somatic hybridization. Some selection procedures are complex, being based on mutant and transformed cells, or metabolic inhibitors. In contrast, procedures exploiting hybrid cell vigor (heterosis) are simple, and are based on long-established principles in conventional plant breeding. Davey *et al.* (2000, 2004) reviewed the procedures involved in somatic hybridization and discussed some of the agronomically useful traits that have been introgressed into the major crop plants, especially in nonornamental genera, such as *Citrus*, *Brassica*, and *Solanum* (Davey *et al.*, 2005).

Although limited in extent, success has been reported in fusing protoplasts and in generating somatic hybrids of ornamental species. In studies of the viability of fusion products between mesophyll protoplasts of pansy (*Viola tricolor*) and protoplasts from petiole-derived callus of the wild viola, *V. patrinii*, Kwon *et al.* (1992) examined by electron microscopy the products generated by electrofusion. Heterokaryons and homokaryons were observed in the preparations. Mastuti *et al.* (1997) studied the electrofusion of protoplasts isolated from cell suspensions of *Celosia cristata*, a C3 species, with cotyledon protoplasts of *Amaranthus tricolor*, a C4 species. Fourteen compact protoplast-derived tissues were produced, with acid phosphatase analysis confirming their hybridity. However, plant regeneration from these tissues was not reported. Treatment with PEG was used to fuse leaf protoplasts of *Dianthus chinensis* with those of *D. barbatus* (Nakano and Mii, 1993). Unlike the situation in *Viola*, the protoplasts of the two species of *Dianthus* were morphologically identical. Consequently, it was not possible to distinguish heterokaryons from homokaryons or unfused protoplasts. The selection of potential somatic hybrid tissues was based on heterosis, with the most vigorously growing protoplast-derived tissues being selected manually. Shoots regenerated from one of these tissues were analyzed and flower color, chromosome complement, and esterase pattern showed that one plant was an interspecific somatic hybrid. Subsequently, the hybridity of this individual was confirmed by nuclear ribosomal DNA analysis.

VazquezTello *et al.* (1996b) attempted to transfer cold tolerance from *Lavatera thuringiaca* into *Hibiscus rosa-sinensis*, but plants were not regenerated in this intergeneric combination. In a later report, the same research group discussed the potential application of irradiation in asymmetric protoplast fusion for gene transfer (VazquezTello *et al.*, 1996a). Fertile somatic hybrid plants were generated between the oriental hybrid lily cultivars Acapulco and Shirotae and *Lilium* \times *formolongi* cv. Hikuchō (Horita *et al.*, 2003). However, electrofusion-treated protoplasts divided only in the presence of nurse cells of the cultivar Hikuchō. Molecular analyses, using cleaved amplified polymorphic sequence markers and flow cytometry, confirmed the somatic hybrid

nature of the regenerated plants. Asymmetric and symmetric somatic hybrids between cultivated sunflower (*Helianthus annuus*) and the wild species, *H. maximiliani*, were characterized using RAPD markers (Binsfeld *et al.*, 2001; Binsfeld and Schnabl, 2002). Microprotoplasts from the perennial *Helianthus* species were also fused, using PEG, with protoplasts of the annual species, *H. annuus* (Binsfeld *et al.*, 2000). Both types of hybrids showed recombination to different extents of the two parental nuclear genomes, making some of these plants exploitable for inclusion in conventional breeding programs. These studies extended earlier investigations involving the fusion of protoplasts of *H. annuus* with those of *H. maximiliani*, *H. giganteus*, and *H. nuttallii*, in which fertile hybrids were recovered (Henn *et al.*, 1998). The asymmetric and symmetric fertile somatic hybrids in *Helianthus* exhibited a range of variability (Binsfeld *et al.*, 2003). These authors reported that such hybrids were backcrossed to sunflower prior to assessments of their disease resistance and agronomic performance.

Whilst the genus *Helianthus* is primarily of agronomic importance because of its seed and oil production, interest in "sunflowers" as ornamental plants has gained considerable momentum in recent years, emphasizing the relevance of these studies to create novelty in terms of floral form, pigmentation, and plant stature. The genus *Mentha* is grown in gardens not only for its culinary and medicinal uses, but also as a genus of ornamental value, particularly as a sensory plant. Sato *et al.* (1996) fused protoplasts of peppermint (*M. piperita* cv. Blackmint) with those of gingermint (*M. gentilis* cv. *variegata*) to generate somatic hybrids that synthesized the major volatile oil components menthone, menthol, and linalol, the last two being from peppermint and gingermint, respectively. Subsequently, Krasnyanski *et al.* (1998) fused protoplasts of peppermint (*M. piperita* cv. Black Mitcham) with those of spearmint (*M. spicata* cv. Nature Spearmint) in an attempt to combine the oil quality of spearmint with the disease resistance of peppermint. Modification of secondary product biosynthesis was one of the primary objectives of studies involving the fusion of protoplasts of tansy (*Tanacetum vulgare*) with those of pyrethrum

(*T. cinerariifolium*), to improve the oil content and composition of tansy in order to stimulate the production of environmentally benign pesticides, the pyrethrins (Keskitalo *et al.*, 1999). As already discussed for mint, it will be interesting to see whether or not plants of *Tanacetum* modified genetically by protoplast fusion, will become ornamentals in their own right.

Wang and Binding (1993) characterized somatic hybrids and cybrids between the ornamental plants, *Senecio fuchsia*, and ragwort, *S. jacobaea*, through investigations of chromosome complements, isozyme profiles, and DNA RFLPs (restriction fragment length polymorphisms). Nine plants had the amphidiploid chromosome number of 80; three hybrids had reduced chromosome complements of 60–76, indicating karyotypic instability. Plastomes originated from one or both parents, but chondriomes were from either parent. Eight plants were putative cybrids in which only the mitochondrial DNA of *S. jacobaea* was detected in seven of these plants, with mitochondrial DNA fragments of both parents in the remaining cybrid plants. Three cybrids had the nucleus and plastids of *S. fuchsia*, with mitochondrial DNA of *S. jacobaea*. In other detailed analyses of somatic hybrid plants, Barbosa and Vieira (1997) focused their attention on microsporocytes of somatic hybrids between *Passiflora edulis* f. *flavicarpa* and *P. amethystina*. These workers reported univalents, bivalents and quadrivalents, laggard chromosomes and anaphase bridges. Pollen viability and multivalent pairing in four somatic hybrid plants that were analyzed indicated this material may be exploited in the genetic improvement of yellow passion fruit, following the introgression of genes for resistance to pests and diseases. Whilst *P. edulis* is cultivated commercially, mainly as a source of fruit for the extraction of juice, passion flowers, in general, are cultivated extensively as ornamentals because of the beauty of their flowers.

Nicotiana species have become popular as ornamental plants, with genetic manipulation of this genus being important in relation to floral morphology, plant stature, and disease resistance. Ilcheva *et al.* (2000) documented the morphology and cytology of somatic hybrids between *N. tabacum* and *N. megalosiphon*. Both parents were transgenic, enabling the hybrids to be selected on

the basis of their dual resistance to bialaphos and kanamycin. There was no preferential loss of one of the parental genomes in such hybrids. Subsequently, Farbos *et al.* (2001) studied an alloplasmic male-sterile tobacco plant generated by protoplast fusion that consisted of the nuclear genome of *N. tabacum* and the cytoplasm of *N. repanda*. Flowers were morphologically abnormal and developed short, poorly pigmented petals and abnormal sterile stamens that were often fused with the carpel wall. Such abnormalities were attributed to reduced cell division and the size of floral meristems. Defects in cell proliferation were correlated with morphologically modified mitochondria. The same parental species of tobacco were also studied by Sun *et al.* (2005), the selection procedure used to generate the cybrid plants involving inactivation of mesophyll protoplasts of *N. tabacum* var. K326 by rhodamine-6G, before PEG fusion with wild-type protoplasts of *N. repanda*. Thirty-one plants regenerated following fusion treatment resembled the *N. tabacum* parent, apart from their floral morphology and male sterility, the latter confirming the observations of Farbos *et al.* (2001). Cybrid plants had the nuclei of *N. tabacum* and mitochondria from *N. repanda*, in agreement with the earlier report. Backcrossing to *N. tabacum* revealed a strict maternal inheritance of the male-sterile phenotype. Sun *et al.* (2005) emphasized that this methodology is effective for introducing CMS through mitochondrial transfer from wild species, and that this source of CMS is of considerable value in breeding programs.

Other *Nicotiana* species have been used to generate cybrid plants, as in the case of protoplasts of *N. tabacum* with those of *N. suaveolens* (Fitter *et al.*, 2005). Molecular analysis confirmed these cybrids contained mitochondria with both parental DNA sequences, although not all of the parental mitochondrial DNA was present. As earlier, the cybrid plants developed morphologically abnormal flowers, these being similar to the flowers of *N. tabacum*, but with a split corolla and the stamens replaced by a whorl of carpel-like structures. The authors emphasized that such cybrids will facilitate analysis of the role of mitochondrial DNA sequences in floral organ identity. Such information may be applicable to ornamental plants in genera other than *Nicotiana*.

Cybrids have also been generated by fusing protoplasts of tobacco with protoplasts of other members of the Solanaceae, as in *N. tabacum* with *Petunia hybrida* (Dragoeva *et al.*, 1998). Regenerated plants were phenotypically similar to tobacco, were male and female sterile and possessed the nuclear genome of *N. tabacum*, the chloroplasts from *P. hybrida* and recombinant mitochondrial DNA. Zubko *et al.* (2003) investigated the morphology of CMS cybrids possessing the *N. tabacum* nuclear genome, the plastome from *Hyoscyamus niger*, and recombinant mitochondria. Such studies followed earlier work on cybrids of the same genera and those of *N. tabacum* with *Scopolia carniolica* (Zubko *et al.*, 1996). Backcrossing of cybrids with tobacco generated plants with floral modifications, including the conversion of stamens to filamentous structures, modification of petals, and a reduction in most floral organs. Such results indicated that cytoplasm transfer by protoplast fusion, with subsequent conventional backcrossing, generated novel nuclear-cytoplasmic combinations that could be exploitable, in a longer term, in generating useful floral novelty. Undoubtedly, protoplast fusion has resulted in considerable modification of floral morphology in tobacco. Whilst some alterations are detrimental, several are novel and exploitable in achieving long-term breeding objectives. Knowledge accrued from these investigations relating to the genetic control of flower characteristics, should be applicable to future breeding programs not only in tobacco, but also in other ornamental plants.

Most effort has been focused on somatic hybridization in dicotyledons, but progress has been reported in the somatic hybridization of monocotyledonous ornamentals of the genus *Iris*, permitting hybridization that cannot be achieved by conventional breeding. Thus, Shimizu *et al.* (1999) electrofused protoplasts of *I. ensata* with those of *I. germanica*. The selection of symmetric hybrid tissues was based on the growth of protoplasts in culture; selection of asymmetric hybrids employed iodoacetamide inactivation of metabolism. RAPD analysis confirmed the hybrid nature of regenerated plants. It is likely that the genus *Iris* will continue to be targeted in future somatic hybridization programs.

2.3.2 Genetic modification of ornamental plants by DNA uptake into isolated protoplasts

Isolated protoplasts can be induced to take up DNA by chemical and/or physical procedures. These procedures have been important in transforming plants that are not amenable to other methods of gene delivery, particularly *Agrobacterium*-mediated transformation (Rakoczy-Trojanowska, 2002). Both supercoiled and linearized plasmids have been used to transform isolated protoplasts. Treatment of protoplast-plasmid mixtures with PEG and/or electroporation is normally employed to induce DNA uptake into protoplasts, but only about one in every 10 000 protoplasts develops into stably transformed tissues. This emphasizes the necessity for reproducible protoplast-to-plant systems, combined with efficient procedures to select transformed cells and tissues. Protoplasts may be co-transformed with more than one gene carried on the same or separate plasmids. Several parameters influence protoplast transformation, including the plant genotype and the stage of the cell cycle of the recipient protoplasts. Heat shock and irradiation of recipient protoplasts both enhance the transformation frequency (Davey *et al.*, 2000, 2005).

There are limited examples, with no recent reports, of the transformation of protoplasts from ornamental species. Ling and Binding (1997) generated transgenic plants of *Linum usitatissimum* (flax) and the ornamental rockery perennial, *L. suffruticosum* ssp. *salsoloides*, following PEG-mediated uptake of DNA carrying the hygromycin phosphotransferase (*hpt*) gene for hygromycin resistance. Direct DNA uptake was compared with *Agrobacterium*-mediated transformation of protoplast-derived cells, and the advantages of these procedures discussed in relation to previously reported *Agrobacterium* transformation of seedling hypocotyls. As discussed earlier, mint and tobacco are considered as ornamentals in this review. Peppermint (*M. piperita* cv. Black Mitcham) has received attention in terms of gene transfer through both somatic hybridization and transformation. For example, protoplasts from stems were transformed with a binary vector carrying the neomycin phosphotransferase (*nptII*) and β -glucuronidase (*gus*) genes to establish the DNA delivery system. Later, the *gus* gene was

replaced with a limonene synthase gene and transformed plants analyzed for their oil profiles (Krasnyanski *et al.*, 1999). The retention of tissue specificity at the protoplast level is important, because it permits gene constructs to be evaluated in both homologous and heterologous systems. Thus, Martens *et al.* (2002) used protoplasts of tobacco to investigate heterologous expression of dihydroflavonol 4-reductases (DFRs) from various plants, particularly those from sexual hybrids of the ornamental genus *Gerbera*. DFRs catalyze the stereospecific reduction of dihydroflavonols to their respective 3,4-diols (leucoanthocyanidins). Synthesis of functional DFR protein was obtained in protoplasts of *N. tabacum*. Such a protoplast-based approach will be useful for investigating less-understood reactions downstream of DFR in the biosynthesis of anthocyanin, catechin, and proanthocyanidin.

2.4 Genetic Modification of Ornamental Plants by *Agrobacterium*-Mediated Gene Delivery and Particle Bombardment

2.4.1 General background

Details of the molecular biology of *Agrobacterium*-mediated gene delivery to plants is presented in excellent reviews by Newell (2000), Gelvin (2003), Sharma *et al.* (2005), Lacroix *et al.* (2006), and Tzifira and Citovsky (2006). Rakoczy-Trojanowska (2002) has discussed other procedures not based on *Agrobacterium* for delivering DNA to plants, while Altpeter *et al.* (2005) have published a critique of particle bombardment (Biolistics, gene gunning) for DNA delivery. In summarizing the literature, Brand (2006) presented the basic procedures of gene delivery to plants and concluded that more than 40 ornamental genera have been transformed, with *Agrobacterium*-mediated gene delivery being the procedure generally adopted. The cauliflower mosaic virus (CaMV) 35S promoter and the β -glucuronidase (*uidA*, *gus*) genes have featured in many of the reports, particularly in the development of transformation systems. Leaf explants and stem portions have been targeted in most cases, generally with expression of the *nptII* gene and the antibiotic kanamycin in the culture

medium as the system to select transformed cells, tissues, and regenerated shoots.

2.4.2 Expression of genes from the transfer-DNA of *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* in ornamental plants: modification of plant stature

Plant stature is an important characteristic of ornamental plants. Dwarf plants may be more attractive as container-grown specimens, while longer stems may be an important trait for introduction into cut flowers. As Duan *et al.* (2006) indicated, large quantities of synthetic growth regulators with auxin, cytokinin, and abscisic acid (ABA) activities are used in the horticultural industry to control plant height. Of the genes that naturally elicit a transformation response in plants, the *rol* genes from the transfer-DNA (T-DNA) of *A. rhizogenes* have received considerable attention in modifying the characteristics of ornamentals. Casanova *et al.* (2005) reviewed the literature and discussed the general effects of specific *rol* genes from the T-DNA of *A. rhizogenes*, on plant growth, including *rolA* + *B* + *C*, *rolA* + *B*, and *rolB* or *rolC*, with the latter being under the control of its own promoter, or the CaMV 35S promoter. Of the *rolA*, *B*, *C*, and *D* genes, *rolC* has been investigated most extensively, plants expressing *rolC* showing a range of phenotypes with the most dramatic changes induced by plants expressing *rolC* under the 35S promoter. Generally, *rolC* leads to dwarfism and increased lateral branching giving a compact, bushy phenotype. Smith *et al.* (2006) also discussed the use of the *rolC* gene in dwarfing ornamental plants. *RolC*-transformed plants of *Osteospermum* flowered earlier with more flowers per plant, although the head diameter of the flowers was reduced. *RolC*-transformed carnation plants developed more extensive root systems with an increase in the number of shoots compared to their wild-type counterparts (Zuker *et al.*, 2001). Importantly, the same gene reduced the time to flowering in *Pelargonium* (Boase *et al.*, 2004). *RolD* also promoted flowering in tobacco. However, the transformation response often depends upon the target plant, since flowers, for example, may be reduced or increased in size. The severity of the *rolC* phenotype was related to *rolC* mRNA

(messenger-RNA) (Gardner *et al.*, 2006). Cheng *et al.* (2006) discussed the application of wild-type strains of *A. rhizogenes* and isolated *rol* genes, particularly *rol A* + *B* + *C* and *rolB*, to stimulate adventitious root development in hardwood cuttings. As indicated by these authors, the effects of *rolD* expression in ornamental plants are less well understood. Sterility may not be a problem in ornamental plants transformed by *rol* genes or, indeed, by other genes, since many ornamentals, such as carnation, are propagated mainly vegetatively.

Genes from the T-DNA of the Ti (tumor-inducing) plasmid of *A. tumefaciens* have been expressed in ornamental plants. Khodakovskaya *et al.* (2006) demonstrated the use of the isopentenyl transferase (*ipt*) gene to manipulate cytokinin biosynthesis in transgenic plants. For example, *ipt* under the control of a fragment of the 1-aminocyclopropane-1-carboxylic acid oxidase gene from tobacco increased flower bud initiation, altered branching and prolonged retention of leaf chlorophyll in *N. tabacum*. The auxin synthase genes, *iaaM* and *iaaH*, also from the T-DNA of the Ti plasmid of *A. tumefaciens*, have been used to induce root formation in transgenic plants. *IaaM* encodes tryptophan monooxygenase that converts tryptophan to indoleacetamide (IAM); *iaaH* encodes indoleacetamide hydrolase, converting IAM to indoleacetic acid (IAA). The *iaaM* gene increased the number of flowers per inflorescence and the number of inflorescences per plant in some members of the Rosaceae. Down-regulation of auxin concentration has been exploited to confer dwarfing with increased flowering, as in chrysanthemum (McAvoy *et al.*, 2003) transformed with the *iaaL* gene that encodes IAA-lysine synthase. Modification of plant stature by reducing growth is also possible by down-regulation of cytokinin synthesis through cytokinin oxidase/dehydrogenase genes, with expression of such a gene cloned from *Dendrobium* reducing shoot growth. Antisense expression of the gene also stimulated the development of short, lateral adventitious roots (Yang *et al.*, 2003).

Since gibberellins (GAs) control several aspects of plant development (Hedden, 2003), it is not surprising that regulation of GA metabolism can be exploited to control plant characteristics, including stature. However, the effect of such genes depends upon their origin. For example,

Mino *et al.* (2006) indicated a GA20 oxidase gene from pumpkin seeds produces physiologically inactive GAs as it catalyzes the conversion of GA19 and GA24 to GA17 and GA25, respectively, resulting in reduced stem growth in transgenic plants. In contrast, overexpression of GA20ox from *Arabidopsis thaliana* in transgenic plants of the same species, which did not divert active GA to GA17 and GA25, increased GA synthesis with a GA-overproduction phenotype. GA2 and 3 oxidase genes have been used to generate dwarf phenotypes in *A. thaliana*, hybrid poplar, and rice. Currently, there is little information relating to the expression of GA biosynthetic genes in ornamentals, but it is clear that the resulting phenotype may depend upon the target species.

2.4.3 Modification of floral morphology, pigmentation, scent, and longevity

Modification of flowering by increasing the number of flowers, reducing the period to flowering and creating novelty, are prime targets for conventional plant breeding and can also be addressed through somatic hybridization and transformation. Vainstein (2002) emphasized that the limited knowledge of the genetics of many ornamental plants restricts their breeding. Several genes are involved in flower induction and development and the changes that they elicit have been discussed by Giovannini *et al.* (2002) and Giovannini (2006). Floral morphology is important in ornamental plants, with Feng *et al.* (2006) investigating the molecular mechanisms underlying the development of zygomorphic flowers in angiosperms using *Lotus japonicus* as a model. Although this plant is not an ornamental, the information from these investigations may be of relevance, in a longer term, to ornamental plants. Tsai *et al.* (2006) studied gene expression in native diploid orchids of the genus *Phalaenopsis* from Taiwan to increase understanding of floral form in the orchidaceae. The time of flowering can be controlled. For example, Boss *et al.* (2006) delayed flowering by overexpressing a *TERMINAL Flower 1* homolog from grapevine (*VvTFL1*) in tobacco. Such information may be crucial in future gene introduction to modify floral development in orchids and other ornamental plants.

Modification of floral pigmentation is, undoubtedly, one of the most dramatic effects of genetic manipulation. Tanaka *et al.* (2005) discussed the range of floral colors generated in carnation following introduction of flavonoid genes from petunia, and the introduction of delphinidin genes for blue pigmentation from pansy to rose. Other reports have focused on alteration of the carotenoids of marigolds, as the latter are a source of natural pigments for the poultry feed industry to enhance the color of egg yolks. Harriman *et al.* (2006) provided the references relevant to these investigations. Modification of flower color demands knowledge of the complex biochemical pathways associated with pigmentation. Flavonoids and carotenoids are major plant pigments, but the final flower color may be influenced by betalains, chlorophylls, and indigo-related compounds (Rosati and Simoneau, 2006). These latter authors provided examples of target plants, gene constructs, and the effect of specific transgenes on flower pigmentation. Modification of flavonoids has involved down-regulation by gene silencing through expression of antisense RNA, homologous sense RNA (co-suppression), and RNA interference (RNAi). Overexpression of flavonoid genes to increase synthesis of existing pigments is also feasible and has been exploited to modify petal color, although the effects of vacuolar pH and the effects of other pigments often influence final petal pigmentation (Forkmann and Martens, 2001).

Aurone flavonoids give plants, such as those of the genera *Antirrhinum* and *Dahlia*, their bright yellow colors. Changes from white to yellow, orange, and pink colors have been induced in petunia. In modifying flower pigmentation, Ono *et al.* (2006) co-expressed chalcone 4'-O-glucosyltransferase and aureusidin synthase genes in *Torenia hybrida* to give this plant yellow petals. They concluded that chalcones are 4'-O-glucosylated in the cytoplasm, their 4'-O-glucosides being transported to the vacuole where the glucosides are converted enzymatically to aurone 6-O-glucosides. The authors emphasized that the metabolic pathway is unique and opens the way to engineer yellow flowers for major ornamental plants that currently lack this color.

Pigment distribution determines color patterns of flowers, with Morita *et al.* (2006) using

Ipomoea nil and *Dianthus caryophyllus* to investigate transcriptional regulation for anthocyanin biosynthesis. Koseki *et al.* (2005) reported that corolla sectoring in the petunia cv. Red Star was attributed to post-transcriptional gene silencing by double-stranded RNA (dsRNA). Flavonoid 3' hydroxylase (*GtF'H*) and flavone synthesis II (*GtFSII*) genes were isolated from *Gentiana triflora* and introduced into tobacco. *GtF3'H* transformation increased anthocyanin synthesis and flower color intensity, while *GtFSII* transformants had reduced anthocyanin (Nakatsuka *et al.*, 2006a, b).

Generally, carotenoids have received less attention than flavonoids in ornamental species; Davies *et al.* (2003) discussed some of the difficulties of targeting carotenoids. An interesting concept is the introduction of genes for green fluorescent protein (*gfp*) into flowers to generate fluorescent colors when the flowers are irradiated with UV or blue light (Mercuri *et al.*, 2001). Whilst such *gfp* expressing plants are novel in their own right, their longer term value may be questionable.

Floral scents are important in attracting pollinators and enhancing the aesthetic appeal of cut flowers. Loss of floral scent is one of the detrimental features of the conventional breeding of ornamentals, since scents are generally complex mixtures of mainly low molecular weight terpenoid, phenylpropanoid, and benzenoid compounds. Dudareva and Pichersky (2006) discussed the implications of loss of scent, but indicated that it is feasible to engineer plants to synthesize and to emit volatiles from their flowers. These authors presented a comprehensive list of the genes that have been isolated and characterized that are involved in the synthesis of volatile compounds. It is interesting to note that a circadian emission of scents is common in many plants.

Alteration of existing biosynthetic pathways, or the introduction of enzymes to generate novel compounds not normally present in host plants by reduction or up-regulation of metabolic flux through existing biosynthetic pathways, are possible strategies to engineer floral scents. The synthesis of volatiles depends upon the availability of substrates. Thus, petunia plants transformed with the rose alcohol acetyltransferase (*RhAAT*) gene were able to use phenylethyl alcohol and benzyl alcohol when these compounds were supplied in the culture medium, to synthesize the corresponding acetate esters. The latter were

not produced by flowers of nontransformed plants (Guterman *et al.*, 2006). It seems likely, because of the complex biosynthetic pathways involved, that multiple gene transfer may be essential for success, preferably with flower-specific promoters to drive transgene expression. Clearly, as Dudareva and Pichersky (2006) indicated, the genetic manipulation of floral scent is not a trivial issue and its longer term success will demand improved knowledge of existing biosynthetic pathways.

Postharvest vase life is extremely important, particularly for cut flowers. Since ethylene influences plant growth and development and triggers petal senescence, Shibuya and Clark (2006) considered the manipulation of ethylene biosynthesis and signaling to be one of the most promising approaches to improve crops through genetic engineering. Commercially, considerable use is made of chemical treatments to decrease the ethylene sensitivity of plants. However, the cloning of genes involved in ethylene biosynthesis and signaling now enables genetic manipulation to be an alternative approach. Thus, Shibuya and Clark (2006) summarized the gene constructs used in these two approaches in *Dianthus caryophyllus*, *Torenia fournieri*, and *Petunia hybrida*, with the CaMV 35S promoter driving gene expression in most of these constructs. Strategies to block ethylene biosynthesis include suppression of genes encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase or ACC oxidase, and degradation of ethylene precursors by expression of genes not involved directly in ethylene synthesis. The gene for ACC deaminase from *Pseudomonas* has been used in this respect. Serek *et al.* (2006) concluded that an efficient way of suppressing ethylene signaling is by expressing a dominant mutant ethylene receptor gene, such as *etr1-1*, from *A. thaliana* in carnation, extending the vase life of cut flowers threefold. Manipulation of ethylene signaling by expression of the *EIN2* gene, using co-suppression or RNAi technology, has been applied to petunia (Shibuya *et al.*, 2004). As already indicated, the *ipt* gene from *A. tumefaciens* has been used to manipulate cytokinin biosynthesis in transgenic plants. An important commercial application of the expression of this gene results from its control by the SAG12 senescence-specific promoter from *A. thaliana*. The activity of a PSAG12-*ipt* construct occurred only during senescence, delaying this

process in flowers of transgenic plants of petunia (Chang *et al.*, 2003).

Whilst floral morphology is a prime target for genetic manipulation, leaves also contribute to the aesthetic appeal of ornamental plants. Leaf form demands control of cell division and polarity-dependent differentiation of cells. Kim (2006) listed the genes that control leaf shape, although there does not appear to be any publications in which such genes have been targeted to specific plants to induce a definite change in leaf morphology.

2.4.4 Improved tolerance to abiotic stresses

Drought and soil salinity are major problems in many parts of the world with salinity being common in marginal regions and areas of intense, irrigated agriculture. Borsani *et al.* (2003) discussed the ways of improving salt tolerance by transgenic technology, while Shen and Wang (2006) listed the genes exploited in transgenic technology to increase drought, salinity, and freezing tolerance in several crops. Such genes could also be introduced and expressed in horticulturally important plants. Drought response pathways are either dependent or independent of the stress-induced plant growth regulator, ABA. As in the case of cold tolerance, background information relating to the cloning of genes involved in ABA induced responses have been summarized (Shen and Wang, 2006). Similarly, the genes employed during the last decade to engineer cold tolerance in *A. thaliana* and crops such as alfalfa, tobacco, rice, and wheat, have been presented by these authors. It is interesting to note that some genes are multifunctional and confer resistance not only to drought, but also to salinity and freezing in plants. The choice of promoters, matrix attachment regions/scaffold attachment regions, the genes themselves and their terminators, are important parameters that must be considered during transformation, as are the sizes of gene constructs, the cellular targets for transgenes (nuclear/organelle), and the procedures for selection of transgenic plants.

Improved cold tolerance results in increased productivity and quality of horticultural plants, since cold causes considerable economic losses, particularly when plants are cultivated outside

their normal geographical range. Chilling injury occurs above 0°C, while freezing stress is associated with temperatures below 0°C because of ice crystal formation within cells. The extent of chilling injury depends on the species, the stage of plant development, the organs, and the duration of chilling. Physiological changes associated with the response to chilling include alteration in membrane lipids, inhibition of photosynthesis, photo-oxidative damage, and oxidative stress (Park and Chen, 2006). Freezing injury is generally more easily recognized with desiccation or burning of foliage, necrosis, and death. Damage is frequent on early flowering trees, as in members of the Rosaceae. Park and Chen (2006) provided a comprehensive table summarizing the genes already used to introduce chilling tolerance into a range of target plants. Interestingly, expression of the *ipt* gene under a cold-inducible promoter, *cor15a*, from *A. thaliana* prevented leaf senescence in petunia and chrysanthemum under storage in dark and cold conditions (Khodakovskaya *et al.*, 2005). Transformation technology to introduce cold tolerance into plants is restricted to some of the major crops and ornamentals such as petunia, although it is likely that this technology will be applied to other ornamental plants in the future.

The timing of vegetative growth and the initiation of flowering are governed by parameters such as day length, light quality, particularly the red to far-red ratio, and temperature. Consequently, manipulation of growth conditions is often required to ensure plant production for the consumer market at the required time. Genetic manipulation of plants may be an alternative means of circumventing the need to alter growth conditions. For example, Nie *et al.* (2006) introduced, by *Agrobacterium*-mediated gene delivery, the meristem identity gene *API* from *A. thaliana* into protocormlike structures of the orchids *Dendrobium phalaenopsis* and *D. nobile*. Manipulation of endogenous phytochromes is an energy-efficient strategy for modulating plant development and can be instigated at any time of the year (Franklin and Whitelam, 2006). Genes have been identified that control plant responses to light quality and duration (Frankhauser and Stainger, 2002). Expression of phytochrome biosynthesis (*PHY*) genes has been used to change the time of flowering in aster (Wallerstein *et al.*, 2002). In addition to modifying development in

this way, alteration of phytochrome biosynthesis may also affect plant stature, but with little effect of transgene expression on other plant characteristics, as in the expression of the tobacco *PHYB1* gene in chrysanthemum (Zheng *et al.*, 2001).

2.4.5 Disease resistance

Diseases induced by fungi, bacteria, and viruses cause major losses of all crop plants, including ornamentals, and reduce plant quality, which is evident at the market stage (Hammond *et al.*, 2006). Indeed, complete crop losses may occur due to viral and fungal infections. The transformation of ornamental plants for disease resistance has lagged behind that of major field crops, this being related to the broad spectrum of genera exploited for the cut flower market. Hammond *et al.* (2006) reviewed the literature pertaining to the approaches for engineering ornamental plants for pathogen resistance. Approaches for virus resistance include strategies that exploit viral coat protein genes in sense and antisense constructs, replicase sense viral genes, defective interfering RNA and DNAs, ribozymes, antiviral peptides, ribonucleases, and ribosome-inactivating proteins. Similarly, approaches for inducing fungal resistance into ornamental plants include the use of ribosome inactivating proteins, expression of plant and fungal chitinases, glucanases, thaumatins, and thionins. Plants transformed for fungal resistance include petunia, carnation, African violet, and rose. The latter, a popular cut flower globally, has been engineered, for example, for resistance to leaf black spot induced by *Diplocarpon rosae* using a rice chitinase gene (Marchant *et al.*, 1998). A similar technology may be adopted for other fungal pathogens that incite leaf spot diseases and cankers. Approaches to induce bacterial resistance involve transformation with genes for antimicrobial peptides and proteins, enzymes that detoxify or desensitize plants to bacterial toxins, and genes that enhance the natural disease resistance of plants. Wen *et al.* (2006) showed that chestnut rose (*Rosa roxburghii*) is resistant to powdery mildew, the most destructive fungal disease of rose, and that this plant is an excellent source of resistance to the fungus that can be exploited in rose breeding. Root-

knot nematodes account for considerable losses, particularly of field-grown woody ornamentals. Atkinson *et al.* (2003) indicated that a strategy that combines partial host plant resistance and partial transgenic resistance by a cysteine protease inhibitor may confer full nematode resistance.

2.4.6 Phytoremediation

The removal of toxic pollutants, such as phenol and chlorophenol, represents an important aspect of environmental improvement in which ornamental plants may play a key role, especially in the future. In this respect, de Araujo *et al.* (2006) evaluated the ability of several plant genera to accumulate pollutants and suggested, from their results, that the ornamental plant *Solanum aviculare* is an excellent candidate for exploitation in this way.

2.5 Plastid Engineering

In general, transformation studies have targeted the nuclear genome of recipient cells. However, it is likely that the transformation of plastids will assume considerably more importance as plastome engineering progresses from model systems, such as tobacco, to major crops (Maliga, 2003). In recent investigations, Li *et al.* (2006b) used biolistics to transform tobacco chloroplasts with the plastid expression vector pTRVP1 containing the foot-and-mouth disease virus *VPI* gene and the selectable *aadA* marker gene for resistance to the antibiotics streptomycin and spectinomycin. The advantages of plastid transformation compared to nuclear transformation, include high foreign protein synthesis in plastids and the absence of epigenetic effects in regenerated plants (Bock, 2001; Daniell *et al.*, 2002). Most transplastomic plants have been generated by particle bombardment of leaf tissues (Klaus *et al.*, 2003), although DNA uptake into isolated protoplasts has also been employed. The former procedure negates the requirement for labor-intensive protoplast-to-plant systems. The merit of plastid engineering is the apparent lack of transmission through pollen of transgenes to nontransformed plants of the same or different genera or species (Ruf *et al.*, 2001), although Stegemann *et al.* (2003)

demonstrated that escape of genetic material from chloroplasts to the nucleus occurs more frequently than was once believed. This DNA transfer may contribute to intraspecific and intraorganismic genetic variation.

2.6 Generation of Marker-Free Transgenic Ornamentals

Although the antibiotic resistance markers commonly used to select transgenic tissues and plants do not pose health risks to humans or animals, it is clear that legislation will demand the removal of such genes from transgenic plants. Currently, marker genes may be removed from transgenic plants by co-transformation followed by segregation of the resistance marker gene(s), homologous recombination and recombinase-mediated excision (Goldstein *et al.*, 2005). Klaus *et al.* (2004) summarized the procedures to generate marker-free transplastomic plants following DNA delivery to cells. An interesting concept was that reported by Tinoco *et al.* (2006) who used γ -radiation to physically remove marker genes from the nuclei of transgenic plants. Although demonstrated in soybean, there is no reason why this technology cannot be applied to ornamental plants. However, there is the possibility that novel mutations may be introduced by this approach.

2.7 Somaclonal Variation in Tissue Culture-Derived Plants: A Simple Form of Genetic Engineering?

Reliable procedures are available to combine interspecific and intergeneric traits by protoplast fusion, and the specific modification of genomes by transformation. Somaclonal variation, or protoclonal variation in the case of protoplasts, may be superimposed on these procedures. Such variation involves expression of naturally occurring genetic variation as a result of the culture procedure, or variation may be induced by the culture conditions *per se*. Such variation may be regarded as a simple form of genetic manipulation (Kantharajah and Golegaonkar, 2004) of relevance to crop improvement (Jain, 2001), and is frequently superimposed on the procedures of somatic hybridization and transformation. Several

examples of exposure of somaclonal variation exist in the literature relating to ornamental plants. Chaput *et al.* (1996) regenerated plants from leaf protoplasts of *Mentha* \times *piperata* and *M.* \times *citrata* and analyzed regenerants for their monoterpene composition. Their results revealed a decrease in menthone and menthol, but an increase in carvone in all protoplast-derived plants. Physiological changes have also been recorded in protoplast-derived plants, Pan *et al.* (2005) detecting more than a threefold variation in antioxidant activity in *Hypericum perforatum*. As in the case of mint, such chemically distinct germplines appeared morphologically normal.

It is not unexpected that most emphasis has been directed to changes in the morphology of ornamental plants. In a detailed investigation, Mizuhiro *et al.* (2001) reported that plants derived from cell suspensions of *Primula malacoides* cv. Lovely Tokyo and *P. obconica* cv. Aalsmeer Giant White, showed alterations in morphological characteristics compared to the plants from *in vitro*-germinated seeds. Specifically, such changes involved significantly reduced petioles and variation in floral morphology, particularly the lengths of pedicels and peduncles, inflorescence number and corolla size. In general, protoplast-derived plants formed compact inflorescences with multiple small flowers. Red pigmentation at the center of flowers was paler than in controls, while the periphery of some petals had green pigmentation. Such plant germplasm may be useful in future breeding programs to increase the phenotypic novelty in *Primula* species that are grown extensively as house and glasshouse pot plants. Somaclonal variation was also recorded in protoplast-derived plants of the Oriental hybrid lily cv. Acapulco, regenerated plants resembling their parents except for brown pigmentation of their anthers (Horita *et al.*, 2002).

Alstroemeria is a popular ornamental plant with demand for new cultivars. Subjecting isolated protoplasts to a period of culture may expose variation and contribute to enhancing the genetic base of this ornamental. In this respect, Kim *et al.* (2005) recognized variation in the morphology of leaves and flowers of *Alstroemeria* plants regenerated from protoplasts isolated from friable callus, including more elongate and rounded leaves, flowers with an increased number of petals, or petals with aberrant shapes. Flowers had either

increased pigmentation, or conversely, less intense pigmentation and an albinolike appearance. This protoplast-to-plant system for *Alstroemeria* is important not only in exposing genetic variation, but should facilitate the application of somatic hybridization to develop genotypes that are potential parents for breeding programs. Other workers have targeted *Iris fulva* and *Tricyrtis hirta*. In studies with the former genus, Inoue *et al.* (2006) regenerated tetraploid protoplast-derived plants, which the authors compared with diploid wild-type plants. Tetraploid plants had larger flowers of different morphology compared to parental plants. Subsequent experiments involved sexual hybridization of both diploid and tetraploid plants with *I. ensata* and *I. laevigata* to introduce the unique brown pigmentation from *I. fulva* into these other species. Nakano *et al.* (2006) studied somaclonal variation in *T. hirta* (Japanese toad lily), a popular pot and garden plant, and reported the regeneration of tetraploid ($2n = 4x = 52$) individuals amongst the normal diploid plants ($2n = 2x = 26$). Tetraploid regenerants had several attractive features, including longer shoots, thicker stems, and larger flowers. Chen *et al.* (2006a) analyzed the genetic differences of 19 cultivars selected from somaclonal variants of *Syngonium podophyllum* and six other aroids using AFLP markers generated by 12 primer sets. The authors concluded that much of the plant material was genetically similar, and emphasized the need to search for greater genetic diversity in this genus.

Where maximum genetic change is required, somaclonal variation provides a useful adjunct to the more technologically demanding approaches of transformation and somatic hybridization. Importantly, somaclonal variation does not require information of the genetic basis of specific traits, does not involve knowledge or practice of recombinant-DNA techniques, and may be exploited through simple routine culture procedures.

3. CONCLUDING REMARKS

Increased understanding of the genes involved in flowering will be of considerable benefit in floriculture, but interaction between biotechnological approaches and conventional plant breeding will be essential to improve ornamental crops

with the minimum of inputs (Giovannini, 2006). Brand (2006) emphasized that there still exists a considerable number of technical challenges of generating transgenic ornamental plants. However, the greatest challenge to realizing the potential benefits of transgenic technology remains the difficulty of gaining public acceptance of genetically manipulated crops and the prohibitive costs of generating environmental impact data to gain regulatory clearance. Klingeman and Hall (2006) concluded that the issues relating to the genetic manipulation of ornamental plants have been overshadowed by the debate on genetically manipulated foods. Studies by the same group involved surveys of the perception of gardeners to the effects of genetically manipulated ornamental plants on the environment and human health (Klingeman *et al.*, 2006). Participants stressed the need for genetically manipulated plant products to be clearly labeled at their point of sale. Undoubtedly, there is a requirement to improve consumer knowledge of genetically manipulated products and, likewise, of scientists to understand consumer perception and acceptance, since there remains a critical knowledge gap at the present time. Perhaps, as Klingeman *et al.* (2006) suggest, genetically manipulated ornamentals may play a crucial role in bringing positive public attention to the products of current biotechnology.

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Turfgrass

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1. INTRODUCTION

Turfgrasses are grasses that provide vegetative ground cover, which is usually mowed routinely. Turfgrasses are used worldwide for lawns of home and office buildings, athletic fields, other recreational facilities, and roadsides. In the United States, there are more than 50 000 000 lawns and 14 000 golf courses and the turfgrass area was estimated in 1990 to be 30 million acres (12 million hectares) (Emmons, 1995). The turfgrass industry is a multibillion dollar a year business. Turfgrasses consist of a remarkably diverse group of species, mostly perennial grasses from the *Poa* family, which are selectively used based on the applications and/or environmental conditions. In general, based on their origins and geographical distributions, turfgrasses can be divided into two groups: cool-season grasses and warm-season grasses. Major cool-season turfgrasses include tall fescue, Kentucky bluegrass, perennial ryegrass, creeping and colonial bentgrasses, and fine fescues. Warm-season grasses used for turf contain bermudagrass, St. Augustinegrass, centipedegrass, Japanese lawngrass, buffalograss, seashore paspalum, and bahiagrass. Most of the cool-season grasses are propagated by seeds while a majority of the warm-season grasses are vegetatively propagated. Many of these species can also be used as forage crops.

Tall fescue (*Festuca arundinacea* Schreb.) is an outcrossing, allohexaploid species, with $2n = 6x = 42$. The genome size of tall fescue is estimated as 7.5×10^9 bp $1C^{-1}$. Tall fescue is of bunch-type growth and primarily spreads by tillering. It is more tolerant to heat and drought among the cool-season grasses and is widely used in the transition zone and the southern regions of the cool season zone. Tall fescue is also an important forage crop (Meyer and Watkins, 2003).

Kentucky bluegrass (*Poa pratensis* L.) is the most widely used turfgrass in the cool-season zone, and spreads with strong rhizomes to produce a dense turf. The reproduction mode and the cytology of Kentucky bluegrass are complicated issues. Kentucky bluegrass is aneuploid and produces seeds both asexually through apomixes and sexually. The level of apomixes among cultivars could vary from 25% to 96%, and the chromosome numbers within the species range from 24 up to 124 (Huff, 2003). The chromosome number among somatic cells within a genotype could vary too. An average chromosome is estimated to be 0.13–0.14 pg (picogram) of DNA (Huff, 2003) or approximately 0.13×10^9 bp/chromosome, and the genome size varies according to the chromosome numbers. A popular cultivar, Midnight, which has the chromosome number of 58–62, was estimated to be approximately 4.1×10^9 bp $1C^{-1}$.

Perennial ryegrass (*Lolium perenne* L.) is an outcrossing species although forced selfing can be achieved (Thorogood, 2003). It is diploid with $2n = 2x = 14$, and has an estimated genome size of approximately $2.1\text{--}2.5 \times 10^9 \text{ bp } 1\text{C}^{-1}$. It has a bunch-type growth habit, and is often a choice for athletic fields for its good wearing resistance. Perennial ryegrass is also widely used for winter overseeding of warm-season grass turf in the subtropical zone partially because of its rapid establishment.

Creeping bentgrass (*Agrostis stolonifera* L. or *Agrostis palustris* Huds.) has a fine texture and tolerance to mowing as low as 3 mm. Requiring high maintenance, the stoloniferous growth habit of creeping bentgrass provides carpetlike, soft, dense sod, and is mostly used for high quality turf on golf course putting greens, tees, and fairways. The outcrossing creeping bentgrass is allotetraploid with $2n = 4x = 28$ (Warnke, 2003) and has an estimated genome size of $2.7 \times 10^9 \text{ bp } 1\text{C}^{-1}$.

Colonial bentgrass (*Agrostis capillaris* L. or *Agrostis tenuis* Sibth.) is mostly used as a fairway grass and for erosion control. Outcrossing is the primary mode of sexual reproduction. The species is allotetraploid with $2n = 4x = 28$ (Ruemmele, 2003) with an estimated genome size of $2.8 \times 10^9 \text{ bp } 1\text{C}^{-1}$ (based on Bonos *et al.*, 2002).

Fine fescues are a group of fine-leaved *Festuca* species, containing two main species complexes: the red fescue (*F. rubra*) complex and the *F. ovina* complex (Ruemmele *et al.*, 2003). Being important turfgrasses in Europe and the United States, fine fescues are mostly used as turf in dry, shady, and low-fertile soils. Fine fescues are primarily open-pollinated species. The chromosome number of *F. rubra* complex species is $2n = 28, 42, 56$, and 70 with most of the plants being hexa- or octoploids, whereas *F. ovina* complex is $2n = 14, 28$, or 42. A hexaploid hard fescue (*Festuca trachyphyllas* or *Festuca longifolia*) cultivar Aurora in this complex has an estimated genome of $6.1 \times 10^9 \text{ bp } 1\text{C}^{-1}$.

Turf-type bermudagrass mainly includes common (or seeded) bermudagrass (*Cynodon dactylon* (L.) Pers. var. *dactylon*), a fertile tetraploid ($2n = 4x = 36$) species, and the interspecific hybrid (*C. dactylon* \times *Cynodon transvaalensis* Burt-Davy), a sterile triploid ($2n = 3x = 27$) (Taliaferro, 2003). The genome sizes of the two are estimated to be $0.94 \times 10^9 \text{ bp } 1\text{C}^{-1}$, and $0.66\text{--}0.78 \times 10^9 \text{ bp } 1\text{C}^{-1}$,

respectively. Hexaploid ($2n = 6x = 54$) of *C. dactylon* was also reported (Hanna *et al.*, 1990). Bermudagrass is the most important warm-season turfgrass found from temperate to tropical regions and forms good quality turf. The vegetatively propagated hybrid cultivars Tifgreen, Tifway, and the new TifEagle are widely used in golf greens and fairways in the warm-season zone. Common bermudagrass is outcrossing and propagated by seeds.

St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) is a major turfgrass species in subtropical and tropical regions. In 2001, 70% of the lawns grown in Florida were St. Augustinegrass. The grass spreads by branching stolons and, in general, has coarse leaves. St. Augustinegrass is vegetatively propagated. Although artificial crossing can be readily performed, attempts for seed propagation were not successful. St. Augustinegrass is often $2n = 2x = 18$, although sterile triploid ($2n = 3x = 27$) and aneuploid were also reported (Busey, 2003). The genome size of a diploid cultivar, Raleigh, was estimated to be $0.52 \times 10^9 \text{ bp } 1\text{C}^{-1}$.

Centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) is a turfgrass species requiring only low maintenance and still provides acceptable turf quality (Hanna and Liu, 2003). It spreads by stolons, and is used in lawns and roadsides. The grass is seed propagated with $2n = 2x = 18$ chromosomes. The genome size of centipedegrass is $0.8 \times 10^9 \text{ bp } 1\text{C}^{-1}$.

Japanese lawngrass (*Zoysiagrass japonica*) is a turfgrass used for lawns, golf fairways, and athletic fields. It spreads by rhizomes and stolons, and can be either vegetatively or seed propagated. The chromosome number of the species is mostly reported as tetraploid with $2n = 4x = 40$ although diploidy was also recorded (Engelke and Anderson, 2003). The tetraploid genome size was estimated as $0.42 \times 10^9 \text{ bp } 1\text{C}^{-1}$.

Buffalograss (*Buchloe dactyloides* (Nutt.) Engelm) is a native North American grass used extensively as a turfgrass. Requiring minimum care, the grass spreads by branching stolons, is drought tolerant, and has excellent erosion control. Being a warm-season grass, buffalograss is also quite cold tolerant. Buffalograss is a dioecious species with separate male and female flowers that often occur in separate plants. The grass is mostly propagated vegetatively, and sometimes

by seeding (Riordan and Browning, 2003). Di-, tetra-, and hexaploidy ($2n = 2x, 4x, 6x = 20, 40, 60$) have been reported in buffalograss. Cultivar 609 is a tetraploid with a genome size of 0.77×10^9 bp $1C^{-1}$.

Seashore paspalum (*Paspalum vaginatum* Swartz) is a very unique, good-quality turfgrass species. It has high tolerance to salinity, drought, waterlogging, and low light intensity; adapts to a wide range of pH (3.6 to 10.2); and requires low maintenance (Duncan, 2003). It has been used as a salt-tolerant turfgrass in golf courses, sports fields, and for general landscaping, as well as utility turf for erosion control at coastal regions. The grass spreads by stolons and rhizomes, is outcrossing, but propagated vegetatively. Seashore paspalum is predominantly diploid with $2n = 2x = 20$ although tetra- and hexaploidy were reported. The genome size of the grass is not determined.

A chapter in the series is dedicated to bahiagrass (*Paspalum notatum* Flugge). Thus, the species would not be further discussed in this chapter.

Unless otherwise specified, the genome sizes were estimated based on the nuclear DNA content of individual species reported by Arumuganathan *et al.*, 1999, and the information that 1 pg DNA $\approx 0.965 \times 10^9$ bp (Arumuganathan and Earle, 1991).

Methodical turfgrass breeding probably started around the turn of the 20th century, and was substantially intensified in the past few decades (Casler and Duncan, 2003). The passage of the Plant Variety Protection (PVP) Act in the 1970s by the US Congress greatly encouraged and inspired the private businesses in the United States to breed new crop cultivars, including those of turfgrasses. A more uniform, dense turf with finer and greener leaves is a major target trait for improvement in turfgrass breeding. In addition, it should be realized that, although turfgrasses arose from natural habitats, the environments for them to be used as turf are “human-defined”, and often much more stressful than their original habitats, including many biotic and abiotic stress factors (Casler and Duncan, 2003). Thus, main breeding efforts are also toward resistance to (mostly fungal) diseases and insects, and tolerance to drought, extreme temperatures, shade, and in some cases, mowing. Recurrent selection of a population resulted from intercrossing of several individual parent clones, when possible, is probably the mostly

used approach for new cultivar development in turfgrass species. Interspecific hybridization and irradiation mutagenesis also play important roles in turfgrass breeding (Casler and Duncan, 2003). Transgenic technology was first applied to turfgrasses in the early 1990s, and so far transgenic plants have been successfully generated in nearly a dozen turfgrass species. Transgenic technology can break the barriers of gene exchange between species, and even kingdoms. It also can up- or down-regulate an individual endogenous gene for improvement of trait(s). For example, an herbicide or disease resistance gene of bacterial origin is readily introduced into a turfgrass species using the technology while no such gene can be found in the germplasm pool of the particular species. However, because of the perennial growth habit of turfgrasses and the outcrossing nature of most of the turfgrass species, the risk of transgene escape is a major concern in releasing transgenic turfgrass cultivars. Since tissue culture is usually a prerequisite for successful genetic transformation of turfgrasses, we will examine the achievements in tissue culture of various turfgrass species first in the chapter, which will be followed by the reported successes in transformation technologies in turfgrasses, improved traits by genetic transformation, regulatory issues on release of transgenic turfgrass, and future perspectives of turfgrass improvement through biotechnology.

2. GENETIC TRANSFORMATION OF TURFGRASSES

2.1 Tissue Culture Studies of Turfgrasses

With few exceptions (Clough and Bent, 1998), almost all plant transformation has to go through a tissue culture stage to deliver genes to embryogenic cells and to eventually regenerate transgenic plants from those cells. Thus, optimization of tissue culture responses of a plant species, especially improvement of regeneration ability, often plays a key role in the success of transformation efforts. Although not all the turfgrass species mentioned above have been transformed, tissue culture studies have been reported for each of them. In addition to obtaining transgenic plants, tissue culture also serves purposes such as rapid propagation of plants or creation of somaclonal variation

for breeding efforts (Brown and Thorpe, 1995). Tissue culture responses are generally affected by genotype and explant type of the species, the composition and supplements of the culture media, the phytohormones added in the media, and, sometimes, other factors. In the following description, we try to highlight the major approaches and the accomplishments in tissue culture found in the literature for each major turfgrass species, so the readers can have an historical view of the progresses in the field.

Krans (1981) obtained calli of tall fescue by culturing mature caryopses at 5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and plantlets from regeneration medium containing 0.01 mg l^{-1} 2,4-D and 0.2 mg l^{-1} kinetin. Lowe and Conger (1979) cultured mature embryos of tall fescue (cv. Kentucky 31) on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 9 mg l^{-1} 2,4-D and observed almost 100% callus induction. About 18% calli regenerated shoots and the regeneration ability declined along with subculture on 5 mg l^{-1} 2,4-D. Dale and Dalton (1983) cultured immature inflorescences (1–20 mm in length) in MS medium containing 2 mg l^{-1} 2,4-D with or without 0.2 mg l^{-1} 6-benzylaminopurine (BAP), and obtained 10.2 plantlets per inflorescence. The shoots could directly form from floret primordia or through callus. Eizenga and Dahleen (1990) studied culture responses of partially emerged inflorescences of various genotypes within cv. Kentucky 31 and observed significant variations among genotypes in callus induction and plant regeneration. The explants were cultured on Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) medium with various auxins. Callus production was significantly higher when 2,4-D was used as the auxin source over para-chlorophenoxyacetic acid (pCPA) or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Bai and Qu (2000) compared tissue culture responses of mature caryopses among 25 turf-type tall fescue cultivars on MS medium containing 9 mg l^{-1} 2,4-D, and observed a wide range of callus induction rates (from 4.4% to 51.9%). The callus regeneration rates were from 16.7% to 58.8% on MS regeneration medium containing 2.5 mg l^{-1} BAP. The overall plant regeneration rates (regeneration callus over explants cultured) varied from 1% to 22%, reflecting substantial differences among cultivars. In another report, the same group studied effects

of medium supplements on culture responses of an elite turf-type cultivar, Coronado (Bai and Qu, 2001a). They found inclusion of BAP (0.1 mg l^{-1}) in callus induction medium significantly improved callus regeneration for explants of immature embryo and mature caryopses. Casein hydrolysate (CH), L-proline, and myo-inositol improved callus induction of immature embryos but not mature caryopsis. These supplements had no effect on callus regeneration. Longitudinally slicing mature caryopses into two halves improved their callus induction by three- to sixfold. The optimal supplement combination for the sliced caryopses was 5 or 9 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP, which yielded about 30% overall plant regeneration. Dalton (1988) developed suspension cultures in MS-based medium from embryogenic calli induced from chopped mature embryos of tall fescue. Up to 0.4% of protoplasts isolated from the suspension culture formed colonies when cultured in glucose-containing medium, and more than 100 colonies formed green shoots. By using agarose bead method and nurse culture, Takamizo *et al.* (1990) also obtained green plants from protoplasts isolated from a suspension line that was derived from a young leaf-base culture. In another direction, Kasperbauer *et al.* (1980) obtained anther-derived haploid plants from culture of panicle sections containing several florets after a cold temperature pretreatment. Later on, doubled haploid plants were recovered after subculturing the haploid calli for 15 weeks to reach an “aged” stage and then transferred to a regeneration medium (Kasperbauer and Eizenga, 1985). Somaclonal variation among the regenerated tall fescue plants was observed in traits such as total biomass, seed yield, and leaf shape (Roylance *et al.*, 1994; Bai, 2001). Garcia *et al.* (1994) reported great alteration in flowering response among regenerated plants: some plants flowered without vernalization treatment, and a plant did not flower after 2 years with vernalization. Chromosome number usually did not change but chromosome aberrations were observed. Since maintenance of suspension culture is labor intensive and the regeneration ability of the suspension lines usually declines along with time, Wang *et al.* (1994) explored conditions of cryopreservation of the suspension cells, and found that high-osmotic preculture and cryoprotectant containing 10% dimethyl sulfoxide (DMSO) and

0.5 M sorbitol were the most suitable for storage of tall fescue suspension cells in liquid nitrogen. Up to 71% of the cells could regrow after cryopreservation, and embryogenic suspensions were re-established. Bai (2001) found that a cryoprotectant recipe used for rice and barley, which contains 0.5 M DMSO, 0.5 M glycerol and 1 M sucrose, was the best among the tested, and did not require osmotic preculture treatment. The cells, after being stored for 30 days in liquid nitrogen, increased by 65% in fresh weight after 4 weeks of culture on a solid medium.

Krans (1981) reported callus induction and root formation of Kentucky bluegrass. McDonnell and Conger (1984) used mature embryos as explants and a modified SH medium, and observed that 20 μ M dicamba or 60 μ M picloram was most effective in callus growth and in inhibiting leaf growth. The regeneration rates were very low (0–3.1%) but were improved by culturing at a low temperature (15 °C), and further enhanced (up to 18%) by a week of cold treatment (4 °C) before transferring to the regeneration medium. Boyd and Dale (1986) used mature embryo culture to evaluate 50 cultivars for their callus induction and plantlet regeneration ability, and found great variations among the cultivars. The authors considered that MS basal medium supplemented with 0.2 mg l⁻¹ 2,4-D, 0.1 mg l⁻¹ BAP, 100 mg l⁻¹ CH, and 25 g l⁻¹ sucrose was good for both callus growth and plantlet regeneration. Wu and Jampates (1986) obtained plantlets from calli induced from shoot tips. Van Der Valk *et al.* (1989) studied tissue culture responses of 15 cultivars, and found that calli from immature inflorescences of 14 cultivars regenerated plantlets and the regeneration rates were often over 50%, whereas regeneration of calli from mature caryopses were very low: plantlets were recovered from only 3 cultivars with regeneration frequencies below 3%. Van Ark *et al.* (1991) found regeneration frequency was doubled when Gelrite was used as a gelling agent over agar, and abscisic acid (ABA) enhanced somatic embryogenesis although regeneration rate was not affected. In 1995, two groups reported high frequency of regeneration from mature caryopsis-induced calli by including BAP in the callus-induction medium (Griffin and Dibble, 1995; Van Der Valk *et al.*, 1995). Ke and Lee (1996) investigated culture responses of tissues from young seedlings, and observed that calli

induced from coleoptiles had highest regeneration frequency (32%) when the regeneration medium contained 0.2 mg l⁻¹ picloram and 0.01 mg l⁻¹ α -naphthalene acetic acid (NAA). Salehi and Khosh-Khui (2005) observed that the callus induction rate from mature caryopses was 100% when 40 μ M (8.85 mg l⁻¹) 2,4-D was added to the induction medium and 100% regeneration was achieved when 60 μ M 2,4-D (13.3 mg l⁻¹) and 12.5 μ M (2.8 mg l⁻¹) of BAP was used in the regeneration medium. Hu *et al.* (2006) cultured shoot apices of seedlings in a medium containing low 2,4-D (0.9 μ M or 0.2 mg l⁻¹) and 8.9 μ M (2 mg l⁻¹) BAP to induce multiple shoot clumps, which came from meristematic cell clumps originated from embryonic axis, especially the hypocotyls in the culture. In addition, green plantlet regeneration from embryogenic suspension cell culture (Nielsen and Knudsen, 1993) and protoplast (Nielsen *et al.*, 1993) were reported. The suspension lines were derived from calli induced from mature caryopses or mature embryos of cv. Geronimo. Using the agarose-embedding technique, the protoplast plating efficiencies were demonstrated to vary from 0.004% to 1.5%, and 0.4% to 2.7% of the protoplast-derived microcolonies were able to regenerate.

Krans (1981) also first reported perennial ryegrass plants regenerated through tissue culture using an approach similar to he did with tall fescue. With mature caryopses as explant, Torello and Symington (1984) found subculture of calli on medium containing 5 mg l⁻¹ of 2,4-D was preferred over 10 mg l⁻¹, with 0.1 mg l⁻¹ BAP in regeneration medium being optimal for shoot regeneration. The same group (Zaghmout and Torello, 1992a) reported advantages to include cefotaxime (60–200 mg l⁻¹) in culture medium for improved green plantlet formation. The authors observed that “cefotaxime reduced the inhibitory effect of 2,4-D on precocious germination of somatic embryos.” Dale and Dalton (1983) cultured immature inflorescences (1–20 mm in length) in MS medium, and found 50% more shoots were formed directly or through callus when the medium containing 2 mg l⁻¹ 2,4-D was supplemented with 0.2 mg l⁻¹ BAP and 100 mg l⁻¹ casein hydrolysate (CH). In mature caryopsis culture, Bradley *et al.* (2001) observed substantial variations in callus induction (2.3–21%) and regeneration (0–58%) among 13 elite turf-type

cultivars. Slicing of the caryopsis longitudinally increased callus induction rate by more than fivefold when 2,4-D level was 2 mg l^{-1} . Inclusion of BAP (0.5 mg l^{-1}) in callus induction and subculture medium enhanced its regeneration rate by more than threefold. Salehi and Khosh-Khui (2005) observed 98% callus induction rate from mature caryopses (cv. Barbel) when 2,4-D concentration in the callus induction medium was as high as $150 \mu\text{M}$ (33.3 mg l^{-1}). They obtained 100% callus regeneration when the regeneration medium contained $60 \mu\text{M}$ 2,4-D (13.3 mg l^{-1}) and $7.5 \mu\text{M}$ (1.7 mg l^{-1}) BAP. In suspension culture, Olesen *et al.* (1996) examined suspensions from 21 commercial varieties and could regenerate green plantlets from 18 of them. In a multifactorial experiment to optimize suspension culture conditions, Altpeter and Posselt (2000) observed that replacement of sucrose by maltose, dicamba by 2,4-D (5 mg l^{-1}), and a stepwise increase of BAP to 0.25 mg l^{-1} enhanced green shoot regeneration ability of the suspensions, but addition of CH did not. Using this medium, together with the best-performed genotype, L6, 488 green plantlets were regenerated from per gram fresh weight suspensions. Dalton (1988) reported recovery of green plantlets from protoplast culture of perennial ryegrass without agarose embedding and nurse culture. Creemers-Molenaar *et al.* (1989) observed that protoplast from "nonmorphogenic" suspensions had higher plating rate than the ones from "morphogenic" suspensions, and also recovered green plantlets from protoplast culture. The same group (Creemers-Molenaar *et al.*, 1992) reported that conditioned medium was "indispensable" for plating of protoplasts from younger, regenerable suspensions. O-acetylsalicylic acid and some antioxidants also helped improve plating efficiency. Wang *et al.* (1993) established single genotype-based embryogenic suspension lines and cultured protoplasts using agarose bead and nurse cells. Protoplasts from cryopreserved suspensions performed similarly to the ones isolated from the fresh suspensions and dozens of green plantlets were regenerated. Moreover, Olesen *et al.* (1988) reported successful anther culture of perennial ryegrass after cold pretreatment without nurse culture, and found that recovery of green plantlets were mostly from 2 genotypes. Perezvicente *et al.* (1993) cultured vegetative apices ($0.05\text{--}0.3 \text{ mm}$) consisting of the meristematic dome and 1–4 leaf

primordia in MS medium containing 0.01 mg l^{-1} 2,4-D and 0.2 mg l^{-1} kinetin under dim light condition. About 99% of the explants formed plantlets. Plants regenerated from suspension cell cultures of perennial ryegrass were compared to the seed-grown plants in the field (Stadelmann *et al.*, 1998). Quite often, the regenerated plants showed a delay in inflorescence emergence, a reduced seed yield, and other morphological variations. Sometimes, alterations in DNA sequences were detected by random amplified polymorphic DNA markers. Plants with similar or superior performance to the seed-grown plants were also observed.

Successful tissue culture of creeping bentgrass was first reported by Krans (1981) using caryopses as explants. The greatest callus induction came from cultures in medium containing 1 mg l^{-1} 2,4-D under light, or containing 1 mg l^{-1} 2,4-D and 0.01 mg l^{-1} kinetin in the dark. Calli cultured on 1 mg l^{-1} 2,4-D maintained good regeneration ability and the best regeneration medium contained 0.1 or 1 mg l^{-1} kinetin. Using a 24-week-old stock callus, Blanche *et al.* (1986) observed that callus growth was twice greater after being cultured for 12 or 24 days in suspension, whereas suspension culture greatly reduced plantlet formation. They also determined that the callus growth rate and regeneration frequency were correlated to the size of callus aggregates and the plating density. Zhong *et al.* (1991) evaluated various plant growth regulators on embryogenesis of creeping bentgrass caryopses culture, and observed that MS basal medium containing $30 \mu\text{M}$ (6.63 mg l^{-1}) dicamba and $2.25 \mu\text{M}$ (0.5 mg l^{-1}) BAP was optimal, in which 91% calli were embryogenic and over 80% of the somatic embryos formed plantlets after transferring to the regeneration medium. Terakawa *et al.* (1992) reported microcolony formation and 0.36% plating efficiency from protoplasts of creeping bentgrass, cv. Penncross, cultured in a conditioned medium. Thirty percent of the microcolonies formed green plantlets. Lee *et al.* (1996) developed an efficient system for protoplast isolation, culture and regeneration from 7 cultivars. Using feeder cells, the plating frequencies ranged from 0.05% to 0.32%.

Torello *et al.* (1984) observed different culture responses of mature caryopses of two cultivars in red fescue, and investigated the effects of auxin sources on callus growth. They found $20 \mu\text{M}$ (4.4 mg l^{-1}) 2,4-D was the best in callus

growth, and used 0.1 mg l^{-1} BAP for regeneration medium. In general, regeneration ability of a long-term callus culture declined. The same group found that addition of activated charcoal to the callus maintenance medium prior to callus transferring to the regeneration medium significantly enhanced the regeneration ability of calli cultured for over 5 years (Zaghmout and Torello, 1988). They believed that the activated charcoal could adsorb toxic levels of 2,4-D and other morphogenesis inhibitors. These authors later developed suspension cultures using a modified MS medium containing 4 mg l^{-1} 2,4-D and 3 g l^{-1} CH with the latter enhancing culture growth significantly. Removal of fast-growing, nonembryogenic cell clusters before initiation of suspensions was thought to be critical, and green plantlets were regenerated from the suspensions (Zaghmout and Torello, 1989). In addition, the same authors found that elevating sucrose level from 60 mM up to 120 and 180 mM in suspension as well as regeneration media helped restore regeneration potential of long-term suspension cultures, and increased green plantlet formation (Zaghmout and Torello, 1992b). Protoplasts were isolated from the embryogenic suspension cells and plated in agarose squares surrounded by nurse cells. The maximum plating efficiency was 1%. The microcolonies produced embryos, which germinated to form plantlets in regeneration medium (Zaghmout and Torello, 1990). Altpeter and Xu (2000) tested tissue culture responses of mature embryos from 7 turf-type red fescue cultivars, observed callus induction rates ranged from 57% to 78.4% in a medium containing 5 mg l^{-1} 2,4-D, and found significant difference in callus regeneration ability (from 0% to 22%) on a medium containing 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP. Recently, Salehi and Khosh-Khui (2005) reported that very high 2,4-D concentration ($200 \text{ } \mu\text{M}$ or 44.4 mg l^{-1}) enhanced callus induction rate (78%) of red fescue (cv. Shadow), and a combination of $60 \text{ } \mu\text{M}$ (13.3 mg l^{-1}) of 2,4-D and $12.5 \text{ } \mu\text{M}$ (2.8 mg l^{-1}) of BAP resulted in the highest callus regeneration rate (34%) of red fescue.

The only report on colonial bentgrass tissue culture (Wang *et al.*, 2002) evaluated 14 MS-based culture media containing various phytohormones and other supplements for mature caryopses culture of cv. Exeter. Eleven media that contained 2,4-D as an auxin had high callus induction

rates (98–100%). In general, embryogenic callus induction was low. Although the best callus induction medium was the MS medium containing 5 mg l^{-1} 2,4-D and 500 mg l^{-1} L-proline, which had 100% callus induction and yielded 19% “embryogenic calli”, they regenerated poorly. On the other hand, the media containing dicamba (6.63 mg l^{-1}), BAP ($0.5\text{--}2 \text{ mg l}^{-1}$), and CH (500 mg l^{-1}) had low callus induction rates (20–37%), but relatively high embryogenic callus frequencies (6–10%), and the embryogenic calli were more regenerable. CH by itself seemed to promote callus regeneration. Media containing 2,4-D ($2\text{--}5 \text{ mg l}^{-1}$) and CH had 5–7% embryogenic callus formation and high regeneration rates from those calli.

In warm-season turfgrasses, callus induction of common bermudagrass was first reported by Krans (1981). Ahn *et al.* (1985) observed that calli had significantly more fresh weight when growing on N6 medium (Chu *et al.*, 1975) over the MS medium. The best culture conditions in the report were immature inflorescence of 0.5 cm in length on N6 medium containing 1 mg l^{-1} 2,4-D and 60 g l^{-1} sucrose, from which 84% of calli were embryogenic. Green plantlets were obtained from hormone-free N6 medium and, subsequently, grown to maturity. It seemed the explant type and age played an important role in recovering regenerated plants since no plantlets were regenerated when nodes, root tips, young leaves, mature caryopses or inflorescences longer than 1.5 cm were used as explants. A follow-up report from the same group demonstrated genotype dependence of the regeneration ability of the induced calli. In addition, a regenerable suspension cell culture was established (Ahn *et al.*, 1987). Artunduaga *et al.* (1988) investigated 2,4-D concentrations required by different genotypes of common bermudagrass for best embryogenic callus formation from immature inflorescence. While all the induction medium (a half-strength MS medium) also contained 1 mg l^{-1} indole-3-acetic acid (IAA), the authors found 1 mg l^{-1} 2,4-D was optimal for embryogenic callus formation from an accession while 3 mg l^{-1} was more suitable for two other accessions. Plantlets were regenerated from two out of the three accessions on a half-strength MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} zeatin although about 90% of the plantlets from an accession were

albino. In another correspondence (Artunduaga *et al.*, 1989), the same group studied 2,4-D concentration and supplement of CH on culture responses of immature inflorescence of *C. dactylon* cv. Zebra. The maximum fresh weight and embryogenic callus induction came from the medium containing 3 mg l^{-1} 2,4-D and 200 mg l^{-1} CH. The albino plants were as high as 31% of the regenerated plants. Chaudhury and Qu (2000) observed that inclusion of 1 mg l^{-1} 2,4-D and very low concentration of BAP (0.01 mg l^{-1}) in the callus induction/subculture medium substantially improved the regeneration ability of the calli from common bermudagrass cv. Savannah and hybrid cv. Tifgreen. The effect was more obvious when younger immature inflorescences ($<0.75 \text{ cm}$) were used as explants. All 96 plantlets obtained were green and morphologically normal. Scanning electron microscopy (SEM) revealed embryogenesis was the major pathway for plantlet regeneration in bermudagrass. Li and Qu (2002) found that supplement of ABA ($2\text{--}5 \text{ mg l}^{-1}$) to the above BAP-containing medium further improved formation of somatic embryo clusters (SEC), and tracked the whole process of SEC formation microscopically over a period of 70 days. In addition, secondary somatic embryogenesis was observed in this species. Moreover, they found that bermudagrass plantlet regeneration was greatly enhanced by addition of GA_3 in the regeneration medium. Li and Qu (2004) further developed highly-regenerable callus lines, including suspension lines, by adjusting BAP level in the medium and carefully selecting calli. Salehi and Khosh-Khui (2005) tested a wide range of 2,4-D concentrations in MS callus induction medium for mature caryopsis culture of common bermudagrass (the cultivar was only identified as "California origin") and BAP concentrations in regeneration medium, and found that $40 \text{ }\mu\text{M}$ (8.85 mg l^{-1}) 2,4-D had 100% callus induction frequency, and $60 \text{ }\mu\text{M}$ 2,4-D (13.3 mg l^{-1}) and $7.5 \text{ }\mu\text{M}$ (1.7 mg l^{-1}) BAP led to 100% callus regeneration with 3.2 plantlets per piece of callus. The widely used hybrid bermudagrass cultivars, Tifgreen (Burton, 1964), Tifway (Burton, 1966), TifEagle (Hanna and Elsner, 1999), and TifSport (Hanna *et al.*, 1997), although all being *C. dactylon* \times *C. transvaalensis*, seem to be very divergent in their responses to tissue culture. Goldman *et al.* (2004b) reported 36% embryogenic callus induction rate from nodes

of TifEagle plants using an MS medium containing $4.5 \text{ }\mu\text{M}$ 2,4-D, $0.044 \text{ }\mu\text{M}$ BAP, and 40 g l^{-1} (instead of the routine 30 g l^{-1}) sucrose. In contrary, no embryogenic calli were produced from nodes of TifSport. The best culture of TifSport using very young immature inflorescence yielded 16% of embryogenic calli on the same medium. A great range of somaclonal variation, in terms of plant height, leaf width, leaf length, and number of stolons, was observed from regenerated plants of TifEagle, but not from TifSport. Tifway, on the other hand, may be the most recalcitrant cultivar among the four, with tissue culture responses more similar to a parent, *C. transvaalensis* (Chaudhury and Qu, unpublished data). Although calli were ready to be induced from vegetative tissues (nodes, internodes, and young leaves), and were not prone to browning, they were not regenerable. Severe browning, caused by secretion of phenolic compounds, was encountered when immature inflorescences were cultured, which resulted in little callus induction. Qu and Chaudhury (2001) overcame the problem by treating the inflorescence segments in liquid MS medium containing 0.2% L-ascorbic acid, an antioxidant, followed by culture on an MS callus induction medium supplemented with 6 g l^{-1} polyvinylpyrrolidone (PVPP), which is a polyphenol adsorbent. By doing so, calli were induced from over 50% of immature inflorescences smaller than 1.5 cm, and green plantlets were regenerated.

St. Augustinegrass tissue culture was first reported by Kuo and Smith (1993). Immature embryos isolated from inflorescences, 10–14 days after emergence from the sheath, of cv. Texas Common were cultured on MS medium supplemented with various concentration of 2, 4-D for callus induction. Calli induced on medium containing 1 mg l^{-1} 2,4-D grew faster than the calli on 5 mg l^{-1} 2,4-D medium, whereas no calli were induced on medium having 10 mg l^{-1} 2, 4-D. Four weeks later, the calli were subcultured on MS medium containing 0.5 mg l^{-1} 2,4-D and 0.25 mg l^{-1} kinetin for another 4 weeks before they were transferred to the MS medium supplemented with 0.25 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin. After 1–2 weeks, somatic embryos became obvious and shootlike structures were observed. The calli were then transferred to the hormone-free, half-strength MS medium for further growth of the plantlets. Approximately 33% of the induced calli were able

to regenerate plantlets. The authors considered it was critical to have timely transfer of callus at each stage for successful regeneration. Li *et al.* (2006) examined callus induction of 11 types of explants of St. Augustinegrass (cv. Raleigh) and the effect of BAP in callus induction medium. The explants were cultured on MS medium supplemented with 1 mg l^{-1} 2,4-D and various concentrations of BAP. Although they observed that five types of explants had high callus induction frequencies (71–100%), only calli from three kinds of explants were embryogenic and can regenerate plantlets. They are immature embryos harvested at various stages (3 days and 7–14 days) and young shoot base. However, the callus induction of the 3 days immature embryos was below 20%, and the regeneration rate of calli induced from young shoot base was under 10%. In general, addition of BAP in callus induction medium did not affect callus induction but significantly improved callus regeneration ability. The best combination the authors found were the use of 7–14 days immature embryo as explant and a callus induction medium (with 1 mg l^{-1} 2,4-D) supplemented with 0.5 mg l^{-1} BAP, which had 97.7% callus induction frequency and 47.6% regeneration rate. SEM study revealed details of somatic embryogenesis in the species. Approximately 8000 plants were regenerated in a breeding effort to induce somaclonal variations (Li *et al.*, submitted).

Tissue culture of Japanese lawngrass was first reported in 1989 (Alkhayri *et al.*, 1989; Asano, 1989). In Asano's report, calli were induced from 4-day-old seedlings cultured on a modified N6 medium containing various concentration of 2, 4-D or picloram. More fresh weight of callus was recovered from picloram-containing medium. Only 0.3% of calli developed embryogenic sectors, from which green plantlets were obtained on a hormone-free, half-strength MS medium. No obvious morphological variations were observed from the recovered plants. Protoplasts were isolated from callus lines and cultured in the same modified N6 medium supplemented with 5 mg l^{-1} picloram. Plating efficiency was about 20–30%, but no regeneration was observed. Alkhayri *et al.* (1989) had somewhat different observation. They cultured mature embryos separated from caryopses on MS or N6 medium with various 2,4-D concentrations. Over 90% of the explants produced calli in all treatments.

The best combination was MS medium with 1 mg l^{-1} 2,4-D; the calli had more fresh weight and higher regeneration frequency (42–59%) after transferred to 2,4-D-free regeneration medium. Calli from N6 medium did not regenerate. Asano *et al.* (1996) found that cytokinin, especially BAP and thidiazuron (TDZ), and thiamine HCl were essential for embryogenesis. In their presence, supplement of riboflavin or α -ketoglutaric acid, a key metabolic intermediate of TCA cycle, further enhanced formation of embryogenic calli. Inokuma *et al.* (1996) observed that LS medium (Linsmaier and Skoog, 1965) had been superior over MS or N6 medium in inducing embryogenic calli in this species. Protoplasts were isolated from the suspensions and the best plating efficiency was 0.15% in medium solidified with agarose. Green plantlets were recovered from the protoplast culture.

In vitro regeneration of buffalograss was first reported in 1997 (Fei *et al.*, 1997). Female immature inflorescences of cv. 609 were cultured in MS medium containing a combination of 2, 4-D ($1\text{--}4 \text{ mg l}^{-1}$) and BAP ($0\text{--}0.4 \text{ mg l}^{-1}$). Seventeen percent calli from medium containing 2,4-D only ($1\text{--}3 \text{ mg l}^{-1}$) regenerated shoots. Interestingly, no calli from BAP-containing medium regenerated. The tissue culture responses depended on genotype and the season when the inflorescences were harvested. Later on, the same group reported that inclusion of silver nitrate (10 mg l^{-1}) in the callus induction medium significantly improved embryogenic callus formation and subsequent regeneration for cultures of immature inflorescences from male plants of two genotypes but not the ones from female plants. The stimulatory effects were environment and genotype dependent (Fei *et al.*, 2000). In another correspondence (Fei *et al.*, 2002), the authors reported that lowering 2, 4-D concentration and addition of BAP enhanced shoot regeneration for 2-month-old calli induced on a medium containing $9 \mu\text{M}$ (2 mg l^{-1}) 2,4-D from male immature inflorescences of cv. Texoka. The best regeneration medium contained $2.25 \mu\text{M}$ (0.5 mg l^{-1}) 2,4-D and $0.44 \mu\text{M}$ (0.1 mg l^{-1}) of BAP, on which 36.4% embryogenic calli regenerated shoots. It was also found that gelling agent Gelrite significantly improved somatic embryo formation over agar. However, these effects also seemed to be genotype dependent since no such enhancement was observed for immature inflorescences collected

from female plants of genotype 315. In addition, embryogenic calli were obtained from leaf base and leaf segments of seedlings from germinating caryopsis of cv. Cody. The induction frequency was relatively low (around 10%) and depended on seedling age and the concentration of 2,4-D in the induction medium. Plantlets were recovered from the embryogenic calli (Fei *et al.*, 2001).

Seashore paspalum is a recently promoted turfgrass species and its tissue culture was not reported until 1997. Cardona and Duncan (1997) used immature inflorescences as an explant from 9 ecotypes of seashore paspalum. The explants were cultured on a medium consisting of the MS basal salts and B5 vitamins. In a factorial experiment designed to test the effects of growth regulators 2,4-D and BAP, it was observed that the effect of BAP or the interaction between BAP and 2,4-D was not significant in callus induction efficiency whereas the effect of 2,4-D was. Responses to 2,4-D concentrations varied among the ecotypes tested. However, a low concentration of 2,4-D (1 to 2 mg l^{-1}) was recommended for embryogenic callus formation. The callus first emerged about a week after culture initiation while “precocious” somatic embryos were observed 3 weeks later. Callus induction frequency varied from 0% up to 55%. Five-month-old calli were cultured on a half-strength MS medium containing different combinations of BAP and NAA for regeneration, and numerous green plantlets were recovered from all the ecotypes. Although the optimized combination of BAP and NAA varied among the ecotypes, the combination of 1 mg l^{-1} BAP with NAA between 0.5 and 2 mg l^{-1} often had the highest regeneration frequency. Ecotypes HI-1, Mauna Key, and PI299042 were among the best in regeneration ability.

Centipedegrass tissue culture was first reported by Krans’ lab (Krans, 1981; Krans and Blanche, 1985). One mg l^{-1} 2,4-D or 100 mg l^{-1} IAA was determined to be the optimal concentration for callus induction on MS medium based on fresh callus weight. Fresh callus weight from a single explant was greater using immature inflorescence than mature caryopses. When the calli were transferred to regeneration medium, either hormone free or containing 0.5 mg l^{-1} kinetin, plantlets were obtained with less than 1% of albinos. “Explant source had no affect on frequency or quality of plantlets.” Ma *et al.*

(2004) induced calli from mature caryopses on MS medium supplemented with 4.5 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP, and subcultured them on MS medium containing 4 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, and 5 mg l^{-1} ascorbic acid. The MS regeneration medium included 2 mg l^{-1} BAP, 1 mg l^{-1} NAA, 5 mg l^{-1} CoCl_2 , and 0.5 mg l^{-1} TDZ. The regeneration frequency was over 90%. The authors thought that CoCl_2 , an ethylene inhibitor, helped in callus regeneration.

2.2 Development of Transgenic Turfgrass Plants

As in most monocot transformation cases, there are three major approaches to transform turfgrasses: protoplast (by polyethylene glycol (PEG) treatment or electroporation), particle bombardment (also known as microprojectile bombardment or the biolistic method, and can be performed with a Du Pont PDS-1000/He device or a particle inflow gun), or *Agrobacterium tumefaciens* infection. The most frequently used selection systems include *Escherichia coli* hygromycin B phosphotransferase gene (*hph* or *hpt*) and hygromycin B (*hyg B*) (Gritz and Davies, 1983), or *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene (*bar*) and bialaphos (active ingredient of herbicide Herbace[®]) or phosphinothricin (PPT or glufosinate, active ingredient of herbicides BastaTM, Liberty[®], Finale[®], and Challenge[®]) (Thompson *et al.*, 1987). The selectable marker genes are usually under control of cauliflower mosaic virus (CaMV) 35S promoter, rice *Act1* promoter, or maize *ubil* promoter. Quite often, the *E. coli* β -glucuronidase gene (*uidA* or *GUS*) (Jefferson *et al.*, 1987) or the jellyfish green fluorescent protein gene (*gfp*) (Chalfie *et al.*, 1994) is used as a reporter gene to help evaluate the transformation efforts. Polymerase chain reaction (PCR) and reporter gene expression are usually employed to preliminarily screen for putative transgenic plants. However, the genome integration of the transgene in the selection agent-resistant plants has to be confirmed by Southern blot analysis. The Southern hybridization patterns often help distinguish independent transformation events. Expression of the transgene should be demonstrated by

Northern and/or Western analysis, and/or the activity of the enzyme encoded by the transgene.

Wang *et al.* (1992) first reported tall fescue transformation by introducing DNA vectors through PEG treatment into protoplasts isolated from embryogenic suspensions. After transformation, the protoplasts were cultured with agarose bead-type method with nurse cells, and were later selected with either hyg B (200 mg l⁻¹) or PPT (100 or 200 mg l⁻¹). The transformation frequency ranged from 10⁻⁶ and 10⁻⁵. A total of 38 independent, morphologically normal transgenic plants were obtained. Plants with *bar* transgene were resistant to herbicide BastaTM. In another report, Ha *et al.* (1992) electroporated protoplasts and recovered regenerated transgenic plants with hyg B selection. They also demonstrated *GUS* reporter gene expression in the transgenic plants. The same group later (Penmetsa and Ha, 1994) studied and optimized the conditions (including DNA concentration, protoplast density, age of the suspension, and field strength used for electroporation) for electroporation transformation of tall fescue protoplasts using *GUS* transient expression assays. Kuai and Morris (1995) found that the age of the suspension, the stage of the growth cycle, the time length of cell wall digestion, and the osmolarity of the transformation medium all affected transient and stable *GUS* reporter gene expression in PEG-mediated protoplast transformation. Dalton *et al.* (1995) tested different hyg B selection schemes in PEG-mediated protoplast transformation and observed that continuous selection at a low concentration (50 mg l⁻¹) yielded the highest transformation frequency, and continuous selection at high level biased selection of transgenic plants with multiple transgene copies. The same group (Bettany *et al.*, 2002) reported the highest co-expression frequency (38%) of two co-transformed vectors when the molar gene ratio was 4:1. Spangenberg *et al.* (1995a) obtained transgenic plants by particle bombardment of embryogenic suspensions and hyg B selection. They observed a twofold improved efficiency in recovering resistance calli when the initial selection (hyg B, a step-up selection scheme from 30 up to 150 mg l⁻¹) was performed in liquid culture medium, compared to a continuous solid medium selection (150 mg l⁻¹). About 35% of the hyg B-resistant calli regenerated into plants. Kuai *et al.* (1999) recovered a transgenic plant

line of the *bar* gene by protoplast transformation and PPT selection (1.5–3 mg l), which showed resistance to PPT spray and was fertile. However, with limited number of seeds set by crossing with nontransgenic plants, they did not observe transmission of the *bar* gene to the T₁ progenies through pollen. Cho *et al.* (2000) adjusted concentrations of 2,4-D, BAP, and CuSO₄ in the culture medium and obtained regenerative tissues containing “multiple, light green, shoot-meristem-like structures” from mature caryopses (cv. Kentucky 31) cultured under dim-light conditions. They co-transformed the tissue with *hpt*, *bar*, and *GUS* transgenes by particle bombardment, and recovered 8 independent transgenic plant lines from hyg B selection (30–100 mg l⁻¹). Co-expression of two transgenes (*hpt* and *bar* or *GUS*) were 50–75%, and 25% transgenic plants had expression of all the three genes. Bai and Qu (2001b) reported transformation of two elite turf-type cultivars (Coronado and Virtue) by bombardment of embryogenic suspensions and hyg B (250 mg l⁻¹) or bialaphos (3–5 mg l⁻¹) selection. Dalton *et al.* (1998) employed silicon carbide “whiskers” method to transform suspensions and obtained 8 transgenic plants. Bettany *et al.* (2003) first reported *Agrobacterium* transformation of tall fescue. They infected suspensions with *Agrobacterium* strain LBA4404 harboring a “super-binary” vector pTOK233 and recovered two independent transgenic plant lines. Later on, more efficient *Agrobacterium*-mediated transformation was reported by three laboratories using 5–8 weeks of embryogenic calli induced from mature caryopses. Lee *et al.* (2004) found that a combination of strain EHA101 (pIG121Hm), 100 μM acetosyringone, and a 30-min vacuum treatment during co-cultivation yielded the best results: over 20% transformation frequency was achieved when hyg B selection was performed at 50 mg l⁻¹ level (cv. Kentucky 31). Wang and Ge (2005a) broke embryogenic calli into small pieces (cv. Jesup and Kentucky 31) and infected them with *Agrobacterium* strains LBA4404 or EHA105 harboring pTOK233 or a pCAMBIA binary vector, and selected the transgenics at 250 mg l⁻¹ hyg B. An average of 5.5% overall transformation efficiency (independent transgenic plant lines over the number of intact callus pieces infected) was achieved. No advantage of pTOK233 over pCAMBIA vectors was observed.

Fertile plants were obtained and Mendelian inheritance of transgenes was observed at T_1 generation by PCR analysis. Dong and Qu (2005) transformed calli of elite turf-type cultivars Matador and Coronado, and infected them with *Agrobacterium* strains LBA4404 or EHA105 containing binary vector pCAMBIA1301 with or without pTOK47, a vector similar to pTOK233. Approximately 34% of the calli infected were resistant to hyg B selection (250 mg l^{-1}), and 8% overall transformation frequency was achieved. They observed that a higher level of 2,4-D (5 mg l^{-1}) during callus culture and co-cultivation helped improve the transformation efficiency while inclusion of pTOK47 did not. In all the three reports, low transgene copy number in transgenic plants was observed. Recently, Cao *et al.* (2006) obtained highly regenerative callus lines from mature caryopses culture (cv. Triple A) on a modified N6 medium and found higher pH (6.2) enhanced both calli induction and plant regeneration. After co-cultivation with strain EHA105 (pCAMBIA1301), the calli were cultured on a nonselective preregeneration medium containing 400 mg l^{-1} L-cysteine for 7 days followed by regeneration on a nonselective medium. The selection pressure was imposed at the rooting medium (70 mg l^{-1} hyg B), and putative transgenic plants developed roots. In studies of transgene stability and inheritance in transgenic tall fescue plants, Bettany *et al.* (1998) investigated two transgenic plants in their GUS expression after vegetative propagation (tillering). The plants either had multiple *GUS* gene copies or the transgene had deletion or rearrangement. Considerable variations in GUS enzyme activity, among the tillers and from generation to generation, were observed mostly in the first four generations, and GUS expression seemed to be stabilized by the fifth generation. Southern and DNA methylation analysis could not identify obvious difference between the tillers that had various GUS activities. On the other hand, Z.Y. Wang *et al.* (2003a) studied *hph* and *GUS* transgene inheritance in transgenic plants obtained by particle bombardment through reciprocal crosses. Among over 40 T_1 plants from each cross, an 1:1 segregation ratio was observed by PCR analysis, indicating that both of the transgenes were inherited as a genetic locus, and the transgenes were transmitted by both egg cells

and pollen grains. Southern analysis showed the same hybridization pattern among T_0 , T_1 , and T_2 plants, suggesting a stable inheritance of the transgenes through sexual reproduction. To facilitate inheritance study, Z.Y. Wang *et al.* (2003b) developed a protocol for vernalization of transgenic tall fescue plants. In addition, the same group (Z.Y. Wang *et al.*, 2003c) investigated the field performance of transgenic tall fescue plants in comparison with nontransgenic, regenerated plants through tissue culture and nontransgenic, seed-derived plants. Although the T_0 transgenic and the tissue culture plants were inferior to the seed-derived nontransgenic plants in terms of height, tiller number, and seed yield, no major differences were observed among their progenies. In general, transgenic plants performed similarly to the tissue culture plants, and no major effect was observed from transgene introduction.

Kentucky Bluegrass transformation was first reported by Ha *et al.* (2001). The authors used an approach similar to what they did with tall fescue (Cho *et al.*, 2000) to obtain highly regenerative tissues from mature caryopses (cv. Kenblue) and co-bombarded the explants with three transgenes (*hpt*, *GUS*, and *gfp*) and the bombarded tissues were subjected to hyg B selection, first at 100 mg l^{-1} and later at 30 mg l^{-1} . It took quite a few months of subculture before the resistant, light-green tissues were observed. A transformation efficiency of 2.2% was achieved and 70% of the resistant tissues regenerated into plantlets. Co-expression frequency of two transgenes was 30–40%, and for all three genes was 20%. Gao *et al.* (2006) reported an efficient transformation protocol for Kentucky bluegrass using particle bombardment. The authors induced calli from immature embryos (breeding line DP-37-61), of which 90% were embryogenic. They achieved 22% transformation efficiency with the *hpt* transgene and hyg B (100 mg l^{-1}) selection, or 7.5% with the *bar* transgene and bialaphos selection (2 mg l^{-1}). The *bar* gene transgenic plants were resistant to herbicide BastaTM. The authors attributed their high transformation efficiency to a combination of the genotype and explant they employed, and the careful selection of the embryogenic callus lines used for the transformation experiments. *Agrobacterium* transformation of Kentucky bluegrass has been reported. Chai *et al.* (2003) found supplement of cupric sulfate enhanced induction

of embryogenic calli from mature caryopses (cv. Md). Embryogenic calli were infected with strain AGL1 (pDM805) and selection was carried out with bialaphos ($1.5\text{--}3\text{ mg l}^{-1}$). Four transgenic lines were obtained and the transgenic plants showed resistance to BastaTM spray.

Stable transformation of perennial ryegrass was first achieved with particle bombardment of a nonembryogenic suspension line, and hyg B resistance callus lines were obtained (Van Der Maas *et al.*, 1994). Spangenberg *et al.* (1995b) optimized conditions of bombardment of single-genotype derived embryogenic suspension lines (cv. Citadel) using a particle flow gun. They found that initial liquid selection and a stepwise increase of hyg B selection (from $50\text{--}200\text{ mg l}^{-1}$) had higher transformation efficiency with 26% bombarded plates yielded resistant calli, of which 23% regenerated. Wang *et al.* (1997) performed PEG-mediated protoplast transformation using a selection system containing the *nptII* gene and kanamycin/G418, which is more often used in dicot transformation. The transformed protoplasts were embedded in agarose with no nurse culture and first subjected to selection at 25 mg l^{-1} kanamycin, followed by selection at 25 and 40 mg l^{-1} G418, respectively. A transformation frequency of 5×10^{-6} was achieved, and 20 green plants were obtained. Fertile plants set seeds after crossing and the transgenes were inherited to T1 progenies. Folling *et al.* (1998) found that the nuclease activity released from protoplasts degraded DNA vectors and decreased transformation frequency. Raising the pH of the transformation buffer to 9 and performing transformation at 0°C reduced the nuclease activity and improved transformation frequency by nearly fivefold. Similar to what they did in tall fescue, Dalton *et al.* (1998) also described perennial ryegrass transformation using silicon carbide, and recovered a transgenic plant. The same group later found that the transformation efficiency of the particle inflow gun was “considerably higher”, and used the approach to transform the suspensions of an agronomically more important diploid cultivar (Dalton *et al.*, 1999). They employed liquid culture selection at the first 2–3 weeks at 50 mg l^{-1} hyg B and then solid culture selection at 75 mg l^{-1} of hyg B, and eventually recovered 6 transgenic plants. Altpeter *et al.* (2000) described a selection scheme using *nptII* gene and paromomycin (100 or 200 mg l^{-1})

after particle bombardment of calli induced from mature and immature embryos and immature inflorescences. Dozens of fertile transgenic plants were generated, and the transformation efficiency ranged from 3.7% to 11.4%. Recently two articles described high efficiency transformation of perennial ryegrass by the *Agrobacterium*-mediated approach. Bajaj *et al.* (2006) induced calli from the longitudinally sliced meristematic regions of seedlings. Among 250 lines, 6 highly regenerable callus lines were selected and maintained as plants *in vitro*, and the calli induced from the meristematic regions of these plant tillers were infected with *Agrobacterium* strain EHA101. Transformed calli were selected with hyg B at $94.8\text{ }\mu\text{M}$ (50 mg l^{-1}) for the first 2 weeks and $151.6\text{ }\mu\text{M}$ (80 mg l^{-1}) for the second round. About 7% transformation frequency was achieved and more than 1000 transgenic plants were obtained. Using a similar *Agrobacterium*-mediated transformation and selection scheme as they did in tall fescue, Cao *et al.* (2006) achieved 23.3% transformation efficiency for perennial ryegrass.

Creeping bentgrass transformation was first achieved by particle bombardment of embryogenic calli and the transgenic plants were identified by the GUS transgene expression without any selection (Zhong *et al.*, 1993). Hartman *et al.* (1994) introduced the *bar* gene into two cultivars of creeping bentgrass through bombardment of embryogenic suspensions, and recovered 55 transgenic plants resistant to herbicide Herbiace[®], suggesting they could be used in the golf greens to help solve the *Poa annua* weed problems. Lee *et al.* (1996) introduced the *bar* gene into protoplasts using either PEG treatment or electroporation, and obtained 153 transgenic plants resistant to Herbiace[®]. Xiao and Ha (1997) reported efficient recovery of *hph* transgenic plants using 200 mg l^{-1} hyg B selection after bombardment of suspensions. Asano *et al.* (1998) found that substitution of CaCl_2 with $\text{Ca}(\text{NO}_3)_2$ and elevated pH (9 to 10) in the buffer improved transient GUS activity in protoplast electroporation transformation, and used the altered buffer to recover *bar* transgenic plants. Dalton *et al.* (1998) obtained 6 transgenic plants from silicon carbide transformation of creeping bentgrass. *Agrobacterium*-mediated transformation of creeping bentgrass was first reported by Yu *et al.* (2000) in which the authors introduced a *gfp* gene and recovered the transgenic

plants by detecting the green fluorescence without any selection. Using *Agrobacterium*-mediated transformation and *bar* gene selection, Luo *et al.* (2004b) obtained a large number of transgenic plants (219 independent transformation events) with stable transformation frequency of 18–45%. The *bar* gene was stably expressed in T₁ plants and a Mendelian inheritance was observed among T₁ populations. About two-thirds of the transgenic plants had a single copy of the *bar* transgene. Han *et al.* (2005) reported *Agrobacterium* transformation of creeping bentgrass and *hyg B* selection. A novel *Agrobacterium*-mediated transformation of creeping bentgrass (cv. Penncross) was reported (Wang and Ge, 2005b) in which the nodes from stolons were cut into half and directly infected by *Agrobacterium*. Green shoots were produced from the infected nodes 4–5 weeks under *hyg B* selection in a medium containing 4.5 μ M kinetin and 0.2 μ M 2,4-D. The approach was efficient (6.3–11.3%) and transgenic plants can be quickly recovered. Moreover, this approach bypasses the callus induction and regeneration stages and may save a great deal of efforts in improving tissue culture responses, and has potential to be a way for genetic transformation of recalcitrant grass species. It would be interesting to see whether any transgenic plants obtained this way are chimeric and if not, histologically what kind of cells were transformed and formed the shoots. In other aspects, Fu *et al.* (2006) analyzed the integration sites of the transfer DNA (T-DNA) in transgenic creeping bentgrass and found that, like in other plants, the integration is a rather complicated process and may have diverse mechanisms, such as the microsimilarity-based illegitimate recombinations, and involvement of filler DNA, and/or T-DNA truncation. Fei and Nelson (2004) evaluated four transformation events of roundup-tolerant creeping bentgrass expressing the *cp4 epsps* gene, in fitness-related reproductive traits, such as heading date, anthesis duration, inflorescence length, number of florets per inflorescence, pollen size, and seed set capacity by open pollination. No significant differences were observed between the transgenic plants and the nontransgenic plants obtained through tissue culture.

Agrobacterium-mediated transformation of colonial bentgrass was reported by Chai *et al.* (2004). Embryogenic calli from mature caryopses

of cv. Tiger were induced on a MS medium containing 2 mg l⁻¹ 2,4-D, and infected with *Agrobacterium* strain LBA4404 (pTOK233) in the presence of 100 μ M acetocyringone. The infected calli were selected with *hyg B* first at 50 mg l⁻¹ and later at 70 mg l⁻¹, and the callus regeneration medium contained 50 mg l⁻¹ *hyg B*, and the plantlets were further selected at 20 mg l⁻¹ for rooting. After two cycles of selection, 81.2% of the *hyg B* resistant calli had strong *GUS* expression while roots of all *hyg B*-resistant plants and some leaves also showed *GUS* activity. Southern analysis of four randomly chosen *hyg B*-resistant plants showed integration of the *hpt* transgene in the plant genome.

Spangenberg *et al.* (1994) developed a red fescue protoplast culture system from fresh or cryopreserved suspensions using agarose beads, nurse culture, and regenerated plantlets. They employed the system and introduced the *bar* gene into red fescue by PEG treatment and recovered transgenic plants under selection at high concentration (50 mg l⁻¹) of PPT. The plants survived spray of herbicide BastaTM. Using the embryogenic suspensions, the same group (Spangenberg *et al.*, 1995a) later reported red fescue transformation by the biolistic method. Eighty-five percent of the resistant calli regenerated into plantlets. Cho *et al.* (2000) used an approach similar to what they did in tall fescue and Kentucky bluegrass as mentioned above, by generating highly regenerative green tissues and bombardment, and obtained transgenic red fescue plants with a stepwise increased selection of *hyg B* (30–100 mg l⁻¹). Altpeter and Xu (2000) bombarded embryogenic calli with an *nptII* gene construct and used 100 mg l⁻¹ paromomycin for selection. Between 3% and 5% of the bombarded calli of red fescue produced transgenic plants. No transformation of other fine fescues was reported.

Among the warm-season grasses, bermudagrass transformation was first reported in the triploid, interspecific hybrid (*C. dactylon* \times *C. transvaalensis*, cv. TifEagle). Zhang *et al.* (2003) induced embryogenic calli from stolons and developed suspensions from them. The suspensions were bombarded with a vector containing the *hpt* gene. Seventy-five transgenic plants were obtained under selection of 200 mg l⁻¹ *hyg B*. The integration and expression of the transgene were stable during vegetative propagation. Goldman *et al.* (2004a)

transformed embryogenic calli of TifEagle with the herbicide-resistant *bar* gene, and recovered 89 transgenic plants from at least 9 transformation events using selection of 5–15 mg l⁻¹ PPT. The transgenic plants were resistant to herbicide Liberty[®]. Flow cytometry revealed that most of the plants (82/89) were hexaploid, which often showed significant variation in leaf length and width from the parent cultivar. However, no detectable difference was observed in amplified fragment length polymorphism (AFLP) analysis between the hexaploid transgenic plants and the triploid parent cultivar plants. Hu *et al.* (2005) reported *Agrobacterium*-mediated transformation of TifEagle with the *hpt* gene and hyg B selection. A total of 24 independent transgenic plant lines were obtained. Since the vector used for transformation also contained the *bar* gene, plants expressing the *bar* gene displayed resistance to herbicide Liberty[®]. Li and Qu (2004) developed highly regenerable callus lines from tetraploid common bermudagrass (*C. dactylon*) cv. J1224, and recovered transgenic plants from four independent transformation events by particle bombardment and hyg B selection (200 mg l⁻¹ at callus level and 100 mg l⁻¹ at regeneration stage). Because of the *bar* and *GUS* genes in the vector used for co-transformation, one event had *GUS* activity and three events were resistant to Liberty[®]. The group later reported *Agrobacterium* transformation of the same cultivar using both callus lines and suspension cells, and hyg B selection (Li *et al.*, 2005). Flow cytometry analysis indicated that the ploidy level of the transgenic plants was not altered. Attempts to use *bar* gene selection resulted in transgenic calli but no plants were recovered. Similar to the approach used in creeping bentgrass, successful transformation through direct node infection by *Agrobacterium* was reported in triploid, hybrid bermudagrass (cv. TifEagle, Wang and Ge, 2005b).

Japanese lawngrass transformation was first reported using direct gene transfer to protoplasts mediated by PEG (Inokuma *et al.*, 1998). The authors found that 400 mg l⁻¹ hyg B was a proper level for the selection of resistant cell colonies. The selection seemed to be quite efficient and about 400 transgenic plantlets were recovered without selection at the regeneration stage. Toyama *et al.* (2003) first reported *Agrobacterium* transformation of the Japanese lawngrass. They observed that removal

of 2,4-D and CaCl₂ from the infection medium, and extension of the co-cultivation period to 9 days enhanced transient *GUS* reporter gene expression, and thus used these conditions to perform transformation. Four bialaphos-resistant plants were regenerated per 700 mg calli after a step-up selection at callus and shoot levels (0.5–5 mg l⁻¹) and 10 mg l⁻¹ bialaphos at rooting medium. Ge *et al.* (2006) again, successfully applied their “node-infection-by-*Agrobacterium*” approach to Japanese lawngrass and obtained shoots resistant to hyg B selection (75 mg l⁻¹) in 4–5 weeks. Approximately 50% of the resistant plants were transgenic, and a transformation frequency of 6.8% was achieved.

Genetic transformation of buffalograss has been reported (Fei *et al.*, 2005). Embryogenic calli induced from female immature inflorescences of cv. 91–118 were subcultured for 15 months to obtain soft, friable, and highly regenerable calli, which were bombarded with gold particles of 0.75 mm in diameter. A delay of selection (7 or 28 days) yielded about 2% of calli resistant to glyphosate using a vector containing two *cp4 epsps* gene constructs. In total, 77 pieces (2.2%) of calli were resistant to glyphosate (1 or 2 mM) selection and transferred to regeneration medium containing 0.1 mM glyphosate. Three green shoots developed roots in the selection-rooting medium (0.05 mM glyphosate). Trait RUR Lateral Flow Test strips revealed the resistant plantlets had CP4 EPSPS enzyme activity.

Genetic transformation of centipedegrass was recently achieved (Hanna, personal communication). No transformation of St. Augustinegrass or seashore paspalum was reported. However, Lee and Berg (1999) had a patent (US patent 5948956) on St. Augustinegrass transformation using bombardment of node segments and direct selection of the regenerated plantlets.

2.3 Useful Genes Used in Turfgrass Transformation and Their Effects

Appreciable progress in trait modifications using transgenic technologies in a variety of turfgrass species demonstrates the great potential of genetic engineering in plant breeding, and suggests ways for genetic improvement of turfgrasses at a much accelerated pace than before (Lee, 1996;

Chai and Sticklen, 1998; Wang *et al.*, 2001; Wang and Ge, 2006). Turf varieties with desired traits such as disease, insect, and herbicide resistance and environmental stress tolerance can be expected in the very near future. The use of genetically modified turf varieties on a large scale could benefit the turfgrass industry, lawn owners, and the environment. For example, new genes can be introduced into turf that confer traits such as drought and stress tolerance that will reduce water usage, pest resistance that will reduce pesticide applications, phyto-remediation capability and aluminum tolerance that will improve environmental qualities. So far, a large number of useful genes have been introduced into various turf species to produce transgenic plants with enhanced agronomic traits.

2.3.1 Genes conferring tolerance to biotic stress in turfgrass

Most plants, during their life cycle, frequently encounter biotic and abiotic environmental stresses that adversely affect their growth, development, or productivity. Biotic stresses are imposed to plants either by infection with bacteria, fungi, viruses, insects, and nematodes, or by competition with weeds or other undesirable plant species. Resistance to biotic stress is one of the most important targets in the improvement of turfgrass. Although knowledge of the molecular mechanism involved in biotic stress tolerance of turfgrass is limited, and studies in this area lag far behind other major crop species, information from model species, such as *Arabidopsis* and rice whose genomes have been recently sequenced, and other grass species, provides insights into the complexities of the processes as well as candidate genes to be used in genetic engineering of turfgrass plants with durable resistance to biotic stress.

2.3.1.1 Fungal resistance

Turfgrass species are highly susceptible to a wide range of destructive fungal pathogens and require extensive application of fungicides to maintain turf quality. This not only adds to operational costs, but also raises environmental concerns. Plants have evolved mechanisms to

protect themselves from fungal attacks, including coordinated activation and expression of a number of genes encoding pathogenesis-related (PR) proteins, or the expression of specific resistance (R) genes for certain pathogens. In addition, genes identified from sources other than plant kingdom also have been demonstrated to confer disease resistance in plants. With the rapid development of transgenic techniques for use in different turfgrass species, a great number of different disease resistance mechanisms have been genetically engineered into turfgrass species to strengthen the host resistance to fungal attacks and demonstrated to be quite effective, pointing to the great potential of exploiting more in this path for use in practice.

Chitinase and glucanase Chitinase, identified in a wide variety of plants, is one of the most representative pathogenesis response-related proteins (PR3) that are induced strongly during fungal infection, treatment of plant tissues with fungal cell wall extracts, or wounding (Collinge *et al.*, 1993; Graham and Sticklen, 1994). Plant chitinases can attack pathogens directly by degrading chitin (a fungal cell wall component) to confer disease resistance, or by binding to the chitin of the fungal cell wall to interfere with fungal growth because of the affinity of chitin-binding proteins to nascent chitin, which leads to severe morphological changes in fungi (Nielsen *et al.*, 1997; Theis and Stahl, 2004). Chitinases are monomers of 25–35 kDa, found as acidic or basic forms, and consist of a putative chitin binding domain as well as a catalytic domain (Shinshi *et al.*, 1990). β -1,3-glucan is a major component of fungal cell walls. Plants have evolved to have β -1,3-glucanases (PR2) as one of the major PR proteins in their defense systems (De Lucca *et al.*, 2005). β -1,3-glucanases are believed to be able to directly digest the β -1,3-glucans of fungal cell walls, resulting in cell lysis, and/or release of bioactive cell wall fragments as elicitors for inducing plant defense reactions (Leubner-Metzger and Meins, 1999). Both chitinase and β -1,3-glucanase have been introduced into turfgrass aiming at engineering enhanced fungal resistance. Chai *et al.* (2002) transferred the elm chitinase gene (*hs2*) to creeping bentgrass, a cool-season grass mainly used in golf greens.

Creeping bentgrass is susceptible to several fungal diseases, especially *Rhizoctonia solani* (Kuhn), a basidiomycete fungus and the causal agent of brown patch disease, and *Sclerotinia homoeocarpa*, which causes dollar spot disease. Compared to the untransformed plants, two transgenic lines out of five showed improved resistance to brown patch disease. Interestingly, the same group also demonstrated the prevention of fungal diseases in transgenic creeping bentgrass expressing herbicide-resistant gene, *bar*, after spraying of transgenic plants with bialaphos and glufosinate (Liu *et al.*, 1998). This observation was confirmed in separate studies when both a rice chitinase *RCH 10* gene and an alfalfa glucanase *AGLU1* gene were co-transferred with a *bar* gene into creeping bentgrass. Although the transgenic plants themselves did not exhibit resistance to these two fungal pathogens, they became resistant to both dollar spot and brown patch diseases when the herbicide glufosinate was sprayed before the fungal inoculation (Y. Wang *et al.*, 2003a, b). Chitinase also has been introduced into Italian ryegrass (*Lolium multiflorum* Lam.), one of the most important temperate pasture grasses, and sown for forage and turf, to improve plant resistance to crown rust (*Puccinia coronata*), the most serious foliar fungal disease (Takahashi *et al.*, 2005). An *RCC2* gene encoding rice chitinase (*Cht-2*) (Nishizawa *et al.*, 1993) was used to transform Italian ryegrass. Bioassay of detached leaves indicated increased resistance to crown rust in transgenic plants, which exhibited higher chitinase activity than a nontransgenic plant (Takahashi *et al.*, 2005). A recent study in introducing the alfalfa glucanase *AGLU1* gene into tall fescue (*F. arundinacea* Schreb.), a cool-season turf and forage grass species of great economic importance, has resulted in transgenic plants with enhanced resistance to various fungal diseases (Dong *et al.*, 2007). Among the 12 tall fescue plants containing the *AGLU1* gene, two were resistant to brown patch (*R. solani*), and three were highly resistant to another fungal disease, gray leaf spot, caused by an ascomycete fungus of *Magnaporthe grisea* (T.T. Hebert) Yaegashi and Udagawa.

Thaumatococcus-like proteins (TLPs) Group PR5 of the PR proteins are known as thaumatococcus-like proteins (TLPs) because of their homology to

the sweet-tasting thaumatococcus from *Thaumatococcus daniellii* (Edens *et al.*, 1982). PR5 proteins have been found to have antifungal activity, presumably by disrupting fungal plasma membrane (Vigers *et al.*, 1992). A rice thaumatococcus-like protein gene, *tlpd34*, under the control of maize ubiquitin (*ubi1*) promoter was introduced into creeping bentgrass and the transgenic plants expressing TLPD34 showed improved resistance to dollar spot in the field tests although the same transgenic lines showed no improvement against brown patch under greenhouse conditions (Fu *et al.*, 2005). In a separate study, an *Arabidopsis thaliana* (L.) Henyeh gene encoding for PR5K, a receptor protein kinase that has an extracellular domain with similarity to the PR5 proteins, was also placed under the control of maize *ubi1* promoter, and introduced into creeping bentgrass. Four of the eight transgenic lines expressing PR5K exhibited delays in development of dollar spot disease symptoms by 29–45 days, relative to the control plants after inoculation (Guo *et al.*, 2003).

Plant resistance (R) genes Plant R genes express in response to the invasion of certain pathogens. They consist of common motifs such as a nucleotide binding site (NBS), leucine-rich repeats (LRRs), kinase, coiled-coil domain (CC), Toll/interleukin-1-receptor (TIR), and transmembrane domain (Hammond-Kosack and Jones, 1997). The R gene products recognize corresponding avirulence gene products (elicitor) from the pathogen, which trigger the cascade of plant defense responses, such as hypersensitive response or programmed cell death, and consequently slow down or completely stop the growth of the pathogen (Dangl *et al.*, 1996; Pennell and Lamb, 1997). *Pi9*, an unusual rice blast R gene that exhibited high resistance to all the rice blast-causing races of *M. grisea* tested so far (Qu *et al.*, 2006), was recently introduced into tall fescue as a genomic DNA fragment with its own gene expression regulatory element and transcriptional terminator. Preliminary results obtained from the transformed plant showed that *Pi9* conferred a high level of resistance to the turfgrass isolates of *Magnaporthe grisea* (Dong *et al.*, 2007).

Ribosome-inactivating proteins (RIPs) RIPs are toxic N-glycosidases that cleave a highly conserved sequence of the 28S rRNA (Endo and Wool, 1982; Theis and Stahl, 2004). RIPs identified in many plant species are believed to function as defense proteins (Stirpe *et al.*, 1992; Barbieri *et al.*, 1993; Nielsen and Boston, 2001). Three types of RIPs from pokeweed (*Phytolacca americana*), referred to as pokeweed antiviral proteins (PAPs), PAP-Y, PAP-C, and PAPII (Irvin *et al.*, 1980; Irvin, 1983) were used to transform creeping bentgrass under the control of maize *ubil* promoter. Transgenic plants produced exhibited different levels of PAP expression, some of which displayed resistance to dollar spot disease (*S. homoeocarpa*) albeit toxic effect of highly expressed PAPs on host plants (Dai *et al.*, 2003).

Lysozymes Lysozymes exist widely in microorganisms and animals, and are generally considered to be antibacterial because of their 1,4- β -N-acetylmuramidase activity against peptidoglycan in bacterial cells (Jolles and Jolles, 1984). Some lysozymes can hydrolyze chitin and thus also have antifungal activities. Antifungal activity of T4 lysozyme was first observed after abolishing the muramidase activity by heat treatment, and the C-terminal of the enzyme was considered to confer this “membrane-disturbing” activity (Düring *et al.*, 1999) confer resistance to fungal attack in transgenic rice, where over 80% of the transgenic plants expressing T4 lysozyme were resistant to four isolates of the rice blast pathogen *M. grisea* (Tian *et al.*, 2002). This demonstrates the great potential of T4 lysozyme for use in plants in defending fungal attacks. T4LYS, the T4 phage lysozyme gene driven by maize *Ubi1* promoter was introduced into tall fescue to test its utility in conferring disease resistance to transgenic plants (Dong *et al.*, 2008). Among the 14 transgenic plants obtained, seven showed highly significant resistance to gray leaf spot (*M. grisea*). Moreover, three of these plants were also resistant to brown patch disease (*R. solani*). The results demonstrated the potential to introduce T4 lysozyme gene into plants for fungal disease resistance.

Small antimicrobial peptides Fungi, insects, animals, and humans all possess genes encoding

antimicrobial proteins or peptides. Many antimicrobial proteins/peptides have been identified and some have been introduced into plants for enhanced disease resistance. Small antimicrobial peptides also can be engineered into plants for disease resistance. Typically, these antimicrobial peptides permeabilize the cell membrane or cause osmotic shock to the pathogens (Nissen-Meyer and Nes, 1997). Dermaseptins are small antimicrobial peptides originally isolated from frog skin. Dermaseptins and their truncated analogs were cytolytic to bacteria, yeast, filamentous fungi, and protozoa (Mor and Nicolas, 1994). Immunofluorescence, electron microscopic, and electrophysiological studies indicated that the interactions between the peptide and the lipid bilayer of cells caused changes in membrane functions, which resulted in the imbalance of the osmotic pressure and cell death (Pouny *et al.*, 1992). A derivative of a dermaseptin B gene was reported to confer resistance to fungal pathogens from *Alternaria*, *Cercospora*, *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, and *Verticillium* genera in transgenic potato (Osusky *et al.*, 2005). Recently, a truncated dermaseptin SI gene encoding a peptide of 18 AA residues (ALWKTMLKKLGTMAL-HAG), which was demonstrated to exhibit active antimicrobial activity (Mor and Nicolas, 1994), was introduced into tall fescue under the control of rice *rubi3* gene promoter (Dong *et al.*, 2007). The two transgenic plants expressing the truncated dermaseptin SI gene grew normally and displayed good resistance to gray leaf spot (*M. grisea*). One of them tested was also resistant to brown patch disease (*R. solani*). This preliminary result, together with the results reported in potato (Osusky *et al.*, 2005), suggests the potential of using dermaseptins to render resistance to a wide spectrum of fungal pathogens in turfgrass.

2.3.1.2 Virus resistance

Virus disease is another biotic stress that has big impact on certain turfgrass species. Plant-derived virus resistance genes are often unavailable. Pathogen-derived resistance (PDR) (Sanford and Johnston, 1985) mediated by transgene (virus coat protein genes, replicase genes, or movement protein genes) products, either the proteins (protein-mediated) or the transcripts

(RNA-mediated), have been demonstrated to be very effective in fighting against viruses in plants (Baulcombe, 2005). RNA-mediated virus resistance may operate by targeted degradation of the transgene and homologous virus RNA, resulting in post-transcriptional gene silencing (PTGS) along with inhibition of virus RNA replication. This strategy was used in perennial ryegrass (*Lolium perenne* L.), the most important grass sown in areas with a temperate climate, to genetically engineer resistance to a common perennial ryegrass pathogen, ryegrass mosaic virus (RgMV), which can tremendously reduce plant yield and persistence (Xu *et al.*, 2001). RgMV belongs to the family Potyviridae, genus *Rymovirus*, and is a cytoplasmically replicating virus with a monocistronic ssRNA genome. An untranslatable RgMV coat protein gene (*RgMV-CP*) driven by the rice *Act1* promoter was introduced into perennial ryegrass. Primary transgenic plants and their sexual progeny exhibited resistance against high-dose virion inocula of different RgMC strains over a 9-month monitoring period after inoculation.

2.3.2 Genes conferring tolerance to abiotic stress in turfgrass

Abiotic stresses arise from an unfavorable physical or chemical environment surrounding the plant, such as water deficit or flooding, high or low temperatures, excessive soil salinity, inadequate mineral nutrients, or presence of pollutants, such as heavy metals in the soil and chemicals, such as herbicides. Many factors interact to determine how plants respond to environmental stresses, including the features of the plants (genotype, developmental stages, and organ or tissue identity), the way a stress is imposed (duration, severity, and frequency) and the additive or synergistic effect of multiple stresses (Bray *et al.*, 2000). Although none of the mechanisms by which higher plants perceive abiotic stresses have been elucidated, tremendous progress in understanding plant responses to stress through different mechanisms has been made (Bohnert *et al.*, 1995; Shinozaki and Yamaguchi-Shinozaki, 1999; Bray *et al.*, 2000; Shinozaki *et al.*, 2003). This knowledge allows designing molecular strategies to genetically modify plants of various species,

including turfgrass, for enhanced abiotic tolerance, leading to improved agricultural production.

2.3.2.1 Drought and salt tolerance

Drought and salinity are the most important osmotic stresses affecting plant growth and productivity. Plants respond to water deficit and adapt to drought and salinity conditions by changes in metabolism and development that can often be attributed to altered patterns of gene expression. Many higher plants have evolved mechanisms such as the accumulation of osmoprotectants including amino acids, ammonium compounds, and polyols/sugars to protect themselves from drought and salt stress conditions. This provides tolerance to the cells under stress by stabilizing the quaternary structure of the complex proteins and adjusting the osmotic potential in their cytoplasm to maintain water content. Recent progress has been made in analyzing the complex cascades of gene expression in drought and cold stress responses, especially in identifying specificity and cross talk in stress signaling (Shinozaki *et al.*, 2003; Siobhan *et al.*, 2003).

Several of the stress-induced genes are regulated by ABA, a plant hormone that is increased through *de novo* synthesis in response to water deficit and low temperature. ABA plays a role in several responses to water stress, most notably stomatal closure and induction of gene expression (Crozier *et al.*, 2000; Trewavas, 2000). This suggests the great potential of manipulating ABA levels in plants by enhanced expression of key regulatory genes in ABA biosynthesis for mitigating plant tolerance to water stress, and has been demonstrated in transgenic *Arabidopsis* (Iuchi *et al.*, 2001) and tobacco (Qin and Zeevaart, 2002) plants overexpressing a gene for 9-cis-epoxycarotenoid dioxygenase (Qin and Zeevaart, 1999) that catalyzes the cleavage of 9-cis-epoxycarotenoids, the first committed and presumably the limiting step of ABA biosynthesis. Recently, *VuNCED1*, a 9-cis-epoxycarotenoid dioxygenase gene cloned from cowpea (Iuchi *et al.*, 2000) and placed under the control of the CaMV 35S promoter was introduced into creeping bentgrass (Aswath *et al.*, 2005). Challenge studies performed with transgenic plants by exposure to water stress (up to 75%) and salt stress (up to 10 dSm⁻¹) for

10 weeks, revealed that more than 50% of the transgenic plants could survive drought and NaCl stress whereas wild type was not. ABA levels were measured under drought and normal conditions; endogenous ABA was dramatically increased by drought and NaCl stress in transgenic plants.

The detrimental effects of salt on plants are a consequence of both a water deficit resulting in osmotic stress and the effects of excess sodium ions on key biochemical processes. To tolerate high levels of salts, plants should be able to use ions for osmotic adjustment and to internally distribute these ions to keep sodium away from the cytosol. Vacuolar Na^+/H^+ antiports, the prevalent membrane proteins, found in animals, yeasts, bacteria, and plants (Fukuda *et al.*, 1999), play important roles in this process. They function to pump Na^+ from cytoplasm into vacuole, to maintain a higher K^+/Na^+ ratio in the cytoplasm than that in vacuoles, protecting cell from sodium toxicity (Fukuda *et al.*, 1999). In addition, the vacuolar proton pumps, such as the vacuolar H^+ -pyrophosphatase, also should increase the sequestration of ions in the vacuole by increasing the availability of protons. Although salt tolerance is a complex trait that seems to involve a large number of salt-responsive genes (Zhu, 2000), the overexpression of a single gene, such as the vacuolar Na^+/H^+ antiport gene (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001; Ohta *et al.*, 2002; Fukuda *et al.*, 2004) or the H^+ -pyrophosphatase gene (Gaxiola *et al.*, 2001; Park *et al.*, 2005), has been shown to improve plant tolerance to salt, and in some cases, to drought in transgenic *Arabidopsis*, tomato, and rice plants. A rice Na^+/H^+ antiport gene, *OsNHX1* driven by the CaMV 35S promoter has been introduced into perennial ryegrass (*L. perenne* L.) (Wu *et al.*, 2005). The resultant transgenic ryegrass had better salt tolerance. After stress treatment for 10 weeks with 350 mmol l^{-1} NaCl, transgenic plants survived, while wild-type plants did not. The leaves of transgenic plants accumulated higher concentrations of Na^+ , K^+ , and proline than those of the control plants.

2.3.2.2 Freezing and cold tolerance

Fructans are polyfructose molecules produced from the polymerization of sucrose that accu-

mulate in plants in addition to or instead of starch (Hendry, 1993). Fructans are particularly widespread in the grasses and predominantly stored as highly accessible, nonstructural carbohydrates in the vacuole where they are synthesized (Chatterton *et al.*, 1989; Hendry, 1993). Fructan not only plays an important role in assimilate partitioning (Pollock and Cairns, 1991; Schnyder, 1993), but also has been associated with improved tolerance to cold and drought through osmoregulation (Pontis, 1989; Pollock and Cairns, 1991; Hendry, 1993; Ebskamp *et al.*, 1994; Pilon-Smits *et al.*, 1995; Konstantinova *et al.*, 2002). The *sacB* gene for *Bacillus subtilis* levansucrase, one of the enzymes involved in the synthesis of high-molecular-weight bacterium fructan (levan) was placed under the control of the maize *ubi1* promoter, or the double CaMV 35S promoter and introduced into Italian ryegrass (*Lolium multiflorum* Lam.) (Ye *et al.*, 2001). In order to direct the expressed levansucrase to the vacuole (Ebskamp *et al.*, 1994), the *sacB* coding sequence was fused to the vacuolar targeting signal sequence from the yeast carboxypeptidase Y (*cpy*) or sweet potato preprosporamin (*spor*) genes (Hattori *et al.*, 1985; Valls *et al.*, 1987). Transgenic plants expressing the chimeric *sacB* gene accumulated low levels of bacterial levan and displayed distorted native grass fructan synthesis pattern and slowed growth with the onset of the reproductive phase. It is unclear how this was related to the response of transgenic plants to environmental stresses. In a separate study, transgenic perennial ryegrass (*Lolium perenne*) plants were produced that overexpressed the CaMV 35S promoter-driven wheat fructosyltransferase genes, *wft1* and *wft2*, which encode sucrose-fructan 6-fructosyltransferase (6-SFT) and sucrose-sucrose 1-fructosyltransferase (1-SST) (Kawakami and Yoshida, 2002), respectively. Significant increases in fructan content and freezing tolerance on a cellular level were detected in the transgenic perennial ryegrass plants (Hisano *et al.*, 2004).

Cytokinins, a class of phytohormones, play an important role in the processes of plant development. They have pleiotropic effects on plants when applied exogenously, including shoot initiation from callus cultures, promotion of axillary bud growth, directed transport of nutrients, stimulation of pigment synthesis, inhibition of root growth, and delay of senescence (Medford

et al., 1989). *ipt*, the *A. tumefaciens* cytokinin biosynthetic gene coding for the enzyme isopen-tenyl transferase involved in the rate-limiting step in cytokinin biosynthesis (Akiyoshi *et al.*, 1983), and has been transferred into plants to induce additional cytokinin production as well as a number of modifications in morphology and plant development (Li *et al.*, 1992; Hewelt *et al.*, 1994; Faiss *et al.*, 1997; Rupp *et al.*, 1999), some of which indirectly impacted on stress tolerance (Smigocki *et al.*, 1993; Li *et al.*, 2004). The *A. tumefaciens ipt* gene driven by maize *ubil* promoter was introduced into *F. arundinacea*, a cool season tall fescue turfgrass. Transgenic plants expressing exogenous *ipt* gene showed enhanced tillering ability, higher chlorophyll a and b levels, a longer stay-green period under low-temperature conditions and consequently, greatly improved cold tolerance (Hu *et al.*, 2005).

2.3.2.3 Transcription factors for use in abiotic stress tolerance

Abiotic stress-inducible transcription factors are capable of activating the expression of multiple downstream genes involved in protection against environmental stresses, thus leading to a wide-arrayed altered response. The transcription factor of the C-repeat/dehydration-responsive-element (C/DRE) pathway, DREB1A/CBF3 specifically interacts with the dehydration responsive element (DRE/CRT) of promoters of the responsive genes. Overexpression of *DREB1A* in transgenic plants of various species enhanced tolerance to different abiotic stresses with concomitant expression of target genes (Kasuga *et al.*, 1999, 2004; Jaglo *et al.*, 2001; Hsieh *et al.*, 2002a, b; Oh *et al.*, 2005). These results demonstrate the great potential of transgenic expression of regulatory genes as a more effective approach for enhancing plant stress tolerance. A DREB1A transcription factor ortholog BCBF3 isolated from wild barley and under the control of the stress-inducible barley *HVA1* promoter was introduced into bahiagrass (*Paspalum notatum* Flugge), an important warm season perennial grass to evaluate the effects of stress-inducible transgene expression on the response of transgenic plants to abiotic stress, and demonstrated to confer enhanced freezing and drought tolerance (James *et al.*, 2004).

2.3.3 Genes conferring tolerance to herbicides in turfgrasses

Weeds or other undesirable plant species (including turf species themselves) greatly impact turf quality for a given turfgrass species and may be considered to represent another type of biotic stresses. The use of herbicides to reduce loss in agricultural production due to weeds has become an integrate part of modern agriculture. The application of herbicides for controlling weeds in turfgrass calls for turf varieties with tolerance to different herbicides. Herbicide resistance in turfgrass through genetic engineering provides a very effective tool in golf course management and lawn maintenance.

2.3.3.1 Resistance to PPT, bialaphos, or glufosinate herbicide

Bialaphos herbicide is a tripeptide antibiotic produced by *S. hygroscopicus*, an organism that produces the tripeptide bialaphos as a secondary metabolite. It consists of two L-alanine residues and PPT, an analog of L-glutamic acid, which is an inhibitor of glutamine synthetase (GS). GS plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants. It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation, and photorespiration. Inhibition of GS by PPT causes rapid accumulation of ammonia, which leads to death of the plant cell (De Block *et al.*, 1987). While PPT is an inhibitor of glutamine synthetase in both plants and bacteria, the intact tripeptide has little or no inhibitory activity *in vitro*. In both bacteria and plants, intracellular peptidases remove the alanine residues and release active PPT. The *bar* gene originally cloned from *S. hygroscopicus* is involved in the bialaphos biosynthesis pathway. It encodes a modifying enzyme, phosphinothricin acetyltransferase (PAT) that acetylates the free NH₂ group of PPT and thereby prevents autotoxicity in the producing organism (Thompson *et al.*, 1987). Since its first introduction into plants as a selectable marker in plant transgenic research (De Block *et al.*, 1987), it has been widely used in various plant species, including a number of turfgrass species (Wang

et al., 1992; Hartman *et al.*, 1994; Spangenberg *et al.*, 1994; Lee, 1996; Asano *et al.*, 1998; Liu *et al.*, 1998; Yu *et al.*, 2000; Y. Wang *et al.*, 2003a, b; Luo *et al.*, 2004b). A commercial creeping bentgrass cultivar that has been genetically engineered for glufosinate resistance and male sterility is currently under field trial (Luo *et al.*, 2004a, b, 2005).

2.3.3.2 Resistance to glyphosate herbicide

Glyphosate herbicide (marketed under the trade name Roundup) inhibits 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) within the aromatic amino acid biosynthetic pathway. Disruption of this pathway not only creates a deficiency in protein synthetic precursors, but also affects many other plant cell components that are derived from intermediates and derivatives of this pathway (e.g., auxins, lignans, flavonoids, anthocyanins, and quinones). Therefore, a crop plant must be engineered with a resistant enzyme to maintain flux through this pathway for uninhibited growth and development. A naturally occurring form of EPSPS from *Agrobacterium* sp. strain CP4 provides tolerance to high concentrations of glyphosate. Transgenic expression of CP4 EPSPS within glyphosate-tolerant Roundup Ready[®] soybean, cotton, and canola provided the appropriate support to the aromatic amino acid biosynthetic pathway without negative impact on yield, compositional qualities, and nutritional value of the harvested product. The *Agrobacterium* CP4 EPSPS also has been genetically engineered into creeping bentgrass (*A. stolonifera* syn. *A. palustris*) to create Roundup-resistant transgenic lines that are currently in the process of applying for the United States Department of Agriculture (USDA) deregulation (Gardner *et al.*, 2003, 2004; Fei and Nelson, 2004).

2.3.3.3 Conversion of proherbicide to herbicide for selective elimination of undesirable plants

The *E. coli argE* gene encodes *N*-acetylornithinase in the *E. coli* arginine biosynthetic pathway, which removes the acetyl group from *N*²-acetylornithine and produces acetate and ornithine (Meinell

et al., 1992). The enzyme can also de-acetylate *N*-acetyl-PPT, a nontoxic compound, to produce PPT (Kriete *et al.*, 1996). This property was recently used in turfgrass to develop a strategy to eliminate undesirable plants in golf course management. Perennial ryegrass is widely used for winter overseeding of dormant bermudagrass on golf courses and sports fields in southeastern United States to provide green color and improved playability. Late spring and summer persistence of perennial ryegrass may decrease the quality of the bermudagrass turf and reduce its winter hardiness. To solve this problem, the *E. coli argE* gene under the control of maize *ubi1* promoter was introduced into perennial ryegrass and transgenic plants expressing the *argE* transgene were selectively controlled upon application of the nontoxic proherbicide, *N*-acetyl-PPT. The nontransgenic bermudagrass plants were unaffected by the treatment (Chen *et al.*, 2005). This approach provides a means to selectively remove a group of transgenic plants without affecting other plants growing with them.

2.3.4 Modification of other important traits in turfgrasses

2.3.4.1 Modification of senescence

Senescence-associated gene promoters, whose functional values have been demonstrated in dicot genetic manipulation (Gan and Amasino, 1997), are of considerable interest in grasses because of their probable involvement in economically important traits such as productivity (Thomas and Howarth, 2000), sward color (Thorogood, 1996), and responses to elevated CO₂ (Ludewig and Sonnewald, 2000). The 5' flanking sequence of a maize *SEE1* gene encoding cysteine protease was used to drive the *A. tumefaciens* cytokinin biosynthesis gene, *ipt*, in ryegrass (*L. multiflorum* Lam.). Transgenic analysis demonstrated that the *SEE1* flanking sequence functioned as a senescence-enhanced promoter in ryegrass. The *ipt* transgene was detected in 28 regenerants from five independent transformation events, and the leaves of transgenic plants displayed a stay-green phenotype. Some lines developed spontaneous lesions (Li *et al.*, 2004).

2.3.4.2 Floral inhibition

Flowering in plants is controlled by both environmental (primarily temperature and light) and genetic factors. The genetic factors include genes encoding transcriptional activators and repressors, such as MADS box genes, zinc-finger transcription factors, AP2-domain genes, MYB-domain genes, and also RNA-binding proteins, polycomb-group genes, starch metabolism related proteins, photoreceptors, circadian-clock controlling proteins, GA biosynthesizing enzymes, GA signaling repressors, etc. (reviewed in Levy and Dean, 1998; Simpson *et al.*, 1999). The genes that control the transition from vegetative to reproductive growth called floral meristem identity genes, act either by repressing or promoting flowering. For example, *TERMINAL FLOWER1* (*TFL1*), a floral meristem identity gene, identified in *Arabidopsis* has been demonstrated to specify an indeterminate identity of inflorescence meristems and extend plant vegetative phase. *Arabidopsis* plants overexpressing *TFL1* flower late and produce more secondary shoots (Ratcliffe *et al.*, 1998). Similar functions have been demonstrated in its homolog, *LpTFL1* isolated from perennial ryegrass (*L. perenne* L.). Expression of *LpTFL1* in *Arabidopsis* gave a remarkably strong phenotype with a dramatic delay in flowering (some nonflowering) and extended lateral branching (Jensen *et al.*, 2001).

In turfgrass, the stems that start to emerge during the growth season suppress the formation of new shoots and affect the quality, density, and persistence of the sward. Therefore, prolonged vegetative growth phase through delay of flowering is of major interest in genetic improvement of turfgrass. To manipulate the transition to flowering in turfgrass, the strong floral repressor, *LpTFL1* gene was introduced into red fescue (*F. rubra* L.), a commercially important cool-season turfgrass. Expression of *LpTFL1* with the constitutive maize *ub1* promoter represses flowering in red fescue, and the flowering repression phenotype correlates well with the level of *LpTFL* expression. Transgenic lines showing low to intermediate expression of *LpTFL1* flowered approximately 2 weeks later than the controls, and transgenic lines showing very high *LpTFL1* expression levels still remained nonflowering after exposure to natural vernalization conditions (Danish winter) in two

successive years. There were no other phenotypic effects associated with the *LpTFL* transgene expression during vegetative growth (Jensen *et al.*, 2004).

The *Arabidopsis* TALE-homeobox gene *ATH1* is highly expressed in the shoot apical meristem (SAM) and leaf primordia of the seedlings (Quaedvlieg *et al.*, 1995; Bürglin, 1997). Prior to floral transition, *ATH1* SAM expression is gradually down-regulated to undetectable levels. *Arabidopsis* plants constitutively expressing antisense *ATH1* and *ath1* mutants display a flowering time phenotype that suggests that *ATH1* functions as an inhibitor of floral transition (Van Der Valk *et al.*, 2004). In addition, the tobacco plants constitutively expressing *ATH1* exhibit late flowering as a consequence of impaired GA biosynthesis (Van Der Valk *et al.*, 2004). To delay floral transition in turfgrass, the *Arabidopsis* *ATH1* gene driven by the maize *ub1* promoter, the rice actin *Act1* promoter, or the rice *OSH1* promoter, respectively, was introduced into perennial ryegrass. In *ATH1*-expressing plants heading was delayed, and in a number of cases the plants never flowered at all. Such non- or late heading was accompanied by the outgrowth of normally quiescent lateral meristems into extra leaves, resulting in a leafy growth habit. When eventually heading, these plants generally produced a reduced number of inflorescences (Van Der Valk *et al.*, 2004).

2.3.4.3 Down-regulation of pollen allergens

Perennial ryegrass (*L. perenne* L.) and Italian ryegrass (*L. multiflorum* Lam.) are major sources of allergenic pollen causing hay fever and seasonal allergic asthma in humans during the flowering period in spring and summer in cool temperate climates. To reduce the allergic potential of ryegrass pollen, the expression of three main allergens of ryegrass pollen, proteins Lol p 1 (35 kDa), Lol p 2 (11 kDa), and Lol p 5 (31 kDa), have been down-regulated in transgenics. The introduction of the *Lol p 1* and *Lol p 2* transgenes in antisense orientation under control of the maize pollen specific *Zm13* promoter into perennial ryegrass and Italian ryegrass led to a reduction in accumulation levels of Lol p 1 and Lol p 2 allergens in pollen of transgenic plants, as

demonstrated by immunoblots using polyclonal antibodies raised against the recombinant Lol p 1 and Lol p 2 allergens as well as with sera from grass pollen-sensitized patients (Petrovska *et al.*, 2004). Similarly, down regulation of the Lol p 5 allergen in annual ryegrass *L. rigidum* L. was achieved with an antisense construct under control of a pollen-specific promoter, *Ory sl*. Immunoblot analysis of proteins with allergen-specific antibodies did not detect Lol p 5 in the transgenic pollen. The transgenic pollen showed remarkably reduced allergenicity as reflected by low IgE binding capacity of pollen extract as compared with that of control pollen. The transgenic ryegrass plants in which *Lol p 5* gene expression is perturbed showed normal fertile pollen development (Bhalla *et al.*, 1999).

2.4 Site-Specific DNA Recombination for Genome Modification in Turfgrass

Site-specific DNA recombination is a precisely defined DNA rearrangement between two appropriate target sequences. A number of site-specific recombinases have been identified in bacteria and yeast that catalyze DNA recombination between specific target DNA sites (Ow and Medberry, 1995), producing various recombinant molecules according to the orientation of their specific target sites. Recombination between directly oriented sites on a circular molecule leads to excision of the DNA between them, whereas recombination between inverted target sites causes inversion of the intervening DNA. Recombination between sites on separate molecules produces a co-integration event. Some site-specific recombination systems have been shown to function efficiently in heterologous cellular environments. For example, FLP/*FRT* from the 2 μ m plasmid of *Saccharomyces cerevisiae* (Broach *et al.*, 1982), or Cre/*lox* from *E. coli* phage P1 (Austin *et al.*, 1981) have been demonstrated to catalyze DNA recombination in a large number of plant species, including agriculturally important crops (reviewed in Luo and Kausch, 2002; Hu *et al.*, 2006), providing excellent genetic tools for controlled modification of plant genomes including chromosomal deletions, inversions, transpositions (Ow, 1996; Luo and Kausch, 2002), or site-specific gene targeting (Albert *et al.*, 1995; Vergunst and

Hooykaas, 1998; Vergunst *et al.*, 1998; Srivastava and Ow, 2002).

To study the feasibility of using FLP/*FRT*, a site-specific DNA recombination system originally identified in yeast, for controlled genome modification in plants of turfgrass species, suspension cell cultures of creeping bentgrass (*A. stolonifera* L.) and Kentucky bluegrass (*P. pratensis*) were co-transformed with a FLP recombinase expression vector and a recombination-reporter test plasmid containing β -glucuronidase (*gusA*) gene, which was separated from the maize *ubi1* promoter by an *FRT*-flanked blocking DNA sequence to prevent its transcription. GUS activity was observed in co-transformed cells when conducting transient assays. PCR and Southern analyses indicated that FLP-mediated excision of the blocking sequence had brought into proximity the upstream promoter and the downstream reporter gene, resulting in GUS expression. Functional evaluation of the FLP/*FRT* system using transgenic creeping bentgrass plants stably expressing FLP recombinase confirmed the observation from the experiment using suspension cell culture (Luo and Kausch, 2002; Hu *et al.*, 2006). These results indicate that FLP/*FRT* system is a useful tool for genetic manipulation of turfgrass, pointing to the great potential of further exploiting the system for genome modification in perennials.

2.5 Molecular Strategies for Transgene Containment in Turfgrass

To realize the full potential of agricultural biotechnology, the ecological, commercial, and public interest concerns about transgene escape to wild and nontransformed plants through the dispersal of viable pollen and/or dissemination in seed must be addressed (Wipff and Fricker, 2001; Adam, 2002; Dale *et al.*, 2002; Daniell, 2002; Watrud *et al.*, 2004). This is especially important when the target plant species are perennials and outcrossing, such as turfgrasses. Field trial (Wipff and Fricker, 2001) and landscape-level studies (Watrud *et al.*, 2004) on pollen-mediated gene flow from genetically modified creeping bentgrass (*A. stolonifera* L.), one of the first wind-pollinated and highly outcrossing transgenic perennial crops being developed for commercial use have presented evidence that documents multiple instances

at numerous locations of long-distance viable pollen movement from multiple source fields of genetically modified creeping bentgrass. A recent study using glyphosate-resistant creeping bentgrass plants expressing the *CP4 EPSPS* transgenes demonstrates that transgene flow from short-term production can result in establishment of transgenic plants at multikilometer distances from genetically modified source fields or plants. Selective pressure from direct application or drift of glyphosate herbicide could enhance introgression of *CP4 EPSPS* transgenes and additional establishment. Obligatory outcrossing and vegetative spread could further contribute to persistence of *CP4 EPSPS* transgenes in wild *Agrostis* populations, both in the presence or absence of herbicide selection (Reichman *et al.*, 2006). These findings suggest that implementation of strategies for gene containment in transgenics might be useful for field release of any transgenic turfgrass species in the near future.

2.5.1 Male sterility for gene containment

Male sterility resulting from the lack of significant numbers of viable pollen grains, when linked to the genes of interest that are to be transferred into the target species, provides an effective way for interrupting gene flow. The feasibility of using cell-specific expression of cytotoxic molecules and an antisense gene that controls male fertility to block pollen development in transgenic turfgrass has been studied (Luo *et al.*, 2004a, 2005). To induce male sterility in turfgrass, a 1.2-kb regulatory fragment (TAP) of the rice *RTS* gene that is exclusively expressed in the anther's tapetum during meiosis (Luo *et al.*, 2006) was fused with two different genes. One is a ribonuclease gene for barnase from *Bacillus amyloliquefaciens* (Hartley, 1988) and the other, the antisense of the rice *RTS* gene essential for male fertility (Luo *et al.*, 2006). Both chimeric genes were linked to the *bar* gene for selection by resistance to the herbicide glufosinate. *Agrobacterium*-mediated transformation of creeping bentgrass (cv. Penn A-4) with both constructs resulted in herbicide resistant transgenic plants that were also 100% pollen-sterile. Mendelian segregation of herbicide resistance and male sterility was observed in T₁ progeny derived from crosses with wild-

type plants. Controlled self- and cross-pollination studies showed no gene transfer to nontransgenic plants from male-sterile transgenics. Thus, male sterility can serve as an important tool to mitigate transgene escape in bentgrass. It could also provide a tool in controlling gene flow in other perennial species using transgenic technology for trait improvement.

2.5.2 Controlled total sterility for gene containment

Another molecular strategy, which combines manipulation of genes involved in the transition from vegetative to reproductive growth and the use of site-specific DNA recombination systems for controlled total vegetative growth has been proposed for mitigating transgene escape in perennials (Oliver *et al.*, 2004; Hu *et al.*, 2006). In this method, a controlled total vegetative growth can be achieved by using down-regulation of a plant gene, the *FLORICAULA/LEAFY* (*FLO/LFY*) homolog, which determines the vegetative to reproductive developmental transition of meristems (Coen *et al.*, 1990; Weigel *et al.*, 1992), together with FLP/*FRT* recombination system for prevention of transgene escape from genetically engineered turfgrass to wild and nontransformed species. Transgenic turfgrass plants containing a construct in which a constitutive promoter is separated from either an RNAi (RNA interference) construction or an antisense of the turfgrass *FLO/LFY* homolog gene by a blocking sequence flanked by directly oriented *FRT* sites can be generated and expected to flower normally to produce seeds. When crossed to a plant expressing transgene of interest together with the FLP recombinase transgene, FLP should excise the *FRT*-flanked blocking fragment thus bringing the RNAi construction or the antisense of turfgrass *FLO/LFY* homolog proximal to the upstream constitutive promoter, activating the constitutive expression of the RNAi or antisense construction for *FLO/LFY*. This in turn will down-regulate the expression of the endogenous *FLO/LFY* genes rendering the plant incapable of producing flowers. The vegetative growth habit of the hybrid retains its commercial application but is incapable of transferring transgenes to neighboring grasses or weedy relatives.

3. REGULATION OF GENETICALLY ENGINEERED TURFGRASSES

As with any genetically engineered (GE) plant in the United States, GE turfgrasses are regulated under the 1986 Coordinated Framework for Regulation of Biotechnology. The US government agencies responsible for oversight of the products of agricultural modern biotechnology are the US Department of Agriculture's Animal and Plant Health Inspection Service-Biotechnology Regulatory Services (USDA-APHIS) (http://www.aphis.usda.gov/biotechnology/brs_main.shtml), the US Environmental Protection Agency (EPA) (<http://www.epa.gov/pesticides/biopesticides>), and the Department of Health and Human Services' Food and Drug Administration (FDA) (<http://www.cfsan.fda.gov>).

The Federal laws currently used to regulate the products of modern biotechnology are the Plant Protection Act (PPA), the Federal Food, Drug, and Cosmetic Act (FFDCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and the Toxic Substances Control Act (TSCA). Regulations have been developed under these statutes as needed to address GE products. New regulations, policy statements, and guidelines continue to be developed as needed.

The responsibilities of USDA, FDA, and EPA in the regulatory oversight of GE crop plants and their products are complementary, and in some cases overlapping. USDA-APHIS has jurisdiction over the planting of GE plants. FDA has jurisdiction over food and feed uses of all foods from plants. EPA has jurisdiction over planting and food and feed uses of pesticides engineered into plants (these are referred to as plant-incorporated protectants, or PIPs). These PIPs would include resistance to diseases and other pests. In addition to PIPs, EPA has jurisdiction over labeling or use of herbicides to be applied on GE herbicide-tolerant plants.

Depending on its characteristics and intended use, a product may be subject to review by one or more of these agencies. A food crop plant being developed using genetic engineering to produce a pesticide in its own tissue provides an example that is reviewed by all three regulatory agencies. A common example of this type of product development is corn into which scientists have inserted a gene isolated from the soil bacterium,

Bacillus thuringiensis (*Bt*). The *Bt* gene encodes a protein that acts as a pesticide for Lepidoptera and when this gene is inserted into the plant, the plant can then produce the *Bt* pesticidal substance.

USDA-APHIS under the PPA would regulate the experimental corn as a "regulated article". USDA-APHIS oversight begins early in the development cycle and continues until the developer applies for and is granted nonregulated status for the plant provided it can be shown that the plant is not a plant pest. Until such time as the developer is granted nonregulated status for the plant, USDA-APHIS authorizes interstate movement, importation, and field-testing of the plant.

EPA regulates the distribution, sale, use, and testing of the pesticidal substance. In the case of *Bt* in corn, EPA regulates the *Bt* because it is a pesticide. EPA generally controls the field testing of pesticides through an Experimental Use Permit (EUP). In order to legally sell or distribute the pesticide in commerce, the company must register the pesticide with EPA. Through the registration, EPA can establish the conditions of commercial use. EPA is also responsible for setting the amounts or levels of pesticide residue that may safely be in food or feed (i.e., establish a tolerance). EPA may allow an exemption from the requirement to set such a tolerance if it can be shown that there is no food or feed safety issues associated with the pesticide.

Developers of the *Bt* corn also consult with FDA about possible other unintended changes to the food or feed, for example possible changes in nutritional composition or levels of natural toxicants. Although this consultation is voluntary, all of the GE food/feed products commercialized to date have gone through the consultation process. The consultation with FDA serves to ensure that safety or other regulatory issues that fall within the agency's jurisdiction, including appropriate labeling of the food, are resolved prior to commercial distribution.

In the case of GE turfgrasses, these plants are not generally developed for food or feed purposes. If this is the case, FDA would not be involved in the review of the GE plant. However, any of the turfgrasses could be used for forage to be fed to animals. This may happen especially during seed production when the straw is removed from the fields after the seed harvest. In many cases, the

straw is fed to animals. Therefore, if there is a possibility that the GE plants will be used for food or feed, it is highly recommended that the FDA review the product.

Disease resistance and insect resistance have always been important goals in the improvement of turfgrasses. Since these characteristics are considered plant-incorporated pesticides (PIPs), both EPA and APHIS would be involved in regulating these plants. EPA requires EUPs for testing an unregistered PIP or an unregistered use of a PIP on a cumulative total of over 10 acres. USDA-APHIS requires permits for all field testing of regulated GE turfgrass plants regardless of the size of the field trial. APHIS and EPA would both review their respective applications and commercial releases for potential impacts on nontarget organisms such as beneficial insects and threatened and endangered species.

Weed control has always been an important management component for high quality turf and, therefore, incorporation of tolerance to a specific herbicide into a turfgrass could be an important goal for turfgrass variety improvement. In the case of GE herbicide tolerance, USDA-APHIS would be the lead agency in regulating these plants with permits required for all field testing, movements, and importations. Since weeds need to be controlled for optimum turf performance for the herbicide-tolerant grass, the herbicide needs to be applied at least occasionally to the turf. Even if this specific herbicide is registered for many different uses, this new use for its application on this new herbicide-tolerant variety needs to be approved by EPA, and this review and approval would be reflected in the labeled use for this herbicide.

Finally, in the case of GE modifications for other desirable turfgrass characteristics such as less vertical growth, tolerance to various stresses (heat, cold, drought, wear, etc.), nutrient use efficiency, etc., USDA-APHIS would be the only agency regulating field testing, movements, and importations assuming it is not used for feed.

3.1 Regulatory Considerations During Field Testing of Transgenic Turfgrasses

Since USDA-APHIS is the regulatory agency most involved in all phases of field testing of GE plants before commercial release, this discussion

will address only USDA-APHIS considerations. APHIS protects agriculture and the environment by ensuring that biotechnology is developed and used in a safe manner. Through a strong regulatory framework, the Biotechnology Regulatory Services (BRS) program of USDA-APHIS ensures the safe and confined introduction of new GE plants with significant safeguards to prevent the accidental release of any GE material. APHIS regulations require that a developer of a GE plant have BRS authorization prior to importing, moving interstate or field testing the GE plant. Applicants must submit all plans for movement, importation, or field testing for thorough review by regulatory scientists. Those scientists will then evaluate the proposed procedures and assess any potential risks. The developer must adhere to certain measures that ensure adequate confinement of the organism. BRS also works closely with states to be sure that they are aware of field tests taking place within their jurisdiction and to allow them to request any additional conditions they may require. To ensure compliance with the permit conditions, BRS inspects field test sites and audits records.

Because each species presents its own unique challenges and opportunities, and regulations (The regulations are in 7 CFR 340 http://www.access.gpo.gov/nara/cfr/waisidx_05/7cfr340_05.html) and guidelines dealing with GE organisms tend to change frequently for this fast changing science, BRS encourages developers of GE turfgrasses to visit the BRS website (<http://www.aphis.usda.gov/biotechnology/brs.main.shtml>) and with BRS scientists to fully understand the requirements and expectations that need to be met before a GE turfgrass can be field tested and eventually commercialized. Permits for field testing GE turfgrasses may require up to 120 days to process so substantial advanced planning is needed. However, if the permit application involves a new species or a novel modification that raises new issues not previously addressed by APHIS, an environmental assessment may need to be prepared which may require additional time for processing the permit. Previously approved field tests and associated environmental assessments can be viewed online at the website for Information Systems for Biotechnology (<http://www.isb.vt.edu>). Examining previous environmental assessments will give the developer

a flavor for the types of information considered for an environmental assessment.

For submitting a permit application, the present requirements call for submitting an APHIS Form 2000 (Since regulations and guidelines change frequently, please check the website noted below. Beginning 2007 the permit application may be submitted electronically.) Following are some key features for submitting a permit for field testing a regulated GE plant: the responsible person needs to be a US resident or designate an agent who is a US resident; confidential business information may be claimed (see the BRS website for detailed guidance); scientific and common names of the donor organisms (The organisms from which the DNA was obtained—the typical construct may have pieces of DNA (promoter, gene of interest, terminators, selectable markers, etc.) from several organisms); scientific and common names of the recipient plant; vector or vector agents (biolistics, disarmed *Agrobacterium tumefaciens*, etc.); names or line numbers of the regulated article; a description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the nonmodified parental plant (morphological characteristics, physiological activities, growth characteristics, etc.); a detailed description of the purpose for the field test including a detailed description of the field test design; the proposed maximum size of the field test; the proposed earliest date of the field planting and the length of time requested for the permit (the length of time that the field test will exist); the proposed number of plantings; a detailed description of the field trial location; a detailed description of the procedures that will be used to prevent the release or movement of the regulated article outside of the test area (this includes monitoring for the regulated article at the end of the field test); and a detailed description of the proposed method of final disposition of the regulated article. For full details in applying for a permit, check the BRS website (http://www.aphis.usda.gov/permits/brs_epermits.shtml).

With the advent of genetically engineered perennial grasses and the availability of single herbicide tolerance genes and other easily detectable molecular markers, extremely precise information is becoming available on pollen flow for perennial grasses. These data are generally

much more precise than the data available based on morphological characteristics of which the precision may be as small as 0.1% (parts per thousand), whereas the data from single herbicide tolerance genes or molecular markers may be as precise as 0.0001% (parts per million). With additional data becoming available on pollen flow for creeping bentgrass (Wipff and Fricker, 2001; Wipff, 2002; Belanger *et al.*, 2003; Watrud *et al.*, 2004; Petition 03-104-01p), tall fescue (Wang *et al.*, 2004), and Kentucky bluegrass (Johnson *et al.*, 2006) and on possible creeping bentgrass seed movement (Reichman *et al.*, 2006), APHIS is updating its guidance for field testing of wind-pollinated perennial grasses. These recent studies have had very similar results. As with most plants, cross-pollination with other plants is most likely between sexually compatible plants that are closest to each other. With wind pollination, the maximum distance that viable pollen may pollinate sexually compatible plants is highly dependent on the speed and direction of air movement. In addition, the amount of viable pollen produced has an effect on the farthest distance that one or more successful pollinations will most likely take place. In all of the pollination studies cited above, only Watrud *et al.* (2004) studied a creeping bentgrass pollen source that greatly exceeded 500 pollen source plants with that study measuring pollen flow from 162 ha (400 acres) with a successful cross-pollination documented at 21 km. The greatest distance found for a successful cross-pollination with one of the smaller studies (Wipff, 2002) was a distance of 426 m with a pollen source of 298 plants.

Perennial grass species used for turf and forage have many characteristics in common. They generally are perennial, wind pollinated, small seeded, easily propagated vegetatively, spread vegetatively by stolons and/or rhizomes (and to some extent by tillers), tolerant of traffic, mowing, grazing, and burning, and tolerant of cold and/or heat stress by becoming dormant. Many of the perennial grass species have some seed dormancy, and many are easily propagated by seeds. These same characteristics are typical of perennial grass species used for forages. In fact, many turfgrass species are used for forage as well as for turf. For the species cited above in the pollen flow studies, creeping bentgrass is used mainly for turf purposes but it has been used frequently for forage

purposes, tall fescue is used for forage and turf, and Kentucky bluegrass is used mainly for turf and is frequently found in pastures and other grazed areas.

All of these characteristics of perennial grasses need to be taken into account when considering confinement procedures for field testing GE turfgrasses. Confinement procedures need to take into consideration any means of gene flow—pollen, seed, and vegetative parts. Testing of turfgrasses is generally conducted in two distinct phases—turf performance and seed production.

When testing its turf performance, it is mowed one to several times a week resulting in the removal of all or most of its flowers. If this is the case, any concerns about pollen flow or seed movement are completely eliminated or greatly reduced, but concerns about vegetative movement and volunteer plants after the removal of the test would still need to be addressed. Various procedures can possibly be used to assure confinement: a nontransgenic border area or fallow zone surrounding the test to allow personnel to identify and control any transgenic plants that may be growing outside of the test; all equipment used in the test area is cleaned inside the test plot or border area before it is moved off the test site; access to the site is restricted to authorized personnel who have been trained to follow suitable procedures to minimize the spread of the regulated article; in addition to the border area, an additional isolation area surrounding the test area which is monitored for the presence of sexually compatible plants (plants that have the potential to cross with the regulated article and produce viable offspring) and if present are destroyed or prevented from flowering; and upon termination of the trial, monitor the regulated trial area for volunteers for an agreed period of time. For clarification, the regulated trial area includes the test area and the surrounding border area.

For testing during the seed production phase, pollen flow and seed movement need to be addressed as well. Possible methods for limiting pollen flow include: using sterile plants that will not produce pollen or seed; using male sterile plants; bagging the flowers/seed heads; covering all plants with a tight mesh netting that will restrict pollen flow; growing plants in an isolated area where no sexually compatible plants are

growing in the immediate area—this includes seed fields of the same or related species, feral plants of the same or related species, and plants of “wild” related species; and restricting access to the site to authorized personnel who have been trained to follow suitable procedures to minimize the spread of pollen on clothes and equipment. For growing the flowering plants in isolation to be effective, scouting for sexually compatible plants at the future site of a proposed trial a year before installing the test is helpful, and then monitoring for sexually compatible plants in the isolation area during the flowering period of the trial itself shortly before or in the early flowering period and destroying any plants found during these monitoring periods would be required.

Possible methods for limiting seed movements include: using sterile plants that will not produce seed; bagging the flowers/seed heads; covering all plants with netting that will restrict seed movement by wind or birds; placing fence around the trial that would restrict animals and unauthorized individuals from walking across the trial; placing harvested seed heads directly into a container instead of laying seed heads down to dry; using dedicated equipment to avoid mixing regulated article with unregulated material; restricting access to the site to authorized personnel who have been trained to follow suitable procedures to minimize the spread of seed on clothes and equipment; conducting the trial in an inhospitable environment so escapes are unlikely to establish and feral populations are unlikely to exist; using a nontransgenic border area or fallow zone surrounding the test to allow personnel to identify and control any transgenic plants that may be growing outside of the test—the border will also provide an area to service and clean equipment, and for a staging area for placing seed prior to planting or placing harvest containers; having a substantial isolation area in addition to the border between the trial and any harvested crop, especially for any seed crop of perennial grasses; and upon termination of the trial, monitoring the regulated trial area for volunteers for an agreed period of time. Again, the regulated trial area includes the test area and the surrounding border area.

All of the confinement measures mentioned above are possible suggestions to consider when

planning to install and conduct trials of transgenic turfgrasses. But for each species, possibly the transgenic characteristic, and the size of the trial, the confinement measures may vary. The length of any recommended monitoring period depends heavily on the seed dormancy characteristics of each species. Therefore decisions on the specific requirements need to be made on a case-by-case basis.

3.2 Obtaining Nonregulated Status and Commercialization of Transgenic Turfgrasses

As with any variety development program, a point is reached where the performance data obtained in various trials appear to justify a commercial release of the developed variety. However, unlike a conventionally developed variety in which no genetic engineering techniques were used, in addition to convincing only the potential buyers or customers of the new variety's advantages, three different government agencies may need to be convinced that it is safe to consume as food or feed (FDA), it has no pesticidal properties that make it harmful to the environment, unsafe to consume, or requires a quantity of a pesticide that is considered to be detrimental to the environment (EPA), and/or it is not detrimental to agriculture or the environment (USDA). For the USDA, a person needs to submit a petition for determination of nonregulated status. If approved, the new variety is considered deregulated, and the product and its progeny would no longer require USDA-APHIS review for movement or release in the United States.

In this petition, the developer should include the following: the purpose or rationale for the petition; a description of the biology of the plant before it was genetically engineered; description of the transformation system; a detailed description of the donor gene(s), other DNA sequences and its products along with the map of the construct used in the transformation; molecular characterization analysis of the DNA insert in the recipient (insert length, number, and partial pieces of the construct, presence of the plasmid backbone, and stability of the insert); a detailed breeding diagram of the selection practices and procedures used to develop each generation,

and population of plants used to provide data in the petition; inheritance of the transgenic trait; differences between the GE plant and the original plant; a description or rationale for the use of the control varieties or lines used in the comparison of the regulated article to the unmodified recipient (this may be the same line as the regulated article without the transgene and/or an array of varieties representative of the species); detailed phenotypic comparisons of the transgenic plant and unmodified recipient for various characteristics—seed germination, seed vigor, seed establishment, seed dispersal, seed dormancy, seed yield, time and length of flowering and seed production period, growth habit, vegetative vigor (horizontal spread, plant height, and/or biomass), tolerance to biotic and abiotic stresses, outcrossing frequency, effect on pollinator species, pollen characteristics (size, shape, amount, stickiness, longevity, etc.), symbionts, asexual reproduction (vegetative and seed), and self-compatibility (<http://www.aphis.usda.gov/brs/canadian/appeannex2e.pdf>, <http://www.cast-science.org/websiteuploads/pdfs/turfbiotech.is.pdf>).

The petitioner may address each of the above topics using the scientific literature and/or test results developed specifically for the petition. These measurements or observations on all of the phenotypic characteristics are useful in the review of the petition for determining the existence of “unintended effects”. From a variety development perspective, almost all of the phenotypic characteristics are noted during the variety testing stages anyway so the documentation of these observations requires only a small additional effort if the tests are well planned in advance of the petition.

All of the field tests conducted under APHIS regulation for this regulated product need to be listed in the petition. All required field-test reports for each of the field tests need to be completed and on file with USDA-APHIS. The petitioner is also required to provide any known information that indicates that the regulated article may pose a greater plant pest risk than that of the unmodified recipient plant.

After reviewing the petition, BRS conducts an environmental assessment in compliance with the National Environmental Policy Act to analyze the potential impacts the GE organism may have on agriculture and the environment. This assessment

includes a wide variety of environmental parameters and looks for possible effects to threatened and endangered species. After the completion of the assessment by BRS, it is posted on the Web along with a copy of the developer's petition (with all confidential business information deleted) and made available for a 60-day public comment period through the *Federal Register* announcement. Following these procedures, BRS then decides to approve or deny the petition. For obtaining details on completing the petition, guidance is provided on the BRS website (http://www.aphis.usda.gov/biotechnology/brs_main.shtml) indicating the type of molecular details and the agronomic data that are helpful in evaluating the petition. The past petitions approved by BRS are also available through the BRS website.

4. FUTURE PERSPECTIVES

The rapid development of turfgrass genetic transformation technologies has made it possible to apply biotechnological approaches for trait modifications in a large number of grass species. The many promising data obtained so far point to the great potential of commercialization of new turfgrass cultivars with genetically improved traits. However, concerns on transgene flow from genetically modified plants to compatible wild species and unintended ecological consequences of potential transgene introgression impose hurdles for the deregulation of any transgenic perennial species. Development and implementation of molecular strategies for gene containment in genetically modified transgenic plants with improved performance may facilitate public acceptance and commercialization of GM products in turfgrass species. Besides the use of strategies involving manipulation of genes that control plant reproductive growth for gene containment, chloroplast genetic engineering to promote maternal inheritance of transgenes is a promising option for prevention of gene flow through pollen dispersion. Maternal inheritance of cytoplasmic organelles is shared by plant (chloroplast) and animal (mitochondria) systems. The prevalent pattern of plastid inheritance found in majority of angiosperms is uniparental-maternal and chloroplast genomes are maternally inherited in

most crops (Daniell, 2002). Maternal inheritance of transgenes and prevention of gene flow through pollen in chloroplast transgenic plants have been successfully demonstrated in several plant species, including tobacco and tomato (Daniell *et al.*, 1998; Ruf *et al.*, 2001). In addition to its potential for gene containment, chloroplast engineering offers several other advantages, including a high level of transgene expression, multigene engineering in a single transformation event, lack of gene silencing, position effect, pleiotropic effects, and undesirable foreign DNA. Chloroplast engineering also can greatly enhance the capability of some turfgrass species as potential bioreactors for large-scale production of industrial enzymes, biodegradable plastics, pharmaceuticals, vaccines, and antibodies. With the success of chloroplast engineering in the first monocot species, rice (Lee *et al.*, 2006), and the availability of creeping bentgrass chloroplast genome sequence, the very first in turfgrass species (Saski *et al.*, 2007), chloroplast engineering in turfgrass species could be expected in the very near future.

Although genomics studies in turfgrass species lag behind that of other agriculturally important food grasses due to the complex genetic constituents of turf species, and the lack of substantial public funding for support, data generated from major crop plants (rice, wheat, and maize), especially the completion of the genome sequencing of the first grass species, rice, provide invaluable information in developing molecular and genomics tools for gene discoveries and for better understanding of molecular mechanisms defining plant resistance to biotic and abiotic stresses as well as other biological processes. This will result in more effective tools and strategies in genetically improving turfgrass for enhanced stress tolerance and desired plant morphologies using biotechnological approaches.

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Poplars

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1. INTRODUCTION

Over the past 20 years, several *Populus* species (commonly known as poplars and encompassing aspens, cottonwoods, and hybrid poplars) have been the most frequently used trees for transgenic research. Among commercially important tree species, poplars are relatively easy to transform and of great economic importance to forest product industries. Although it is difficult to discern from which of the above qualities the preponderance of poplar research activity is a result, poplars are nonetheless regarded as the most suitable model system for tree biotechnology research (Bradshaw *et al.*, 2000; Taylor, 2002; Tuskan *et al.*, 2003). A large number of review articles (e.g., Bradshaw, 1996; Bradshaw *et al.*, 2000; Rishi *et al.*, 2001; Confalonieri *et al.*, 2003) as well as monographs (e.g., Stettler *et al.*, 1996; Klopfenstein *et al.*, 1997) has recently been published on the biology and biotechnology of poplars. This review will attempt to compile and summarize the published literature and research findings in order to serve as an information resource on transgenic poplars.

1.1 History of Poplar Cultivation, Breeding, and Utilization

Historical records indicate that since the late 17th century, there have been poplar plantations across Europe, Asia, and the Middle East, although poplar trees have likely been utilized by humans since prehistorical times (<http://www.cnr.umh.edu/fr/publications/proceedings/papers/Stoffel-Duluth.pdf>). A number of legends, myths, and beliefs are associated with poplars, such as the legend that the cross on which Christ was crucified was made from the wood of a trembling aspen, all of which now tremble with grief and remorse because of the event. A German legend describes the Holy Family as walking in a forest where all trees except aspen bowed before them to show respect. The Holy Child cursed the tree, and the leaves began to, and have ever since, trembled with fear. Poplars were selected as the symbol of liberty during the French revolution of 1789 (McNabb, 1997). In Europe, spontaneous hybrids of the imported *P. deltoides* and the European native black poplar, *P. nigra*, were discovered around 1775 (McNabb, 1997). Initially

named black Italian poplars, they were later renamed *Populus* \times *canadensis* by Mönch in 1795. Hybrid poplars were also depicted by the notable French impressionist painter Monet in such works as *Poplars on the Epte* and *Poplars along the River Epte*. Although naturally occurring poplar hybrids were common in 18th and 19th century Europe, systematic poplar breeding began only in the early 20th century at the Kew Botanical Gardens, England. The first recorded attempt of crossing poplars was made between flowers of *P. angulata* with pollen of *P. trichocarpa*, with the resultant hybrid named as *P. generosa*. Subsequent poplar breeding programs began around 1920 in Canada, United States, Denmark, and France (McNabb, 1997).

Over the past 50 years, hybrid poplars have been planted across the US landscape and windbreak trees. Poplar breeding programs focused on both the hybrid vigor of interspecific poplar hybrids and their clonal selections (Bisoffi and Gullberg, 1996). The spontaneous natural hybrids that were recognized early in 20th century were used as a logical starting point for breeding programs. The relative ease of clonal propagation of poplars assisted in the incorporation of clonal selection in poplar breeding programs. Recently, there has been an increased interest in poplar breeding in the North America. These efforts have focused on producing vigorous *P. tremuloides* and *P. tremula* hybrids (Li, 1995; Bradshaw, 1996). Additionally, there have been cooperative breeding programs between universities and pulp industries in the Pacific Northwest region of North America. These efforts have produced *P. trichocarpa* \times *P. deltoides* hybrids (<http://www.cof.orst.edu/coops/tbgc/index.htm>), and also hybrids of *P. trichocarpa* \times *P. maximowiczii*; *P. trichocarpa* \times *P. nigra*, and *P. deltoides* \times *P. nigra* (Stettler *et al.*, 1996; Zsuffa *et al.*, 1996).

A major consolidation of global poplar research was initiated through the establishment of the International Poplar Commission (IPC) in 1947, which is one of the technical statutory bodies of the Food and Agriculture Organization of the United Nations (FAO). Their mandate is to promote cultivation, conservation, and utilization of members of the family Salicaceae, which includes poplars and willows (<http://www.fao.org/forestry/site/ipc/en>). IPC assists researchers in the direction and coordina-

tion of scientific efforts and promotes conservation and exchange of poplar germplasm among the member nations. The IPC also supports research and management activities that explore issues of concern to its 37 member countries through six working parties—harvesting and utilization; diseases; insect pests; genetics; conservation; and improvement; production systems, and environmental applications. IPC also promotes poplars and willows as fast-growing trees that provide a wide range of goods, including industrial roundwood (sawn timber, peeler logs, and pulpwood), fuelwood, poles and fodder, and services such as soil erosion prevention, and aesthetic value. Moreover, the adaptability of poplars to a wide range of climatic and soil conditions has led to their widespread use in developed and developing countries. Poplars and willows play an important role in rural life in countries with transition economies. Although poplar cultivation has been traditionally integrated into many temperate and subtropical agricultural systems, the use of poplars and willows as biomass for renewable energy is a novel application (Dinus *et al.*, 2001).

Research specific to Short Rotation Woody Crops (SRWCs) such as hybrid poplar has been taking place in the Lake States of the United States for the last 25 years (Dinus *et al.*, 2001). SRWCs are seen as a way to grow more wood fiber on less land in order to reduce potential conflicts between increasing demand for wood fiber and other forest uses. Researchers felt that optimum tree growth could be realized by borrowing the intensive cultivation techniques common in other forms of agriculture, including the use of hybrids. Most of this research involved hardwoods, particularly poplars, because of their rapid growth. Interest in SRWCs intensified in 1977 when the oil embargo provided an impetus for additional funding from the US Department of Energy (DOE) to consider wood as an alternative to fossil fuels. With the current petroleum crisis, there is renewed interest in poplar as bioenergy crops.

1.2 Botanical Classification of Poplars

Typically, all members of the genus *Populus* are fast-growing trees with a single trunk. They multiply naturally through stump sprouts or root suckers. Poplars belong to the family Salicaceae

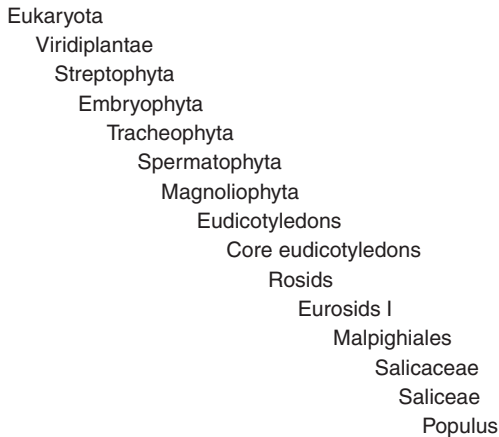


Figure 1 Botanical classification of *Populus*

under the order Malpighiales and are closely related to willows. Figure 1 shows a currently accepted classification system of poplars (Wheeler *et al.*, 2000).

The precise worldwide number of poplar species is difficult to determine. This is due to several reasons: First, the various poplar species are widely distributed throughout the world; second, they actively hybridize with one another, that confounding taxonomic classification; and lastly, they have been profligately imported and exported around the globe, blurring native species boundaries. As per Zsuffa (1975), numerous synonyms exist for the same species, and hybrids and cultivated varieties have often been wrongly named as species. Although the actual number of species belonging to the genus *Populus* is, and continues to be, a highly debatable subject because of intense interspecific hybridizations, Eckenwalder (1996) proposed that the genus *Populus* contains approximately 30 species belonging to six sections. These sections were proposed on the basis of the morphological characteristics of stamens, carpels, seeds, bracts, leaves, and buds (Eckenwalder, 1996). Five of these sections are: *Turanga*, *Leuroides*, *Aigeiros*, *Tacamahaca*, and *Populus*. In the past, a sixth, single-species section has often been proposed to resolve classification problems. For example, section *Abaso* was proposed to accommodate *Populus mexicana* that seems to be weakly related to other species of section *Aigeiros*, in which it was previously placed (Eckenwalder, 1996). Over 90% of the poplar

cultivation throughout the world comes from the species and hybrids in the section *Aigeiros* (Thielges, 1985). This is perhaps due to ease of inter- and intrasectional hybridization within and between the *Aigeiros* and *Tacamahaca* sections, their adaptability in the temperate and subtropical zones, and their ease of vegetative propagation (OECD, 2000). Table 1 shows Eckenwalder's classification of this genus along with distribution of some representative poplar species in each section.

A large number of useful (natural and artificial) intra- and intersectional poplar hybrids are available and being used in cultivation. Table 2 lists some of these poplar hybrids as described in Organization for Economic Cooperation and Development (OECD) report (2000).

As stated previously, there are five major sections of the genus *Populus*: *Populus* (aspens), *Tacamahaca* (Balsam poplars), *Aigeiros* (Cottonwoods), *Turanga* (subtropical poplars), and *Leuroides* (Big leaf poplars). Extensive crossability data exist among species in the *Populus*, *Tacamahaca*, and *Aigeiros* sections, but few data are available for those in section *Turanga* and *Leuroides* (Zsuffa, 1975). Hybrids between members of the same section are produced easily and are often more vigorous than their parents. For example, hybrids between *P. tremuloides* and *P. tremula* are notably vigorous and those between *P. deltoides* and *P. trichocarpa* produce much larger leaves than their parents, thus increasing their potential for biomass production. Crossing success between sections is variable. Crosses between members of *Aigeiros* and *Tacamahaca* sections are simple to complete, while those between sections *Populus* and *Aigeiros*, and sections *Populus* and *Tacamahaca* are most difficult, typically resulting in nonviable seed or dwarfed seedlings (Zsuffa, 1975).

1.3 Origin and Evolution of Poplars

Poplars are hydrophilic trees that grow in high moisture content soils. Their riparian habit and deciduous nature likely helped preserve leaf samples in the fossil record. Some studies suggest poplars originated in the late Paleocene (58 million years ago; Eckenwalder, 1996). A representative of the *Abaso* section related to modern *P. mexicana* was present in North America but disappeared at

Table 1 Classification, nomenclature, and occurrence of some *Populus* species

Section	Scientific name	Common names	Occurrence
<i>Abaso</i> Ecken.			
	<i>P. mexicana</i> Wesmael		Mexico
<i>Turanga</i> Bunge.			
	<i>P. euphratica</i> Oliv.	Euphrates poplar, bahan	Spain, NE Africa, Asia (China)
<i>Leucoides</i> Spach		Large-leaved poplars	
	<i>P. lasiocarpa</i> Oliv.	Chinese necklace poplar	China
	<i>P. heterophylla</i> L.	Swamp cottonwood, swamp poplar,	USA
<i>Tacamahaca</i> Spach		Balsam poplars	
	<i>P. angustifolia</i> James	Narrowleaf cottonwood, narrowleaf balsam poplar	Southern Sask. and Alberta to southwestern US
	<i>P. balsamifera</i> L.	Balsam poplar	North America
	<i>P. ciliata</i> Royle		Himalayas
	<i>P. laurifolia</i> Ledeb.	Laurel poplar	Eastern Asia
	<i>P. trichocarpa</i> Torr. and A. Gray	Black cottonwood, western balsam poplar	Western Canada and US
<i>Aigeiros</i> Duby		Cottonwoods and black poplars	
	<i>P. deltoides</i> Marsh.	Eastern cottonwood (ssp. <i>deltoides</i>), plains cottonwood (ssp. <i>monilifera</i>), Rio Grande cottonwood (ssp. <i>Wislizenii</i>)	Quebec, Ontario Prairie provinces to Texas SW US
	<i>P. nigra</i> L.	Black poplar, European black poplar	Europe, western Asia
<i>Populus</i> L.		Aspens	
	<i>P. alba</i> L.	White poplar, silver poplar	Central and southern Europe to N. Africa, Central Asia
	<i>P. grandidentata</i> Michx.	Large-tooth aspen, bigtooth aspen, aspen, poplar, popple	Eastern North America
	<i>P. sieboldii</i> Miq.	Siebold aspen, Japanese aspen	Japan
	<i>P. tremula</i> L.	European aspen, tremble, Zitterpappel	Europe, Northern Africa, northeastern Asia
	<i>P. tremuloides</i> Michx.	Trembling aspen, quaking aspen	North America

Source: Eckenwalder, 1996. © OECD

the end of the Oligocene (23 million years ago) only to reappear during the Holocene (10 000 years ago). Until the late Eocene period (40 million years ago), when swamp poplars of the section *Leucoides* appeared, members of the section *Abaso* were the only poplar species present in North America. During the Oligocene period, fossils of poplars of the section *Tacamahaca*, (which

may be ancestors of two modern-day sections of poplars, *Tacamahaca* and *Aigeiros*), occurred in both the new and old worlds (Collinson, 1992). The representatives of section *Populus*, however, did not appear in fossil records until the early Miocene (20 million years ago). It is, therefore, possible that poplars have inhabited the earth for more than 60 million years.

Table 2 Nomenclature of some selected *Populus* hybrids

Parentage	Hybrid designation	Common name
<i>P. alba</i> × <i>P. adenopoda</i>	<i>P. × tomentosa</i> Carr.	Chinese white poplar
<i>P. alba</i> × <i>P. tremula</i>	<i>P. × canescens</i> (Ait.) Sm.	Grey poplar
<i>P. angustifolia</i> × <i>P. deltoides</i>	<i>P. × acuminata</i> Rydb.	Lance leaf cottonwood
<i>P. balsamifera</i> × <i>P. deltoides</i>	<i>P. × jackii</i> Sarg.	Jack's poplar
<i>P. deltoides</i> × <i>P. nigra</i>	<i>P. × canadensis</i> Moench	Canada poplar
<i>P. deltoides</i> × <i>P. tremuloides</i>	<i>P. × bernardii</i> Boivin	Bernard poplars
<i>P. deltoides</i> × <i>P. trichocarpa</i>	<i>P. × generosa</i> Henry	Interamerican poplars
<i>P. fremontii</i> × <i>P. trichocarpa</i>	<i>P. × parryi</i> Sarg.	Parry cottonwood

Source: Eckenwalder, 1996. © OECD

1.4 Distribution, Habit, and Habitat of Poplars

The genus *Populus* is widely distributed throughout the northern hemisphere, in both the temperate and colder subtropical zones down to about 30° N of latitude. However, due to intense natural and artificial hybridization and migration across the globe, poplars are now widely planted far beyond their natural range in both the southern and the northern hemispheres.

Poplars are most widely distributed throughout North America, Europe, North Africa, India, China, and Japan (OECD, 2000). *Populus tremuloides* is the most broadly distributed tree species in North America, spanning 110° of longitude and 47° of latitude, and is the second most widely distributed tree in the world. *P. tremula* also has similar wide distribution in Europe. Poplars are a pioneering species that establish following major disturbances like fire. They are also a keystone species that preserves the biodiversity of organisms that inhabit them. Poplars are deciduous trees that are dioecious (i.e., male and female flowers are produced on separate trees), thus resulting in obligate outcrossing. Male and female flowers are produced in pendulous catkins in spring before leaves appear from the flushing vegetative buds. In natural populations, the observed ratio of males to females is generally 1:1, although elevation may affect this ratio. In *P. tremuloides*, females are dominant at lower elevations while the ratio swings to greater than 90% males above 3200 m in the Rocky Mountains (Grant and Mitton, 1979). A continuous stretch of warm temperatures around 12°C for 5–6 days is required for emergence of flowers which are wind-pollinated. Fruiting catkins about 10 cm long are produced on female plants 4–6 weeks after flowering. Each catkin bears several dozen, one-celled capsules, each containing 10–30 small brown seeds, each with tuft of long, white silky hairs attached to one end to aid in long-distance wind dispersal. Poplar trees produce a large number of very small seeds, about 25–50 million. Species in the section *Populus* can produce 6000–8000 seeds per gram, while trees from the *Leucoides* and *Aigeiros* sections produce much larger seeds (Schreiner, 1974). In nature, seeds are viable for a short time and survive only if germinated quickly after dispersal onto moist soil where ample sunlight is available for growth.

Most of the stand growth of poplars is a result of vegetative reproduction from stump sprouts or root suckers rather than from seeds. It has been estimated that in the US, *P. tremuloides* produces 25 000–75 000 suckers per hectares in the Alaska and Great Lakes regions and about half as many in Rockies (Burns and Honkala, 1990). It is believed that in the semi-arid western US, widespread seedling establishment has not occurred since the last glaciation some 10 000 years ago (Einspahr and Winton, 1976). According to Guinness book of world records (<http://www.guinnessworldrecords.com/>), the most massive plant that was ever reported is a network of quaking aspen trees (Pando, i.e., *I spread* in Latin) growing in the Wasatch Mountains, Utah, US, from a single root system, covering 43 hectares (106 acres), consisting of more than 47 000 stems and with a weight in excess of 6 million kilograms, making it the largest known organism (Mitton and Grant, 1996).

Poplars are regarded as small- to medium-sized trees that are fast-growing and short-lived. On average they grow to about 24 m tall with an 18–30 cm d.b.h. (stem diameter at breast height) in about 20 years. Poplars also have a large number of insect, disease, and animal pests ranging from whitetail deer to the poplar willow sawfly to leaf rusts. More information on habit and habitats of a variety of *Populus* species can be obtained at: http://www.na.fs.fed.us/pubs/silvics_manual/volume_2/vol2.Table_of_contents.htm.

1.5 Economic Importance

The economic importance of poplars has been extensively discussed by Balatinecz *et al.* (2001). The most recent and concise information about poplar's worldwide area coverage and wood production is available in the IPC's 2004 report (<http://www.fao.org/forestry/ipc2004>). This report contains a synthesis of progress reports from the 21 member countries and the nonmember country, the Russian Federation, that were present at their 22nd symposium on poplars and willows in Santiago, Chile. According to this report, over 70 million hectares of combined natural poplar populations exist in all these countries. Ninety-seven percent of this growth is present in

three countries: Canada (28.3 million hectares), the Russian Federation (21.9 million hectares), and the US (17.7 million hectares). The next six countries containing significant areas of natural poplar forests are China (2.1 million hectares), Germany (100 000 ha), Finland (67 000 ha), France (39 800 ha), India (10 000 ha), and Italy (7200 ha). The reported global area of poplar plantations, however, was 6.7 million hectares, of which 3.8 million hectares (56%) was planted primarily for wood production and 2.9 million hectares (44%) for environmental purposes. China reported the most planted poplar overall (4.9 million hectares, or 73% of the global total) and India reported 1 million hectares, the second largest area of poplar plantations in the world. The other countries with significant areas of planted poplar included France with 236 000 ha, Turkey with 130 000 ha, Italy with 118 800 ha, Argentina with 63 500 ha, and Chile with 15 000 ha. Only two countries reported significant annual removals of wood from natural stands of poplars: the Russian Federation (100 million m³) and Canada (16 million m³). The five countries that reported annual removals of more than 1 million m³ of poplar wood from planted forests were Turkey (3.8 million m³), China (1.85 million m³), France (1.8 million m³), Italy (1.4 million m³), and India (1.2 million m³).

According to the 2004 IPC report, poplars provide a wide range of wood products (e.g., industrial roundwood and poles, pulp and paper, reconstituted boards, plywood, veneer, sawn timber, packing crates, pallets, furniture); nonwood products (e.g., fodder, fuelwood, bioenergy); and services (e.g., shelter, shade, conservation and protection of soil, water, crops, livestock, and dwellings). Pulp, paper, and cardboard were the most favored end uses in Europe, North America, China, and Argentina. Packaging (pallets, boxes, and crates) was also a favored end use in Europe, the Republic of Korea, the Russian Federation, Canada, China, and India. Reconstituted wood panels were the favored end uses in Germany, Argentina, Canada, the US, Bulgaria, and Italy. Plywood was also a favored end use in India, Italy, Spain, China, Turkey, France, Serbia, Montenegro, and the US. Use of poplars for matches was favored in Chile, the Russian Federation, India, the Republic of

Korea, and Sweden. Furniture manufacturing was generally ranked as a lower priority by most countries except Belgium and Chile. Fuelwood or production of biomass for energy was also generally ranked as a lower priority by most countries; however, it was of significant economic importance in Sweden, the United Kingdom, and Turkey. The other end uses for lumber and general construction were reported as priorities in Turkey, Finland, and Argentina; and handicrafts and wicker work in Chile and the Russian Federation. The Republic of Korea, Serbia, Montenegro, and Sweden highlighted that their principal use of poplar and willow resources was for environmental conservation, thus providing valuable services rather than forest products. In summary, various countries have region-specific uses of poplars, although in most cases the commercial importance of poplars by major forest product industries is regarded as modest as compared to pines and eucalypts (Bradshaw *et al.*, 2000).

1.6 Cytological and Genomic Features of Poplars

Generally, all poplars are diploid species with $2n = 38$ chromosomes (Bradshaw *et al.*, 2000). Polyploid poplar trees have been rarely reported in a few species (Darlington and Wylie, 1956). The first triploid forest tree reported was a clone of *P. tremula* (Müntzing, 1936). Several other natural triploid clones have since been found in both *P. tremula* and *P. tremuloides*, usually exhibiting larger leaves and exceptional growth (Einspahr *et al.*, 1963; Heimbürger, 1968; Einspahr and Winton, 1976). Recently, the whole-genome sequence of the first tree species, black cottonwood (*P. trichocarpa*) was reported (Tuskan *et al.*, 2006). The genome size of poplars is estimated to be little less than 500 Mbp (mega base pairs). By integrating shot-gun sequence assembly with BAC (bacterial artificial chromosome) fingerprinting and genetic mapping, the whole poplar genome was assembled. Strong evidence of whole-genome duplication, as is common among diverse members of the Salicaceae family, was also observed. About 45 000 genes appear to be present in the poplar genome.

1.7 Molecular Genetics of Poplars

A systematic approach of using modern molecular genetic techniques to associate economically important traits with molecular markers located on the poplar genome map was initiated relatively recently (Bradshaw *et al.*, 1994). By taking advantage of the success of interspecific hybridization in poplars, most of the genome mapping studies focused on hybrids that also showed hybrid vigor for growth and had been planted on a commercial scale. Because most of the poplars are diploid with $2n = 38$, F_1 hybrids are fertile and can lead to segregating F_2 progenies that are used for tagging important traits. Ease of vegetative propagation allows further selection of useful clones by reducing the time required to transit from hybridization to commercial production. In addition, ease of clonal propagation also allows side-by-side comparison of performance of parents and their F_1 and F_2 progenies in replicated clonal trials. The physical size of the *Populus* genome is about 500 Mbp and is distributed on 19 chromosomes, giving a combined genetic length of 2400–2800 cM (centimorgans; Bradshaw, 1996). Thus, an average correspondence of physical to genetic lengths of poplars is about 200 kb cM^{-1} , which is similar to that found in *Arabidopsis*. Also, the rich information resources for poplars from a variety of subdisciplines of plant sciences such as physiology, anatomy, biochemistry, genetics, molecular biology, ecology, and pathology have made poplar a very attractive model system for tree biologists (Taylor, 2002). The main traits of interest are stem volume, stem form, crown geometry, spring bud burst, climatic adaptation, disease resistance, wood quality, and wood production (Bradshaw *et al.*, 1994; Bradshaw, 1996).

The earliest poplar genetic map was developed by Liu and Fournier (1993) using an intraspecific cross in *P. tremuloides* employing allozymes and restriction fragment length polymorphisms (RFLPs). However, most of the recent efforts have involved interspecific hybrid poplars (e.g., Bradshaw *et al.*, 1994; Cervera *et al.*, 1997). Most of the quantitative trait loci (QTL) mapping efforts in poplars have focused on a single three-generation pedigree developed from a female black cottonwood (*P. trichocarpa*) clone 93-968 from Washington State and eastern cottonwood

(*P. deltoides*) clone ILL-129 from Illinois. These two trees were crossed in 1981 giving rise to F_1 family 53. In 1988, two of the F_1 hybrids (53-246 and 53-242) were sibmated to generate F_2 family 331. These three-generation mapping pedigrees are maintained as clonal materials by cutting new growth to ground each winter in order to produce multiple sprouts. A genome map developed from F_2 family 331 consists of 250 RFLP markers and ~100 random amplified polymorphic DNA (RAPD) markers covering $2/3$ of the 2600 cM length of the poplar genome. This map has been used to localize QTLs for yield, disease resistance, stem growth, leaf morphology, and sylleptic branch proliferations (Taylor, 2002). (Sylleptic branches are produced directly from axillary buds in response to water, light, and nutrients and contribute to fixed carbon stored in stems.) Additional poplar genome maps have been developed including a Belgian cross between *P. trichocarpa* and *P. nigra* or *P. deltoides* (Cervera *et al.*, 2001) and a French cross between *P. nigra* and *P. deltoides* (Cervera *et al.*, 1997). Wu *et al.* (2000) have also developed a new amplified fragment length polymorphism (AFLP) marker-based map in *P. deltoides*. Additional studies are in progress to map QTLs for leaf rust resistance as reviewed by Taylor (2002). Recently, Cervera *et al.* (2005) have used AFLP markers for discerning relationships among various poplar species and their hybrids.

The problems involved in using traditional breeding for poplar improvement include their undomesticated nature, long generation intervals, late sexual maturity, large physical size, strict outcrossing mating system, lack of inbreeding lines, lack of natural or induced mutant lines, polygenic inheritance of economically important traits (this is common with many other crop plants), and the late start of overall research efforts in this direction (Bradshaw *et al.*, 2000). It is hoped that transformation techniques may mitigate some of the disadvantages of working with this system. In contrast to the decade required for completing one cycle of traditional crossing and selection, genetic engineering can reduce this time between six months and a year. Additionally, genetic engineering may be able to uncover the recessive mutations that normally cannot be recovered from outcrossing. Molecular techniques like RNA interference (RNAi) or antisense suppression are

likely to knock out all copies of a gene, and thus provide a glimpse into the functionalities of that gene. Finally, the process of domestication can hopefully be compressed into decades from the usual centuries using these techniques (Bradshaw *et al.*, 2000).

2. POPLAR TRANSFORMATION

2.1 An Overview of Poplar Transformation

All transformation methods, such as biolistic gun (McCown *et al.*, 1991; Devantier *et al.*, 1993), electroporation (Chupeau *et al.*, 1994), and *Agrobacterium*-mediated methods have been successfully used in poplars (Confalonieri *et al.*, 2003). However, the majority of researchers have chosen *Agrobacterium*-mediated transformation as the preferred method in their studies. Table 3 lists some poplar species in which transformation systems have been established.

Populus was the first woody plant genus that was genetically manipulated. In 1986, the first *Populus* transformation was reported in hybrid poplar (*P. trichocarpa* × *P. deltoides*) using two *Agrobacterium* strains (Parsons *et al.*, 1986). The *Agrobacterium*-mediated method of gene transformation has since been successfully employed in many *Populus* species. However, most

of the successful transformations have been limited to the section *Populus* (aspen type) (Han *et al.*, 2000). Transformation reports of *Populus* species belonging to the section *Aigeiros* (cottonwood type) and *Tacamahaca* (balsam poplar type) are not common. However, Han *et al.* (2000) developed an effective transformation system for a variety of cottonwood hybrids. Recently, Ma *et al.* (2004) reported a transformation protocol for the *P. trichocarpa* clone Nisqually-1, notable for having been the clone used to complete the *Populus* genome sequencing project (Tuskan *et al.*, 2006).

Here, we will discuss some of the important factors that affect the ability to transform various poplar species.

2.1.1 Explants

In poplars, the efficiency of transformation is affected by the physiological condition of the explants, which in turn is affected by the environmental and growing conditions of the mother plants. Mature lignified explants with high phenolics are difficult to regenerate (Civinova and Sladky, 1990). In aspen, young leaf discs are most amenable for regeneration and transformation as compared to older leaf discs. Han *et al.* (2000) compared the rate of callus production and shoot

Table 3 Examples of successful genetic transformation in *Populus* species

Species	<i>Agrobacterium</i> strains	Plasmid construct	Gene	Marker gene ^(a)	References
<i>P. tremula</i>	EHA101, LBA4404, AGLO	pBIG-HYG pBI101 pCGN1559	<i>gus (uidA)</i>	Kan	Tzfira <i>et al.</i> , 1997
<i>P. alba</i>	LBA4404	p35S GUS INT/pCAMBIA 2301	<i>gus (uidA)</i>	Kan	Sánchez <i>et al.</i> , 2004
<i>P. tremuloides</i>	C58	BinSynGus	<i>gus (uidA)</i>	Kan	Tsai <i>et al.</i> , 1994
<i>P. nigra</i>	A281 and 82.139	Several	<i>gus (uidA)</i>	Kan	Confalonieri <i>et al.</i> , 1994, 1995
<i>P. trichocarpa</i> (Nisqually-1)	C58	pGUS-INT	<i>gus (uidA)</i>	Kan	Ma <i>et al.</i> , 2004
<i>P. tremula</i> × <i>P. alba</i>	AGLI	pBINPLUS	Antimicrobial peptide D4E1	Kan	Mentag <i>et al.</i> , 2003
<i>P. ciliata</i> Wall.	LBA4404	PBI121	<i>gus (uidA)</i>	Kan	Thakur <i>et al.</i> , 2005
<i>P. × euramericana</i> (ogy)	EHA101	pRT104	<i>Pin2</i>	Kan	Heuchelin <i>et al.</i> , 1997
<i>P. tomentosa</i>	LBA4404	PBI121	<i>mtlD</i>	Kan	Hu <i>et al.</i> , 2005

^(a)Kan, kanamycin

induction using *in vitro*-grown and growth room-grown explants of stem and leaf. The rate of callus and shoot production was significantly lower in *in vitro*-derived stem and leaf explants as compared to growthroom-derived explants in the black cottonwood hybrids that were tested. *Populus* explants commonly preferred for transformation are taken from *in vitro*-grown plants because they need no additional surface sterilization and have high regeneration capacity (Civinova and Sladky, 1990). The transformation methods used in various poplar species and their regeneration efficiency are dependent on the selected genotype. The same transformation method and growth media may not always work well with other genotypes of the same species. Several studies have shown that significant variations exist among the poplar genotypes (DeBlock, 1990; Riemenschneider, 1990; Confalonieri *et al.*, 1994). Various genotypes of the *Populus* section responded differently to *Agrobacterium tumefaciens* infection (Nesme *et al.*, 1987). In *P. deltoides*, four selected clones regenerated significantly better than 14 other clones examined (Coleman and Ernst, 1990). Transformation has been achieved using various explants, such as leaf discs, apices, petioles, internodes, and stems. The best explant for transformation use depends on the poplar species used, its genotype and its susceptibility to *A. tumefaciens* infection. In cottonwood hybrids, stem and petiole explants were shown to produce more calli and regenerate more shoots than leaf explants (Han *et al.*, 2000). Also, the specific type of preculture media on which the explants were grown influences the subsequent transformation efficiency. Cottonwood hybrid internode explants precultured on BA (benzyladenine; 10 μ M) showed increased shoot production as opposed to those precultured on TDZ (thidiazuron; 0.1 μ M) and zeatin (5 μ M), which led to decreased shoot production. For *P. deltoides*, preculture on auxin-supplemented media improved competence for shoot regeneration (Coleman and Ernst, 1990).

2.1.2 *Agrobacterium* strains and culture induction

Different plants (genotypes and species) exhibit varying susceptibility to *A. tumefaciens* infection.

Therefore, choosing the appropriate *Agrobacterium* strain is very important for the success of the transformation experiment, especially for recalcitrant species. Tree species exhibit better infection rates with nopaline strains of *A. tumefaciens* than with octopine strains (Ahuja, 1987). Earlier, Fillatti *et al.* (1987b) and Pythoud *et al.* (1987) reported that transformation of poplars with octopine strains such as LBA4404 was not successful. For *P. nigra* leaf disc explants, the C58 strain was more virulent as compared to the Ach5 and A281 strains (Confalonieri *et al.*, 1994). The effect of *Agrobacterium* strains on poplar transformation rates has also been studied in the highly susceptible *Populus* section clone-INRA-717-184 (*P. tremula* \times *P. cuba*) where the *Agrobacterium* strains used did not significantly affect the rate of transfer DNA (T-DNA) transfer. However, in *Aigeiros* section clones 24-305 and 184-402, the EHA-105 strain of *Agrobacterium* produced a significant increase in the rate of T-DNA transfer compared to the C58 and LBA4404 strains (Han *et al.*, 2000). Acetosyringone is also known to induce the *Vir* genes in *Agrobacterium* during plant infection and is, therefore, commonly used in transformation of monocots (rice, wheat, maize), which are less susceptible to *Agrobacterium* infection. In several black cottonwood clones, the use of acetosyringone in *Agrobacterium* induction also increased the efficiency of transformation (Confalonieri *et al.*, 1995).

2.1.3 Co-cultivation

Optimum concentration of *Agrobacterium* is required for efficient transformation. Incubation of high concentrations of *Agrobacterium* on poplar explants for a long period generally results in necrosis of the explants. In poplars, co-cultivation of stem explants with approximately 5×10^8 cells/ml of *Agrobacterium* caused necrosis after 2 days of co-cultivation. Co-cultivation of *P. nigra* explants with *Agrobacterium* at a concentration of 7×10^8 cell/ml resulted in improved transformation efficiency based on GUS (β -glucuronidase) expression analysis (Confalonieri *et al.*, 1994). Transformation efficiency is also greatly influenced by co-cultivation conditions, such as the nature of media (solid or liquid), medium composition, light or dark conditions during treatment, and

the number of days of incubation (Mathis and Hinchey, 1994). Transformation efficiency of *P. nigra* was improved significantly by co-cultivating explants for 48 h as compared to 24 h of co-cultivation (Confalonieri *et al.*, 1994). In short, careful monitoring of the entire transformation protocol is essential for the successful outcome of transformation of poplars. Excellent reviews on poplar transformations have been published in the past and can be consulted for the topics not covered here (e.g., Rishi *et al.*, 2001; Bhalerao *et al.*, 2003; Confalonieri *et al.*, 2003; Nehra *et al.*, 2005). Below we discuss some examples of commercially important and environmentally beneficial traits for which poplars have been engineered.

2.2 Genetic Engineering of Poplars for Improved Lignin Production

The removal of lignin from wood for paper making is a chemical- and energy-intensive and environmentally polluting process. The reaction rate of lignin degradation during chemical pulping of angiosperm species is inversely related to lignin content, but directly proportional to the quantity of the syringyl content in lignin (Boerjan *et al.*, 2003). Thus, both lignin quantity and structural composition (i.e., the syringyl-to-guaiacyl (S/G) monolignol ratio), affect pulping efficiency and represent two significant traits targeted in forest tree improvement programs. Unlike agronomic crops, such as maize and sorghum, where natural mutants deficient in enzymes of the lignin biosynthetic pathway have been identified, lignin-mutant trees are rare. To date, only one lignin-mutant tree (i.e., loblolly pine clone 7-56), deficient in cinnamyl alcohol dehydrogenase (CAD), has been identified (MacKay *et al.*, 1997; Figure 2). Genetic engineering of pulpwood species with altered lignification is, therefore, regarded as a promising technology for producing raw materials with improved pulping and bleaching characteristics. Largely because of its economic relevance, the lignin biosynthetic pathway has been investigated for nearly four decades. Thanks to the emerging molecular, genomics and reverse genetics tools, several aspects of the monolignol biosynthetic pathway have been substantially revised, and readers are referred to several excellent recent reviews for an in-depth treatment

(Anterola and Lewis, 2002; Boerjan *et al.*, 2003; Rogers and Campbell, 2004). Here, we focus on the biotechnological applications of lignin modification in *Populus* because it is a fast-growing pulpwood species widely planted across the northern hemisphere.

As was the case for herbaceous model species (e.g., tobacco), *COMT* (caffeate O-methyltransferase) and *CAD* were among the first genes targeted for transgenic manipulation of lignin in *Populus* (VanDoorsselaere *et al.*, 1995; Baucher *et al.*, 1996; Tsai *et al.*, 1998; Jouanin *et al.*, 2000; Figure 2). Mutations in these two genes are the cause of reduced lignin content, altered lignin structure, and discolored vasculature in the brown midrib (*bm*) phenotypes of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) (Vignols *et al.*, 1995; Halpin *et al.*, 1998). In *Populus*, however, lignin content was not reduced by transgenic down-regulation of *COMT* or *CAD*, except in a severe case where *COMT* activity was essentially eliminated, and lignin content was reduced by 17% (Jouanin *et al.*, 2000). Suppression of *COMT* decreased syringyl (S) and increased guaiacyl (G) lignin accumulation in the stem wood (VanDoorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Jouanin *et al.*, 2000). In addition, increased levels of coniferyl aldehyde and incorporation of an abnormal, 5-hydroxyguaiacyl unit into the xylem were observed (VanDoorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Jouanin *et al.*, 2000; Ralph *et al.*, 2001). In the case of *CAD*-deficient transgenic poplars, the lignin S/G ratio remained essentially unchanged, but there was an increase in the incorporation of both coniferyl and sinapyl aldehydes into the lignin (Baucher *et al.*, 1996; Ralph *et al.*, 2001). Wood discoloration reminiscent of the forage *bm* phenotype was also observed in the transgenic trees, but with variations. Wood of transgenic *CAD* plants showed a distinctive red color, while wood color of transgenic *COMT* trees ranged from pale rose to red brown, depending on the means of gene suppression (i.e., antisense vs. sense silencing) (VanDoorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Jouanin *et al.*, 2000).

Lignin reduction in transgenic *Populus* was first achieved by down-regulating 4-coumarate: CoA ligase (4CL), which controls the step at which hydroxycinnamic acids are converted into high-energy hydroxycinnamoyl-CoA intermediates (Hu

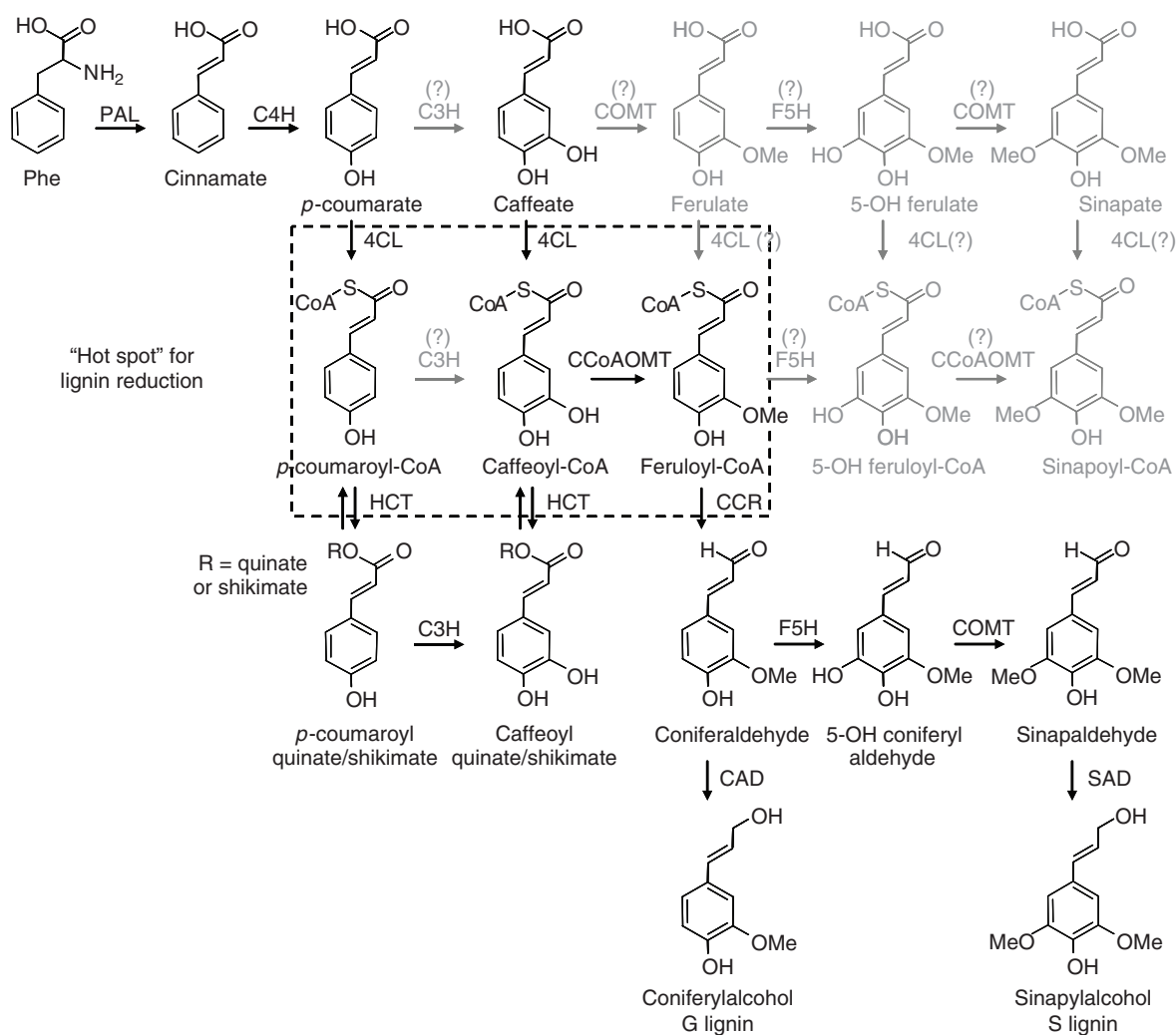


Figure 2 The lignin biosynthetic pathway in plants

et al., 1998, 1999; Li *et al.*, 2003). Substantial reductions of stem wood lignin ($\geq 40\%$) were attained in some lines, with a concomitant increase in cellulose content, resulting in a doubling of the cellulose-to-lignin ratio from ~ 2 in wild type to $\geq \sim 4$ in these transgenics (Hu *et al.*, 1999; Li *et al.*, 2003). Unlike CAD- and COMT-suppressed plants, the lignin S/G ratio was not significantly altered in 4CL-down-regulated aspen. This is consistent with 4CL's restricted involvement at an early stage of lignin synthesis, prior to divergence of guaiacyl and syringyl monolignol biosynthesis (Figure 2). The xylem of 4CL-reduced plants displayed a reddish color intermediate to the colors

observed in CAD-deficient and COMT-deficient poplar/aspen.

Successful lignin reduction has also been reported in transgenic poplars with down-regulated *CCoAOMT* (caffeoyl-coenzyme A O-methyltransferase) and *CCR* (cinnamoyl-coenzyme A reductase; Boerjan *et al.*, 2003) genes. In *CCoAOMT*-suppressed plants, the levels of both G and S monolignols were reduced, but the effect was more pronounced for G lignin, resulting in a slightly increased S/G ratio in the stem wood (Meyermans *et al.*, 2000). Accompanying the lignin alteration in the xylem was an increase of soluble phenolics, particularly the β -D-glucosides

of caffeic acid, vanillic acid, and sinapic acid (Meyermans *et al.*, 2000). Elevated levels of free and wall-bound *p*-hydroxybenzoate were also reported in two independent studies (Meyermans *et al.*, 2000; Zhong *et al.*, 2000). In both cases, a red-brown color was observed in the transgenic wood, although the exact chemical basis of the discoloration remains to be identified.

Increased S lignin content (or S/G ratio) has been achieved in *Populus* by overexpressing *F5H* (ferulate 5-hydroxylase), also known as *CAlD5H* (coniferaldehyde 5-hydroxylase), which encodes a P450 enzyme that catalyzes the conversion of coniferaldehyde to 5-hydroxyconiferaldehyde (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999; Franke *et al.*, 2000; Li *et al.*, 2003). Transgenic aspen showed up to a threefold increase in xylem *CAlD5H* activity, and up to 2.5-fold increase in the S/G ratio of stem wood (Li *et al.*, 2003). Lignin content, on the other hand, was not affected (Li *et al.*, 2003). Co-transformation with antisense 4CL and sense *CAlD5H* constructs led to a simultaneous modification of lignin content and composition (Li *et al.*, 2003), a significant advance toward the biotechnology promise of a more pulp-friendly raw material. Co-transformation can accelerate the genetic engineering and evaluation of multiple traits in tree species, and appears to compare very favorably with other gene stacking or retransformation approaches (Halpin, 2005).

Herbaceous species, such as tobacco, *Arabidopsis*, alfalfa, and maize, have been instrumental in elucidating the function of many of the lignin biosynthetic pathway genes. However, available data from herbaceous models and poplar systems have revealed several interesting distinctions. For instance, overexpression of *F5H* under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter was ineffective in promoting S lignin accumulation in transgenic tobacco stems (Franke *et al.*, 2000), but was effective in transgenic aspen (Tsai *et al.*, 2004). Furthermore, some genes that were successfully targeted for lignin reduction in herbaceous systems could not be manipulated to reduce lignin content in poplars. Examples are COMT-deficient transgenic tobacco (Ni *et al.*, 1994), alfalfa (Guo *et al.*, 2001), and maize (Piquemal *et al.*, 2002), *bm* COMT and CAD mutants of maize and sorghum (Chabbert *et al.*, 1994; Vignols *et al.*, 1995;

Halpin *et al.*, 1998), and *F5H*-overexpressing transgenic tobacco (Franke *et al.*, 2000). Lignin degradability also differed between transgenic maize and poplar with similarly reduced COMT activity: COMT suppression had an adverse effect on pulping performance of transgenic poplar (Pilate *et al.*, 2002), while COMT-reduced maize showed an enhanced *in vitro* digestibility (Piquemal *et al.*, 2002). In both cases, the lignin structure was similarly altered, but, as described above, transgenic maize also had a reduced lignin content which may account for the reported discrepancy in end-use benefit. The results also suggest that positive trait improvement brought about by lignin reduction may offset the negative effect of decreased S/G ratio to have a net positive impact on lignin degradation.

As a corollary, these observations also indicate that some aspects of cell wall formation and lignin biosynthesis in woody perennials are not well modeled using herbaceous species. Analysis of the recently completed poplar genome sequence reveals an expansion in the number of genes involved in phenylpropanoid metabolism and lignin biosynthesis as compared to *Arabidopsis* (Tsai *et al.*, 2006; Tuskan *et al.*, 2006). However, only a small fraction of these genes has been functionally characterized in transgenic *Populus* (Table 4).

Several phenylpropanoid gene families in poplars contain structurally and spatiotemporally distinct isoforms that are differentially associated with lignin and nonlignin phenolic biosynthesis. Examples include the phenylalanine ammonia-lyase (PAL; Kao *et al.*, 2002), 4CL (Hu *et al.*, 1998; Harding *et al.*, 2002), and cinnamate 4-hydroxylase (C4H; Lu *et al.*, 2006) families. Differential expression among members of the HCT (hydroxycinnamoyl-CoA quinate/shikimate hydroxycinnamoyltransferase) family in lignifying versus green/soft tissues of poplar has also been reported (Tsai *et al.*, 2006). Similar expression patterns of the C3H (coumarate 3-hydroxylase) family can also be inferred from the large-scale *Populus* EST (expressed sequence tags) dataset (Sterky *et al.*, 2004). Targeting xylem-specific isoforms of the PAL, C4H, C3H, and HCT families for genetic manipulation should provide further insight into the lignin biosynthetic pathway, and may yield new strategies for transgenic manipulation of lignin.

Table 4 Various lignin pathway genes from *Arabidopsis* and poplar genome

Gene	<i>Arabidopsis</i>			<i>Populus</i>		
	Locus	Expression ^(a)	Mutant or transgenic ^(b)	Gene model	Expression ^(a)	Transgenic ^(b)
Phenylalanine ammonia-lyase (PAL)	At2g37040	+	+	estExt_Genewise1.v1.C.280661		
	At3g53260	+	+	estExt.fgenes4_pg.C.LG.VIII0293	+	
	At5g04230			grail3.0004045401		
	At3g10340			estExt.fgenes4_pg.C.LG.X2023	+	
Trans-Cinnamate 4-hydroxylase (C4H)				gw1.X.2713.1	+	
	At2g30490	+		estExt.fgenes4_pg.C.LG.XIII0519	+	
				grail3.0094002901	+	
	At1g51680	+	+	grail3.0100002702	+	+
4-Coumarate: CoA ligase (4CL)	At3g21240	+		grail3.0099003002	+	
	At1g65060			estExt.fgenes4_pg.C.1210004	+	
	At3g21230			gw1.XVIII.2818.1		
				fgenes4_pg.C.LG.III001773	+	
Hydroxycinnamoyl-CoA quinate/shikimate Hydroxycinnamoyltransferase (HCT)	At5g48930	+	+	fgenes4_pg.C.LG.III001559	+	
				estExt.fgenes4_pm.C.LG.XVIII0344		
				estExt.fgenes4_pg.C.LG.XVIII0910		
				eugene3.00180947		
Coumarate 3-hydroxylase (C3H)				fgenes4_pg.C.scaffold.133000007		
	At2g40890	+	+	eugene3.02080010	+	
				eugene3.36160002		
				eugene3.00160247		
Caffeoyl-CoA O-methyltransferase (CCoAOMT)				fgenes4_pg.C.LG.VI000268	+	
	At4g34050	+		grail3.0001059501	+	+
				fgenes4_pm.C.LG.X000399	+	
	At1g15950	+	+	estExt.fgenes4_kg.C.LG.III0056	+	+
Cinnamoyl-CoA reductase (CCR)	At1g80820			estExt.fgenes4_pg.C.LG.I0389		
				fgenes4_pg.C.scaffold.208000034		
				gw1.208.109.1		
				gw1.208.126.1		
Ferulate 5-hydroxylase (F5H)				estExt.fgenes4_pg.C.2080041		
	At4g36220	+	+	fgenes4_kg.C.LG.I000012	+	+
				scaffold.57:1035361-1038589	+	
	At5g54160	+		LG.VII:11484639-11486746	+	+
Caffeic acid O-methyltransferase (COMT)				LG.XII:3089139-3092252	+	
				LG.XV:255739-258237	+	
	At3g19450	+	+	estExt_Genewise1.v1.C.LG.IX2359	+	+
	At4g34230		+	grail3.0004034803	+	

^(a)Number of genes associated with lignin biosynthesis, based on expression analysis of *Arabidopsis* (Raes *et al.*, 2003) and *Populus* (Sterky *et al.*, 2004; Tsai *et al.*, 2006) genes, or according to the original literature cited in the text

^(b)Number of genes associated with lignin biosynthesis based on characterization of mutant or transgenic plants

^(c)Sinapyl alcohol dehydrogenase (SAD) shown to be involved in S lignin biosynthesis of *Populus* (Li *et al.*, 2001) is included, but its involvement in *Arabidopsis* lignification has not been supported (Sibout *et al.*, 2005). Note two CAD genes are present in *Arabidopsis* versus one in *Populus*

With regard to quantitative lignin modification, a common feature among the three enzymatic steps successfully targeted for lignin reduction in poplar (i.e., 4CL, CCoAOMT and CCR), is their production and utilization of high-energy hydroxycinnamoyl-CoA esters (Figure 2). By the same measure, this “hot spot” for lignin reduction may also include HCT, as boxed in Figure 2, and possibly C3H for its putative role in conversion of *p*-coumaroyl-CoA to caffeoyl-CoA (Wang *et al.*, 1997). Transgenic down-regulation of xylem-specific *HCT* and *C3H* in poplar will help substantiate this hypothesis, as well as help clarify *C3H* function. *F5H* overexpression appears to be the only approach to significantly augment S lignin production. It should be noted that among the many lignin pathway genes reportedly targeted for transgenic modification to date (Table 4), *F5H* overexpression represents the only case in which the sense transgene did not trigger co-suppression or gene silencing (Li *et al.*, 2003). In aspen, *F5H* overexpression was achieved by using either a xylem-specific aspen *4CL1* promoter (Hu *et al.*, 1998; Harding *et al.*, 2002) or a constitutive CaMV 35S promoter, and either a homologous (aspen) or a heterologous (sweetgum, Osakabe *et al.*, 1999) transgene, each producing similar efficacy (Tsai *et al.*, 2004). Because sense transgene-mediated gene silencing is dependent, at least in part, on the level of endogenous expression (e.g., Vaucheret *et al.*, 1998), endogenous *F5H* transcripts may have a very short half-life. The inability to overexpress other lignin pathway genes may account for the general lack of transgenic phenotypes with increased lignin deposition in the studies cited above. Conversely, increased or ectopic deposition of lignin in transgenic plants has only been reported by manipulation of transcription factors (Newman *et al.*, 2004; Goicoechea *et al.*, 2005; Sanchez *et al.*, 2006).

In closing, since the first report of transgenic poplar with modified lignin (VanDoorselaere *et al.*, 1995), the past 12 years have witnessed significant progress in lignin biotechnology for pulpwood species. Traits of commercial relevance (e.g., reduced lignin content and/or more reactive lignin structure), have been successfully introduced into elite *Populus* clones, and their biological and ecological performance have been evaluated in the laboratory and in field settings. For instance, insect herbivory on lignin-reduced aspen has been examined with no direct link apparent

between lignin reduction and laboratory feeding performance of gypsy moth larvae and forest tent caterpillars (Brodeur-Campbell *et al.*, 2006). Elsewhere, a four-year field evaluation of CAD-deficient transgenic poplar at two European sites showed improved Kraft pulping performance, with higher pulp yields and less cellulose degradation as compared to the control (Pilate *et al.*, 2002). Importantly, interactions with leaf insects, pathogens, and soil microbes were not altered (Pilate *et al.*, 2002). These examples point to real promise in reducing the burden of lignin removal to the pulp and paper industry. Commercial-scale use of these trees would translate into significant monetary savings and environmental benefits due to reduced chemical and energy inputs, and reduced outputs of pollutants associated with pulping.

2.3 Transgenic Modification of Gibberellin (GA) Biosynthesis and Signaling

Bioactive forms of GA control diverse plant developmental processes, including seed germination, flower initiation, fruit development, stem elongation, leaf expansion, and trichome development (Davies, 1995). Substantial efforts are underway in a variety of plant species to understand the molecular mechanisms of many of these GA-mediated processes (Olszewski *et al.*, 2002).

Research in the last several years has demonstrated that deficiencies in GA levels or perturbations in the GA signaling pathway are a powerful means to control plant size (Peng *et al.*, 1999; Spielmeier *et al.*, 2002). Low levels of bioactive GA and signaling lead to dwarfism, while conversely higher levels of several bioactive GA forms and increased signaling induce rapid shoot elongation and larger organ size (Davies, 1995). Therefore, transgenic alterations of GA metabolism and/or signal transduction are being viewed as logical approaches for altering growth patterns in trees.

A number of studies substantiate the expectation that based on herbaceous plant studies; GA-related genes will be a powerful means to control the stature and development of woody plants. Early reports considering GAs in woody plants indicated that they stimulate cambium division, whereas auxin is needed for lignification (Wareing,

1958). Using the *Arabidopsis* gene that encodes the GA 20-oxidase enzyme, which catalyzes one of the last steps in GA biosynthesis, GA-overproducing transgenic poplar trees were produced. Transgenic plants displayed enhanced overall growth, as well as longer wood fibers compared to wild-type (WT) plants (Eriksson *et al.*, 2000). Overexpression of a poplar GA 3-oxidase had no effect on transgenic poplar growth and development, suggesting that GA 20-oxidase controls the flux of bioactive GAs in the pathway, a conclusion supported by studies in other annual plants (Israelsson *et al.*, 2004). Thus, it appears that GA can regulate cambium division and some aspects of the differentiation of newly formed vascular initials, and could be an important means of modifying growth and wood quality—critical economic traits in trees.

In trees, dwarfism can be an important mitigation and production trait. The mitigation value is largely considered in the context of a biosafety, limiting spread of transgenes in wild populations (Danielle, 2002). Because competition for light in trees is essential for their survival, slow-growing dwarfing varieties will have a substantial disadvantage compared to their wild relatives. Therefore, these kinds of trees would likely be eliminated early, with little chance for reproduction. However, in an intensively managed plantation where trees are cultured as wood fiber crops, semi-dwarfism may bring about beneficial alterations in tree growth patterns (Bradshaw *et al.*, 2000). For example, domesticated trees that are substantially shorter and stouter may produce less reaction wood (which degrades wood and pulp quality) and give a higher harvest index. Such trees will also have improved harvesting/handling efficiencies and enable greater unit-area fiber yields. Crowns of dwarfed trees will likely be narrower, allowing for a greater number of stems per unit area. Several studies have reported successful induction of dwarfism using transgenic modifications of GA-related genes. Overexpression of a poplar and bean GA 2-oxidase causes dwarfism via reduction in shoot elongation (Busov *et al.*, 2003). GA 2-oxidase is the last step in GA metabolism and is involved in conversion of the bioactive GA_{1/4} into their inactive forms GA_{8/34}. High expression of the gene is associated with increased deactivation of the bioactive GAs that consequently led to decreased elongation and dwarfism. Dwarfism can be rescued by application of a bioactive synthetic form of

the hormone (GA₃) that cannot be catabolized by GA 2-oxidase. Alternatively, dwarfism was successfully induced by overexpression of DELLA (D = Aspartic acid, E = Glutamic acid, L = Leucine, A = Alanine) proteins—strong repressors of GA signaling. DELLA proteins belong to the GRAS family of transcription factors but, in contrast to all other members of this family, have a conserved DELLA domain in the N-terminus that mediates GA-induced sensitivity to photolytic degradation. Complete truncation or nonsynonymous substitution in this domain causes a strong dominant dwarfing effect not only in *Arabidopsis* but also in a number of other species. Similarly, overexpression of DELLA proteins with a mutated DELLA domain induced a strong dwarfing effect in *Populus*. In addition, dwarfed poplars displayed increased root production under *in vitro* conditions and changes in secondary metabolism associated with shifts to storage and defense compounds (Busov *et al.*, 2006). Along with GA, genes involved in the biosynthesis of other plant hormones have also been identified and manipulated. An excellent review by Confalonieri *et al.* (2003) covers those aspects as well as altered growth characteristics of transgenic poplars (e.g., Gallardo *et al.*, 1999). In addition, a number of recent reviews discuss the general applications of genetic-engineering trees in forest biotechnology (e.g., Mann and Plummer, 2002; Merkle and Nairn, 2005). These papers also discuss various aspects of growth alterations in trees.

2.4 Manipulation of Reproductive Development in Poplars

The ability to control flowering in forest trees is desirable for many reasons. The multiple-year juvenile or nonflowering phase of trees is a major impediment to tree improvement and, thus, one goal is the ability to routinely induce flowering in juvenile trees in order to speed breeding (Bhalerao *et al.*, 2003). Prevention of flowering or sterility is desired for forest trees growing in production plantations in order to provide genetic containment (Brunner *et al.*, 2007) and to route more photosynthate into biomass production and not into reproduction (Bradshaw and Strauss, 2001; Ragauskas *et al.*, 2006). Achieving these competing outcomes—inducible early flowering

and suppression of flowering over a multiple-year rotation—is technically challenging, but not an insurmountable paradox. Ideally, a system for transient induction of flowering that can easily be applied to an infinite number of genotypes is desired to advance breeding. In contrast, suppression of flowering should be a stable trait that is introduced into elite clones as the final step in tree improvement.

Various environmental and endogenous signals regulate the transition to flowering via multiple interacting genetic pathways that have been most extensively deciphered in *Arabidopsis* (Boss *et al.*, 2004; He and Amasino, 2005; Ausin *et al.*, 2005). Numerous studies have also identified genes controlling the initiation and differentiation of floral organs in *Arabidopsis* and other annual plants, such as snapdragon and petunia (Jack, 2004). Most studies of genes involved in flowering in trees have dealt with homologs of genes known to control various stages of flowering in *Arabidopsis*. Gene expression studies and heterologous constitutive expression of these tree genes in *Arabidopsis* and tobacco have indicated broad conservation of function in most cases. Studies of flowering gene function in trees have been relatively rare, but the number of analyses completed in transgenic poplar is increasing and these have revealed significant differences in gene function as compared to *Arabidopsis*.

Several *Arabidopsis* genes induce early flowering when constitutively expressed in *Arabidopsis*, but do not accelerate flowering when expressed in poplar (Brunner and Nilsson, 2004; Strauss *et al.*, 2004). Overexpression of the floral meristem identity gene *LEAFY* did induce precocious flowering in some male poplar genotypes, but flowers were abnormal and not fertile (Weigel and Nilsson, 1999; Rottmann *et al.*, 2000). In contrast, constitutive expression of *PtFT1*, a poplar homolog of the *Arabidopsis* flowering time gene *FT*, induced normal flowers in both male and female genotypes in only a few months (Bohlenius *et al.*, 2006). The identification of *PtFT1* as a potent inducer of functional flowering is an important step toward a system of accelerated flowering in order to advance breeding. The study by Bohlenius *et al.* (2006) also revealed differences between poplar and *Arabidopsis*. Whereas *FT* only affects flowering time in *Arabidopsis*, *PtFT* controls both flowering and the short day-induced

growth cessation that occurs in fall as trees prepare for winter dormancy. This result raises the possibility that genes affecting only the flowering in *Arabidopsis* could have additional functions in poplar that reflect the difference between the rapid-cycling annual growth habit of *Arabidopsis* and poplar's perennial woody growth habit. This also indicates that manipulating genes for flowering control in trees may have undesired additional effects on vegetative growth. As described above, induction of flowering should be inducible and transient, which would avoid secondary effects on vegetative growth. However, vegetative effects may be more problematic for engineering sterility.

Even with a strong inducer of flowering in poplar identified, developing an efficient system for “flowering on demand” remains problematic. The alcohol-inducible promoter system is effective in poplar (Filichkin *et al.*, 2006a), and other chemical- or heat shock-inducible promoter systems may also work well in poplar. Perhaps a greater obstacle is developing a simple, rapid method to deliver a transgene to any genotype. *FT* is transcribed in leaves and the *FT* protein is transported to the shoot meristem, where it acts to promote flowering, and transient expression of *FT* mRNA in leaves is sufficient for flowering (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007). Thus, for *FT*-mediated induction of flowering, grafting or an *Agrobacterium*-mediated transient expression system (Johanson and Carrington, 2001), whereby *Agrobacterium* transformed with a *PtFT1* transgene is infiltrated into leaves on an intact plant, might be an effective option. Another approach to induce flowering is to suppress genes that repress flowering, though this has yet shown to be effective in poplar. In this case, virus-induced gene silencing (VIGS) vectors (Burch-Smith *et al.*, 2004) might be an efficient, transient method for inducing flowering in any genotype in a greenhouse. A VIGS vector has recently been developed for poplar (Naylor *et al.*, 2005).

Introduction of genes that cause reproductive sterility or prevent flowering are generally viewed as an enabling technology for commercial use of transgenic forest trees. Dispersal of pollen and seed from transgenic, exotic, or intensively bred forest plantations may cause significant ecological impacts on wild or managed ecosystems. Although genetic containment is also a concern

for some agricultural crops in some locations (Chapman and Burke, 2006; Lee and Natesan, 2006), concerns over transgene escape are greater for forest plantations. This concern is based on the complexity and scale of gene flow that results from the large size and longevity of trees and the frequent proximity of plantations to wild relatives (Williams, 2005). The genes and strategies for engineering reproductive sterility in trees as well as related risk assessment have recently been comprehensively reviewed (Brunner *et al.*, 2007). Here, we will highlight a few of the options available in order to illustrate both the potential and the difficulties of developing genetic containment methods for trees.

Potentially, reproductive development could be blocked at any stage—from the transition to flowering to the final stages of gamete development. Although the functions of only a few flowering genes have been directly characterized in poplar (e.g., Rottmann *et al.*, 2000; Bohlenius *et al.*, 2006), poplar homologs of most of the *Arabidopsis* genes involved in flowering have been identified in (e.g., Brunner and Nilsson, 2004). Many of these genes have expression patterns in poplar that indicate functional conservation (e.g., Rottmann *et al.*, 2000). Blocking the transition to flowering is desired, because this would completely avoid any investment in floral formation, allowing all resources to be directed toward vegetative development. One of the difficulties in demonstrating an effective, stable sterility system in trees is that large trees need to be monitored for many years. Therefore, absence of flowering has the additional advantage of being simpler to confirm than sterility methods that allow formation of floral structures that then need to be examined microscopically to verify sterility. However, prevention of flowering may not be desirable in all cases, such as when a plantation provides important habitat for birds or insects that feed on flower parts. Due to the difficulties in demonstrating a robust sterility system in trees and the genetic redundancy in the flowering control (Irish and Litt, 2005), redundant sterility systems that target different stages of flowering or act via multiple mechanisms might be the best approach to ensure a high level of gene confinement.

Approaches for engineering bisexual sterility fall into three categories: (1) floral ablation, whereby

a floral specific promoter directs expression of a cytotoxin; (2) repression of the transition to flowering by modifying the expression of genes that repress this developmental phase change; and (3) suppression of genes necessary for reproductive development at the DNA, RNA, or protein levels. All have advantages and disadvantages (see Brunner *et al.*, 2007 for a comprehensive description). For example, the ablation approach avoids the complication of genetic redundancy, but low levels of transgene expression in vegetative tissues can have detrimental effects on growth that may not be apparent under greenhouse conditions but appear as a cumulative effect in the field. RNA-mediated gene suppression, also called RNAi, is currently the most widely used method to inhibit gene activity, but it does not completely suppress a gene's expression. Recent advances in targeted gene mutagenesis and replacement using the yeast *RAD54* gene (Shaked *et al.*, 2005) or zinc-finger nucleases (Lloyd *et al.*, 2005; Wright *et al.*, 2005) may eventually lead to efficient methods for engineering null alleles of reproductive genes in poplar.

The long delay to flowering in poplars (3–6 years on good field sites) has been a major obstacle to demonstrating an effective sterility system in trees. Although an early flowering poplar genotype was identified (Meilan *et al.*, 2004b), its transformation efficiency was low and it did not reliably flower in the greenhouse. Screening sterility transgenes in an inducible *PtFT* background (discussed above) might be useful in some cases, such as an ablation transgene targeting reproductive organs or an RNAi transgene causing abnormal reproductive organ development, but induction of *PtFTI* expression confounds the effects of transgenes designed to prevent flowering. Nonetheless, results to date are encouraging. A cytotoxin directed by the tapetal-specific *TA29* promoter from tobacco dramatically reduced pollen production and a *PTLF*-antisense transgene induced abnormal flowers and reduced fertility in a male poplar clone (Brunner *et al.*, 2007). Additional sterility transgenes that use different mechanisms and target different stages of flowering have been introduced into poplar and are undergoing field evaluation. In addition, the genomic platforms available for poplar are facilitating analysis of the gene networks controlling flowering, which, in turn, will enable selection of genes, promoters, and

strategies for manipulating flowering with greater precision and effectiveness.

2.5 Insertional Mutagenesis Using Transgenic Poplars

Genome-scale T-DNA mutagenized populations represent powerful tools for functional genome exploration in model organisms (Alonso *et al.*, 2003). Such resources are most efficient when a genome sequence is available. In the two species where a full, annotated genome sequence is available (*Arabidopsis* and rice), such resources were developed, and continue to be developed, for specific needs as core tools for functional gene characterization (Alonso *et al.*, 2003; An *et al.*, 2003). Except for a few pilot studies (Busov *et al.*, 2003; Groover *et al.*, 2004), such resources are not available in *Populus* or any other tree species. Perennial woody plants have many developmental differences from annual herbaceous plants such as *Arabidopsis* and rice. These include vegetative dormancy, delayed onset of flowering, extended periods of secondary (woody) growth, and gradual vegetative maturation. Therefore, forward genetic approaches using mutagenized populations are likely to uncover many new types of genes, as well as new functions for already discovered genes.

The advantages of using transgenics mutagenesis approaches in trees have already been reviewed (Busov *et al.*, 2005). Three main approaches emerge as feasible in trees—activation tagging, enhancer/gene traps, and transposon tagging, all of which employ transgenics.

Activation tagging is an insertional mutagenesis approach that produces dominant and semi-dominant mutations that can be identified in T₁ (primary) transgenics, a feature that is extremely important in trees because their long generations and inbreeding intolerance precludes selfing for exposing recessive mutations. Activation tagging uses a special T-DNA vector that randomly inserts in a genome location and contains strong enhancers positioned near the left or right borders (Hayashi *et al.*, 1992; Weigel *et al.*, 2000). The vector, when integrated near a gene, causes its up-regulation by the proximity of the enhancers and a gain-of-function, dominant mutation is produced (Hayashi *et al.*, 1992). Integration position is established by recovering a genomic

sequence flanking the insertion sites by employing either TAIL-PCR (thermal asymmetric interlaced-polymerase chain reaction) (Liu *et al.*, 1995) or plasmid rescue techniques (Weigel *et al.*, 2000). Several genes encoding key *Arabidopsis* developmental regulators have been identified via activation tagging. These include the *histidine kinase* gene, whose overexpression bypasses the need for cytokinin in shoot regeneration (Kakimoto, 1996); the flowering time gene *FT*, whose overexpression causes early flowering in *Arabidopsis* (Kardailsky *et al.*, 1999); and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOCI*), whose overexpression suppressed the late flowering phenotype of plants with functional *FRIGIDA* (*FRI*) and *FLOWERING LOCUS* (*FLC*) alleles (Lee *et al.*, 2000). Because of the utility of this method, a special activation tagged, sequenced-indexed stock center was created for *Arabidopsis* (Ichikawa *et al.*, 2003) and rice (Jeong *et al.*, 2006).

In *Populus*, one activation tagging population of 627 lines was generated and planted in a field trial in western Oregon, USA. The first gene tagged via insertional mutagenesis in any tree came from this pilot population (Busov *et al.*, 2003). The gene encodes a GA 2-oxidase (GA2ox), and the activation resulted in a mutant displaying a distinct dwarf phenotype, dark green foliage, and increased trichome production. Using plasmid rescue, the activation tag was positioned approximately 300 bp up-stream of the translation start site, causing hyperactivation of the gene. Using a variety of approaches, the causal relationship between gene activation and phenotype was demonstrated, verifying the function of the tagged gene. This population is still under active study, and many more tagged genes will likely be discovered (Busov *et al.*, 2005). A new population of approximately 2000 events has been produced, resulting in interesting and useful phenotypes that help elucidate unique aspects of woody perennial biology (S. Reagan, personal communication).

Another transgenic strategy for mutagenesis is based on screening for gene expression by using various reporter genes rather than mutant phenotypes (Springer, 2000). Enhancer trapping employs a T-DNA vector carrying a reporter gene preceded by a minimal promoter that typically contains basal sequences required for

transcription and translation, but are not sufficient to drive expression of the reporter gene. As with activation tagging, the enhancer trap vector is inserted randomly into the genome, and when integrated into or near a gene, the reporter gene is expressed in a pattern that reflects native expression of the proximal gene. To identify the nearby gene, flanking sequences of the vector insertion site are recovered using similar methods as described above for activation tagging. Gene traps are designed and act in a similar fashion. In contrast to enhancer traps, gene traps must integrate into a gene in order to be informative. Because of this special requirement, gene traps are usually less efficient than enhancer traps. This technology has been used in *Populus* to identify genes involved in vascular development and wood formation (Groover *et al.*, 2004). Eight percent of gene-trap lines showed marker gene expression in leaves or stems of tissue-culture-grown plants, which corresponds approximately to the tagging efficiency of this method (Groover *et al.*, 2004). Genes related to wood formation have been discovered and are currently under study. Screens not restricted to woody perennial development were also performed and proved to be productive in characterizing genes that were not identified in model annual plants (Filichkin *et al.*, 2006b). Searches for genes expressed during lateral/adventitious root formation identified several enhancer trap lines showing distinct expression of the reporter gene during lateral root initiation. In one line, the insertion was positioned to a gene encoding a DNA-hook transcription factor (Filichkin *et al.*, 2006b). By fusing the promoter of this gene to the same reporter gene, the expression pattern was recapitulated in *Populus* and *Arabidopsis*. This allowed identification of a promoter that directs transcription exclusively in lateral roots.

Finally, delivery of heterologous maize transposon in transgenic poplars has been used as a third strategy for insertional mutagenesis. Transfer, excision, or reintegration of the maize *Ac* transposon has been reported in *Populus* (Howe *et al.*, 1991; Kumar and Fladung, 2003). Because transposons primarily generate recessive mutations, their usage in *Populus* is limited by the requirement of several rounds of selfing to bring the mutation to a homozygous state, thus exposing the phenotype. Haploid cultures have

been proposed to mitigate this problem (Deutsch *et al.*, 2004).

2.6 Field Trials With Transgenic Poplars

Field trials are essential for examining the phenotypic characteristics and the stability of transgenic plants under wild growing conditions. According to the information available at <http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>, where the Animal and Plant Health Inspection Service (APHIS) compiles data on all US transgenic plant field trials (Notifications and Release Permits), over 11 000 such trials have been conducted in the US since 1987 (Figure 3).

The main phenotypic characteristics being tested in these trials include a variety of agronomic properties, pest resistance traits (viral, bacterial, fungal, and insect resistance); herbicide tolerance; and product quality. Of these trials, 144, carried out by various US universities and private companies, used poplars as an experimental system. In 110 of these trials, the poplar species was not specified, 18 used *P. deltoides*, and 12 used a given variety of hybrid poplar. Some of the universities involved in these trials, were Oregon State University, University of Washington, Mississippi State University, University of Connecticut, New York State University, and Michigan Technological University. Private companies involved in poplar field trials were ArborGen, Mead-Westvaco, Monsanto, and Applied Phytogenetics. The first permission for a transgenic poplar field trial was issued in 1989 to Iowa State University to perform a field test for a marker gene, chloramphenicol acetyl transferase or CAT. Since that time, genes for: herbicide tolerance; insect, bacterial, and fungal resistance; agronomic properties like lignin reduction; and flowering traits, have been tested in transgenic poplars. In Europe, data for over 2000 summary notifications using transgenic plants has been compiled for 28 countries. Countries such as France, the Netherlands, Spain, and the United Kingdom have performed over 200 field trials each during the period 1991–2006 (<http://biotech.jrc.it/deliberate/dbcountries.asp>). Of these, only about 20 deal with transgenic poplars, although each study could include

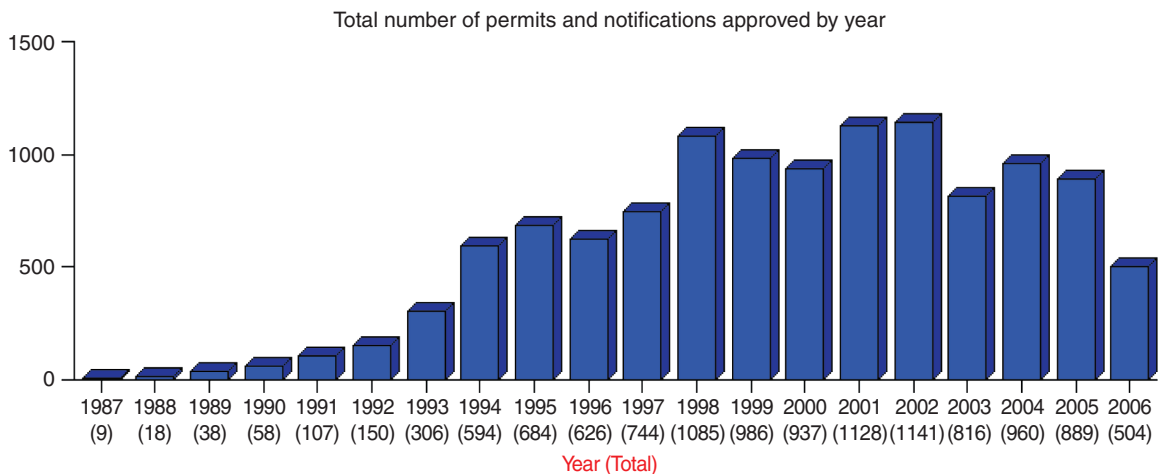


Figure 3 Transgenic field trials in the United States during 1987–2006

one or more organism (<http://biotech.jrc.it/deliberate/dbplants.asp>).

The data on transgenic poplar field trials from other countries are not readily available. The most noteworthy of those is China's ambitious plan to plant one million transgenic poplar trees carrying the insect resistance gene from *Bacillus thuringiensis* (http://www.chinadaily.com.cn/english/doc/2005-04/01/content_430016.htm). China launched the "Great Green Wall" project in 2001, in which a government-sponsored reforestation project aspires to plant a 2800-mile shelterbelt of genetically engineered (GE) trees around the Gobi desert. Transgenic poplars resistant to insects have been planted in northern China, which typically has little rainfall. Chinese researchers believe that there is little chance for escaped transgenic seeds or sprouts to survive in this hostile climate. Only two poplar clones, Poplar-12 and 741 have been approved and planted on about 200 hectares. This falls far below the Chinese government's target of one million trees. The Institute of Science in Society (ISIS), a British organization highly critical of genetically modified organisms (GMOs), however, has criticized this experiment. By their estimates, over 1 million genetically modified (GM) trees have been planted in this particular experiment and 400 000 more such trees have been planted in other parts of China (<http://www.i-sis.org.uk/GMTGL.php>). The concerns of ISIS range from genetic pollution of forests to the negative social impact of such

large-scale experiments. An article in American Chemical Society's technology newsletter of January 26, 2005, paints a less pessimistic picture. They describe the undertaking as a "grand experiment". Brazil appears to be the next country in following with large-scale GE tree production. Sedjo (2006) recently presented a balanced view of the potential of GE trees to resolve the myriad problems faced by the world's forests. He holds that China has released far less than 1 million GE trees. In other parts of the world, transgenic poplars have been field-tested for a variety of introduced traits including herbicide tolerance, insect resistance, and flowering and stature control. These traits will be discussed in detail in the following sections.

2.6.1 Herbicide tolerance

Fillatti *et al.* (1987a) reported the *Populus* transformation results involving the insertion of an herbicide-tolerance gene derived from *Salmonella typhimurium* (*aroA*). A mutation in this gene resulted in the formation of a 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS) that was resistant to inhibition by glyphosate, the active ingredient in Roundup® herbicide (Comai *et al.*, 1983). Ectopic expression (*MAS*, mannopine synthase promoter) of this gene yielded surprisingly low levels of herbicide tolerance (Riemenschneider

et al., 1988). This was thought to be the result of low, cytosolic expression of *aroA*. When the same poplar genotype (NC-5339, *Populus alba* × *Populus grandidentata*) was transformed with *aroA* under the control of the CaMV 35S promoter and a chloroplast transit peptide, the resulting plants showed higher levels of glyphosate tolerance (Riemenschneider and Haissig, 1991; Donahue *et al.*, 1994). Although use of the 35S promoter led to higher expression levels than *MAS*, and the transit peptide directed transgene product to the chloroplast, performance of these *aroA*-containing lines (i.e., unique transformation events) still fell short of expectations (Karnosky *et al.*, 1997). After greenhouse spray tests, chlorophyll content in all transgenic lines was inversely correlated to glyphosate concentration, height growth was arrested following herbicide treatment, and only one line retained live leaves six weeks after treatment (Donahue *et al.*, 1994).

Meilan *et al.* (2002b) tested a construct containing two glyphosate-tolerance genes, *CP4* and *GOX*, for its ability to impart glyphosate tolerance in poplar. *CP4* is an alternative form of EPSPS and was cloned from the *A. tumefaciens* strain CP4. Glyphosate has a low affinity for this form of the enzyme. *GOX* is a gene isolated from *Achromobacter* strain LBAA; it encodes glyphosate oxidoreductase, which is an enzyme capable of metabolizing glyphosate. The initial breakdown product of the reaction catalyzed by *GOX* is aminomethylphosphonic acid (AMPA; Barry *et al.*, 1992). Using an *Agrobacterium*-mediated transformation protocol (Leple *et al.*, 1992; Han *et al.*, 2000), Meilan *et al.* (2000) generated transgenic plants in six clones of hybrid poplar (four clones of *P. trichocarpa* × *P. deltoides*, and one clone each of *P. tremula* × *P. alba* and *P. tremula* × *P. tremuloides*).

Ten percent of their transgenic lines showed no foliar damage or reduced growth after being sprayed with Roundup® at concentrations above usual commercial doses. This was hypothesized to be due to *CP4* expression and not that of *GOX*. Many agronomic crops containing both *CP4* and *GOX* became chlorotic in response to glyphosate treatment, likely resulting from the conversion of glyphosate to AMPA.

To test for undesirable effects of *GOX*, they produced 12 additional lines containing only the *CP4* gene. The performance of these lines was

compared to 12 lines produced in the same genotype, into which both *CP4* and *GOX* had been inserted. Growth of the lines containing only *CP4* was significantly better than those containing both genes and they exhibited less damage in response to glyphosate treatment. This was the first report of transgenic poplars exhibiting high levels of glyphosate tolerance when grown under field conditions (Meilan *et al.*, 2002b).

2.6.2 Insect resistance

Insect pests are a major problem for poplar plantation managers. The two main classes are chrysomelid beetles and lepidopteran caterpillars. Both are susceptible to microbial pesticides derived from different strains of *Bacillus thuringiensis* (*Bt*). The products encoded by *Bt* toxin genes have been used safely as microbial pesticides in numerous crops (Carozzi and Koziel, 1997). These toxins are relatively selective insecticides that have very few nontarget effects (James, 1997). Many different *Bt* strains have been identified, each affecting a select group of insects that are usually closely related phylogenetically (Thompson *et al.*, 1995).

The use of GE trees to produce *Bt* toxins is preferable to spray applications for several reasons. First, vegetation, soil, and water surrounding the crop are not exposed to spray drift. Susceptible, nontarget insects in areas adjacent to the transgenic crop would not be exposed, reducing the potential for development of *Bt* resistance. Second, spray applications quickly degrade, persisting on leaves for, at most, a few days (Thompson *et al.*, 1995; James *et al.*, 1999). GE trees, however, can produce the toxin continuously, thereby avoiding sensitivities to application timing and the costs associated with repeated applications. Finally, because transgenic trees produce the toxin within plant tissues, it is possible to target insects residing in the plant, such as wood borers and leaf folders. For some of these pests, no insecticides are available that target the life stage responsible for the damage.

The cottonwood leaf beetle (CLB, *Chrysomela scripta* Fabricius) is the primary insect pest in poplar plantations. It is a multivoltine insect that has a wide distribution and can produce outbreaks causing severe defoliation, particularly in young plantations (Hart *et al.*, 1996). Significant growth

loss has been shown in poplar after two years of simulated leaf beetle defoliation (Reichenbacher *et al.*, 1996). Unlike weed control, which is only needed for the first few years of a poplar rotation, insect control is often repeated during each year of a rotation, making it costly for growers. James *et al.* (1999) have demonstrated that a *Cry3A Bt* toxin is highly effective against the CLB.

A binary vector containing a *Cry3A* gene under the control of the 35S promoter was used to produce 51 insect-resistant lines in four genotypes of *Populus* (16 lines in 24-305 and nine in 189-434, both of which are *P. trichocarpa* × *P. deltoides* triploids; 17 in 50-197, which is a *P. trichocarpa* × *P. deltoides* diploid; and nine in OP-367, a *P. deltoides* × *P. nigra* diploid). The neomycin phosphotransferase II (*NPTII*) gene under the control of the nopaline synthase (*NOS*) promoter was used as a selectable marker. The T-DNA insert was bordered by matrix attachment region (MAR) elements (Allen *et al.*, 1996). The transgenic trees were field tested in eastern Washington State.

This trial relied on insect pressure from the surrounding, commercial stands to evaluate insect resistance. Trees were evaluated for damage seven weeks after planting and again at the end of the growing season. Records on height and basal diameter were taken on all trees immediately after planting, and at the same time as damage ratings were scored. Virtually all of the *Bt* transgenics showed very low feeding damage, whereas the nontransgenic lines sustained significantly higher levels of defoliation. Moreover, in most cases, the mean growth for transgenic lines was greater than that for the nontransgenic controls within each clone (Meilan *et al.*, 2000).

The potential for insects to develop resistance to GE crops is a major concern (DiCosty and Whalon, 1997; James, 1997; Roush and Shelton, 1997). Before insect-resistant transgenics can be commercialized, a resistance management plan must be developed. Many management strategies have been proposed based on prior experiences with pesticide resistance (e.g., Luttrell and Caprio, 1996; Roush, 1997; Gould, 1998; McGaughey *et al.*, 1998). Combining resistance genes (pyramiding or stacking) is one way of reducing the risk of insects becoming resistant to *Bt* gene products (Nwanze *et al.*, 1995; Maredia and Mihm, 1997; Roush, 1997). This

approach has proven to be an effective strategy for resistance management with the cotton bollworm (*Helicoverpa armigera*; Zhao *et al.*, 1997).

To obtain approval from the US Environmental Protection Agency (EPA) to deploy trees containing a gene encoding a *Bt* toxin, additional studies are needed of beetle dispersal, given that natural refugia are effective buffers for preventing the development of resistance (Matten, 1998).

2.6.3 Flowering control

Before GE trees can be commercialized, the APHIS (a division of the USDA) is likely to require a strategy to mitigate the risk of transgene spread and persistence in the environment. One way to satisfy this need is to control flowering (Meilan *et al.*, 2001). This may help alleviate or at least reduce public and regulatory concerns over the commercialization of transgenic trees. As discussed above, the manipulation of flowering can provide many benefits. Sterility can also reduce genetic pollution from nontransgenic plantations, promote vegetative growth, and eliminate nuisance tissues (e.g., pollen, seed pods). In addition, flowering control could lead to shorter juvenile periods.

While each strategy for engineering sterility has advantages, it is unclear which method will work best with trees. Hence, tests are underway that involve a variety of techniques, such as tissue-specific ablation, dominant negative mutations, and post-transcriptional gene silencing, including RNAi. Employing the first approach, Skinner *et al.* (2003) successfully used the promoter from *populus trichocarpa DEFICIENS* (*PTD*), a poplar homolog of the *Arabidopsis* gene *APETALA3*, to drive the expression of reporter and cytotoxin genes in floral tissues of *Arabidopsis*, tobacco, and poplar.

Recently, Wei *et al.* (2007) tested the utility of an attenuation system designed to avoid the harmful effects of leaky cytotoxin gene expression in the vegetative tissues of transgenic trees. They tested the promoter from the poplar *LEAFY* gene, *populus trichocarpa LEAFY* (*PTLF*), to drive *barnase* expression (*PTLF::barnase*). The same plants were also transformed with *barstar* driven by either the basal 35S promoter (+5

to -72 fragment, 35SBP); 35SBP fused to the tobacco mosaic virus omega element; or the *NOS* promoter. It was not possible to recover transgenic plants using unattenuated PTLF::*barnase*, leading the authors to speculate that this promoter led to substantial vegetative expression. While the *barstar*-attenuated constructs did permit the recovery of transformants, the rate was reduced. Seven percent of the attenuated events had highly abnormal morphology; these events had significantly higher *barnase:barstar* expression ratios, and were identified during the early phases of propagation. A greenhouse study showed that phenotypically normal attenuated plants grew at the same rate as WT and *barnase*-lacking transgenics. In addition, the authors demonstrated a positive correlation between relative growth rate and the *barstar:barnase* expression ratio in attenuated plants. Surprisingly, the appearance and growth rate of the nearly all of the attenuated lines were substantially reduced after one or two growing seasons in the field.

Recently this same group has been working with genes that affect flowering time. RNAi was used to reduce expression of the poplar ortholog of *CENTRORADIALIS* (*PtCENLI*), a gene that plays a key role in maintaining the tree in a juvenile condition. When transgenic poplars containing this RNAi vector were grown under field conditions, four of most strongly silenced lines produced inflorescences or floral buds within two years of planting, which was several years earlier than that seen in WT trees. Surprisingly, overexpression of *PtCENLI* also resulted in delayed vegetative budbreak (Mohamed, 2006). Based on this work, it appears that *PtCENLI* is involved in regulating release from winter dormancy and resumption of growth (Mohamed, 2006). Hopefully, this work will ultimately lead to the development of methods for shortening breeding cycles.

Despite indications that one or more of the strategies can be successfully used to engineer transgene confinement, no single method fulfills the basic requirements for long-term commercial use. Researchers are continuing to determine whether sterility can be complete and stable over several rounds of propagation and growing seasons, successfully identified in juvenile trees, and lack negative growth impacts.

2.6.4 Stature control

In many plants, DELLA-domain proteins, including *GA-INSENSITIVE* (*GAI*), *REPRESSOR OF GAI* (*RGA*), and *RGA*-like 1-3 (*RGL1-3*) have been shown to regulate GA responses. Through the use of transgenesis, Busov *et al.* (2006) demonstrated that DELLA-less versions of *GAI* and *RGL1* led to dramatic shoot dwarfing in *Populus*. This phenotype was associated with reductions in the levels of precursor GAs (e.g., GA_{53,44,19,20}) and dramatic increases in biologically active GAs, such as GA₁ and GA₄. In these same trees, root growth was enhanced twofold to threefold, and adventitious root formation was inhibited. Transgene expression also led to significant changes in the metabolic profiles of both roots and shoots. The observed metabolite changes may cause reduced carbon flux through the lignin biosynthetic pathway, and an increase in the allocation of secondary compounds to storage and defense molecules. These varied responses provide insight into the way in which poplars integrate ecophysiological adaptation. This family of genes could also enable the manipulation of shoot and root architecture and tree metabolism that could affect productivity, flowering, and drought and pest tolerance.

2.6.5 Importance of field tests

Transgenic plants need to be field tested for several reasons. The first is proof of concept—it must be shown that the inserted gene is having the expected effect. *A. tumefaciens* is widely used to insert genes into plant cells, but these insertions occur at random locations within the genome. The DNA surrounding the site of insertion influences the efficiency with which the transgene is expressed (i.e., “positional effects”). In addition, transgenes can be differentially expressed under various conditions (Walter *et al.*, 1992; Neumann *et al.*, 1997; Köhne *et al.*, 1998; Scorza *et al.*, 2001). Thus, it is important to screen all independent lines to verify that the transgene is being expressed at commercially useful levels. It may also be necessary to conduct field trials to determine if there is sufficient value so that end-users are willing to pay a biotechnology premium (i.e., licensing fee) for use of a GM tree. Finally, long-term field

studies are needed to detect somaclonal variations and assess the stability of transgene expression (Meilan *et al.*, 2002a, 2004a), and the ecological implications of deploying transgenic trees across the landscape (Farnum *et al.*, 2007.). The latter will be essential for gaining public and regulator acceptance of tree biotechnology.

3. FUTURE ROAD MAP

In 2006, the poplar research community achieved a very important milestone for the entire field of biotechnology. In this year, the sequencing of the first tree genome was published, adding it to the short list of higher plant genomes that have been fully sequenced, namely, *Arabidopsis* and rice (Tuskan *et al.*, 2006). The genome of a *P. trichocarpa* tree was completely sequenced six times over and this sequence has now been released for public use. Many previously unavailable avenues will now be open for tree researchers. Since we now know that ~45 000 genes are present in the poplar genome, aggressive, genome-wide functional genomics experiments will hinge on the availability of successful transformation protocols. Availability of a whole genome microarray chip, such as those produced for other species by genomics companies like Affymetrix and NimbleGen, will allow researchers to explore the transcriptional road map. This will allow a better understanding of various biological processes in trees (e.g., Mellerowicz *et al.*, 2001) that so far have not been amenable to such experimentation. The poplar research community will also benefit from the availability of these new tools that allow us to pose and resolve previously unanswerable questions. Integration of active proteomics, metabolomics, and phenomics projects will be necessary to interconnect available information and gain a system-level understanding of how trees develop, grow, and respond to the environment (Tsai *et al.*, 2006). Poplars will become a leader in such experiments. Recently, poplars have provided excellent “proof-of-concept” system for employing transgenics toward improving economically important and environmentally beneficial traits in other tree species. These discoveries have laid the foundation for field testing of transgenic trees by universities and forest-products industries

(e.g., Frankenhuyzen and Beardmore, 2004; Nehra *et al.*, 2005). We can predict only a bright future for all poplar research in the years to come.

For achieving such a far-reaching impact, we need to address some road blocks that may stymie future developments. Not all poplar species and genotypes are yet amenable to efficient transformation and regeneration procedures. Special attention should be given to those genotypes that are of commercial importance. It is well known that most of the economically important genetic traits are controlled by multiple genes and so single-gene transformation may not be sufficient. Better protocols for multiple gene transfer must be established and applied. Some success in this has been reported in poplars (e.g., Li *et al.*, 2003) by using co-cultivation methods, but these techniques are ineffective for genes with transcripts greater than 3 kb. Rather than using constitutive expression of genes under the 35S promoter, availability of inducible promoters that regulate expression of genes in a tissue-specific and temporal-manner will be essential for the successful transfer of lab results to field trials (e.g., Filichkin *et al.*, 2006a). Due to a genome duplication event, multiple copies of the same gene may exist in the poplar genome (Tuskan *et al.*, 2006). For example, we have observed that multiple copies of secondary wall-associated cellulose synthase genes are transcriptionally active during wood development. One copy of the gene, however, expressed at a much higher level than the other in most cases. This situation will complicate the functional genomics assays and the application of techniques such as RNAi, which have the potential to silence all copies of a given gene from the poplar genome. Generally, a combination of multiple transcription factors regulates expression of various genes in eukaryotes. Functional dissection of the role of each transcription factor from the poplar genome in biologically and economically important processes is an enormously important undertaking. Genomics advancements such as ChIP-on-Chip will greatly help to further such objectives (Thibaud-Nissen *et al.*, 2006).

A systematically conducted field trial is always necessary for examining the cause–effect relationship between the introduced gene and the phenotypic changes associated with *Agrobacterium*-mediated transformation. However, in countries such as Japan, regulatory authorities require

an extensive risk assessment evaluation before permission for a field trial is given. There is also increasing public concern about the presence of the antibiotic resistance genes commonly used as selectable markers during transformation. Since trees are long-lived species, this aspect is much more important for them than for the crop plants. Moreover, people view trees as a more natural and pristine resource than the feedstocks and crops used to sustain human populations. Lastly, in order to stack more than one gene in any single transgenic tree, we will need to use a variety of selection markers, possibly combining herbicide and antibiotic resistance traits. Ebinuma *et al.* (1997) developed a highly efficient vector system called MAT (multi-auto-transformation) that assists in removal of marker genes and increases the regeneration frequency of transgenic plants without using antibiotic selection. The MAT vector system uses natural oncogenes of *Agrobacterium* such as *ipt* (isopentyl transferase) and *indoleacetamide hydrolase and tryptophan monooxygenase (iaaM/H)*, thus making them hormone independent. This system also possesses the site-specific recombination system, including a recombinase (R) and defined recognition sites (RS). The oncogenes assist in regenerating transgenic plants on hormone-free media but are later removed by the induction of the R/RS system, thus producing marker-free transgenic plants. By manipulating the promoters that regulate the expression of the oncogenes and the R gene, transgenics have been produced for several economically important plant species (Ebinuma *et al.*, 2001), such as hybrid aspen. Examining the stability of introduced genes in transgenic trees is another important issue for tree biotechnologists (Kumar and Fladung, 2004). Insertion of multiple copies of the same gene at the same site in a transgenic plant has been observed to lead to transgenes silencing. Efficient methods for targeted insertion of transgenes at an appropriate position in the genome, therefore, must be developed.

Recently, Professor Steve Strauss co-authored several research articles and reviews concerning the potential utility of forest biotechnology and the need for additional field trials (e.g., Strauss, 2004a, b; Strauss *et al.*, 2004). About 800 poplar trees, including some transgenic trees, were destroyed

or vandalized in their experimental plots in 2001 (Kaiser, 2001). Such attacks jeopardize the basic tenets of science and prevent properly addressing, in a rational manner, the safety and usefulness of GMOs. Vandalism not only demoralizes researchers and deters them from performing important experiments, but is also counter-productive to our shared goal of logically and openly assessing the potential risks of genetically engineered trees.

In the US, the DOE has chosen poplars as a superior short-rotation woody tree in the production of bioethanol (Dinus *et al.*, 2001; Ragauskas *et al.*, 2006). A majority of the carbon in terrestrial ecosystems is sequestered in trees and, given efficient methods can be developed for conversion, can be used for the production of ethanol. Again, genetic improvement of cellulose and lignin production in trees, can, thus have a tremendous impact by producing cellulose with completely altered structural properties. The degree of polymerization, crystallinity, and quantities of cellulose will affect the usefulness efficiency of this process (Joshi *et al.*, 2004). This will require the identification of the poplar genes involved in hemicellulose production, which is a major component of dry wood. Previously identified transcription factors by Mendel Biotechnology that, when modified, produce larger plants in *Arabidopsis* provides optimism for identification of such genes in poplar. The list of such novel targets is always ever-increasing: (<http://www.mendelbio.com/products/yield.html>).

In conclusion, an intelligent combination of modern molecular biological tools and improvement of transformation efficiency in poplars will open many new avenues in tree biotechnology and substantially improve ecologically sound forest tree production.

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Eucalyptus

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1. INTRODUCTION

In the last century, the genus *Eucalypts* became an important multipurpose source of timber in many industrial applications for production of pulp and paper, charcoal, energy, furniture, and housing. The genus *Eucalyptus* is a member of the Myrtaceae family, mainly originated from Australia, comprising of more than 700 species (Brooker, 2000). The trees form tall open forests, woodlands, and occur in environments ranging from areas of high rainfall to semi-arid regions, and from sea level to subalpine altitudes. A few species have been described as occurring outside the Australian territory (Eldridge *et al.*, 1994); *Eucalyptus deglupta* is endemic to the Indonesian islands of Sulawesi and Ceram, Mindanao in the southern Philippines, northern New Guinea, and New Britain; *Eucalyptus urophylla*, *E. orophila*,

and *E. wetarensis* occur in Timor and adjoining islands of the Lesser Sunda group (Pryor *et al.*, 1995). Several factors contributed to the success of *Eucalypts*; the fast growth rate and large biomass production, the ability to grow in a wide range of environments and soils, good wood quality for solid wood products and short cellulose fiber, suitable for pulp production, particularly for paper and tissue. A considerable number of *Eucalyptus* plantations were established in the early 1900s in Brazil and South Africa along the railway tracks providing charcoal for the locomotives. Today, several countries in Asia, South America, southern Europe, and Africa, have an estimated area of planted forest around 16–19 million hectares (FAO, 2000). The predominance species in commercial plantations (around 80%) are *Eucalyptus grandis*, *E. globulus*, *E. camaldulensis*, and their hybrids (Potts, 2004). *E. grandis*,

E. urophylla, and their hybrids are mainly planted in tropical and subtropical regions, while *E. globulus* is preferred in temperate climates (Potts, 2004).

One important factor determining the expansion of large areas of commercial *Eucalyptus* plantations was the development of cloning techniques, initiated in 1975 in the Popular Republic of Congo (Delwaulle *et al.*, 1983). Outstanding genotypes, usually hybrids, having large and straight trunk with good wood properties were selected to make rooted cuttings and planted in large extensions of land. Vegetative propagation has been intensively used by the pulp and paper industry, producing highly uniform timber and allowing further gains in productivity in the pulping process. In the mid 1970s the development of tissue culture techniques and *in vitro* propagation of *Eucalyptus* spp. provided new opportunities for mass propagation, on a commercial scale, of selected genotypes. At the same time, the growing importance of *Eucalyptus* for the pulp and paper industry, particularly in South America and Africa, led to the establishment of breeding programs selecting better hybrids for cloning and to improve the basic populations introduced from Australia.

In the last 10 years, the advances in tissue culture techniques, plant regeneration, and genetic transformation, using mainly the *Agrobacterium* system, allowed the development of the first transgenic *Eucalyptus* trees. Also, advances in the use of molecular markers have played an important role in helping breeders to select *Eucalyptus* trees with better wood quality, disease resistance, and stress tolerance. More recently, the development of genomic approaches, such as major expressed sequence tag (EST) sequencing programs, have increased the interest in the application of biotechnological tools in order to produce better *Eucalyptus* trees.

Our chapter provides an overview of the main topics involved in *Eucalyptus* biotechnology, from cloning and hybrids production, breeding, application of molecular markers, tissue culture and plant regeneration, genetic transformation, main diseases affecting the *Eucalyptus* plantations, wood quality for the pulp and paper industry, energy production and biomaterials, and also the identification of genes involved in disease resistance and wood formation through functional genomics.

1.1 Hybridization, Cloning, and Breeding

1.1.1 Hybridization and cloning of *Eucalyptus* spp.: evolution and its importance for the forest sector in Brazil

The development of forestry plantations for industrial purposes must be oriented to increasing industrial competitiveness in the distinct market segments. In such a scenario, forestry-based companies must take into account the influence that forestry raw material can have on their competitive capacity. The modern concept of competitiveness includes generating products to meet the customer's requirements at low costs, in a sustainable manner and with minimum environmental impact. Therefore, the development of tree breeding programs to obtain quick gains, and a well-established cloning system have become important. Vegetative propagation methods should rapidly transform the genetic gains, obtained through breeding, into benefits for the industry. One of the most efficient tools to acquire these objectives is the combination of interspecific hybridization and establishment of clonal forestry derived from superior hybrid individuals (Assis, 2000).

In this context, hybridization can have a great impact on tree breeding programs, combining superior wood characteristics with tolerance to biotic and abiotic stresses, thus representing a significant source of superior individuals, capable of introducing genetic gains into forest productivity and wood properties. Crossing species with different characteristics allows the production of complementary wood properties in trees to meet special industrial requirements.

The effective and quick integration of genetic gains obtained with hybrids into the industrial process depends basically on the existence of functional large-scale cloning systems. Mass vegetative propagation perfectly complements hybridization for producing clonal forestry and has some advantages over the sexual methods of mass reproduction of selected families, besides being the best way to commercially exploit the heterosis found in several *Eucalyptus* hybrid crosses. By capturing the total genetic variance (Zobel, 1992), vegetative propagation allows for maximum benefits from wood properties and

productivity, besides allowing for the production of more uniform raw material, which from an industrial point of view is highly beneficial to the industrial process and product quality. Therefore, tree breeding programs that focus on these aspects of the forest industry will have a great impact on the three important components of the competitive process: productivity, product quality, and production costs (Assis, 2001).

Recognizing the importance of hybridization and cloning in the context of industrial production has led to a rapid evolution of the techniques and processes that constitute these activities. As a result, methods to produce hybrid seeds and commercial cloning are now well understood and highly effective from the technical and operational perspective in *Eucalyptus* species. Developing the concept of indoor breeding orchards and the creation of artificially induced protogyny (AIP) has made controlled crosses operationally feasible. Hybrid production by controlled pollination is thus a simple and functional process. Indoor breeding orchards do not require flower isolation, saving a lot of time. On the other hand, AIP enables pollination without the need to emasculate the flowers. These two technologies allow large-scale controlled crossing, which is technically difficult to carry out and also economically unfeasible using traditional methods.

In the cloning systems for commercial-scale propagation, the development of microcutting and mini-cutting to replace rooting stem cuttings led to the development of super-intensive systems to produce vegetative propagules, which can now be done in much more controlled environments, allowing greater physiological and phytosanitary control. The use of more appropriate propagation facilities, with better environmental control, has contributed to improve rooting rates. The development of these technologies for *Eucalyptus* marked the beginning of a new cycle in the commercial cloning of vegetatively propagated plants, especially for woody species (Assis, 2001).

In the last two decades, cloning *Eucalyptus* spp. has produced relevant progress for the forestry companies, especially solving problems associated with diseases such as canker (*Cryphonectria cubensis*) and productivity improvement (Campinhos and Ikemori, 1983). Currently, the focus on cloning has shifted to industrial requirements, rather than remaining limited to disease resistance and

increased volume. Wood properties that positively influence industrial processes and product quality are considered, especially where cloning has an important role to play.

1.1.1.1 *Historical and technical evolution of Eucalyptus hybridization and cloning in Brazil*

Hybridization Considering the length of forest production cycles, the commercial use of *Eucalyptus* hybrids is relatively recent in Brazil. Spontaneous hybrids randomly formed at the tree farm in Rio Claro, São Paulo, at the Companhia Paulista de Estradas de Ferro, provided the base for the first plantations for industrial purposes. These hybrids highlighted aspects of resistance to Eucalypt canker (Ferreira, 1997; Alfenas *et al.*, 2004) and to the presence of heterosis at the individual level during the 1970s. Therefore, these hybrids were also the starting point for the introduction of the concept of clonal forestry in Brazil, during the same decade (Campinhos and Ikemori, 1980). In the 1980s, when the importance of hybrids for industry was recognized, hybridization became an essential tool, helping to create a strong and competitive forestry industry (Assis and Mafia, 2007).

The first synthetic *Eucalyptus* hybrids were produced in the 1970s by open-pollination between compatible species (Assis, 1985; Gomes, 1987; Martins and Ikemori, 1987). Since this technique was limited by crosses between species that flowered at the same time, in the 1980s a controlled crossing method was developed in South Africa (Hodgson, 1967; Van Wick, 1977). Although it was difficult to perform, it enabled the expansion of hybrids produced artificially in Brazil (Brigatti *et al.*, 1983; Assis, 1985; Martins and Ikemori, 1987). This method is based on the exploitation of protandry where pollen reaches physiological maturity in anthesis, but the stigma is not receptive for another few days. To make controlled crosses using this technique, the flowers must be emasculated, isolated, and pollinated after 5–7 days. Therefore, the method has a low operational yield, mainly because of the number of visits needed to make the crosses (Harbard *et al.*, 2000). Moreover, relatively high losses are caused by injury to the flower buds during emasculation and

isolation, contributing to reduce the efficiency of the crossings.

In 1996, a new technique for controlled crossing was developed, called AIP. This new technique was initially developed at Riocell S.A. (Assis and Jardim, unpublished results) and its great advantage is higher operational yield and better use of the flower buds. This technique was conceived based on two discoveries made in Chile and in Portugal. OSP (one stop pollination) was developed in Chile (Harbard *et al.*, 2000), allowing receptiveness induced by cutting lengthwise or cutting off the upper third of the pistil of emasculated flowers. In Portugal, it was found that this receptiveness could be achieved before anthesis (Trindade *et al.*, 2001). AIP was developed by combining these two discoveries. This technique consists of the artificial transformation of protandry into protogyny, by cutting the top of the floral bud operculum. At the same time, the upper third of the pistil is cut during the preanthesis stage, i.e., when the flower is still closed, and pollen is applied immediately after induction. Thus, there is no need to emasculate the flowers to make crosses.

Later, this new technique was tested simultaneously in Brazil, at Aracruz Guaíba, and at Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia (Assis *et al.*, 2005) with the same success rate observed at both sites. Besides reducing the number of visits, AIP does away with the need for emasculation and made it possible to obtain more seeds in *Eucalyptus*-controlled crosses. Operational productivity rose from 35 to 400 flowers per man/hour using this technique.

At the same time as AIP was created, the concept of indoor breeding orchard was developed, in screened houses, doing away with the need to isolate individual flowers, umbels or branches, enabling the isolation of whole plants in a collective way (Assis *et al.*, 2005). When some type of contamination is acceptable, this technique can be implemented in open areas that are not isolated. Currently it is the most widely used method for *Eucalyptus*-controlled crosses. The development of indoor breeding orchards was enabled by the development of technologies to induce early flowering in potted plants. Flower production in small plants is necessary to cultivate them within greenhouses and also to allow easier access to the

floral buds. In order to obtain flowered plants in pots, grafting of physiologically adult branches is used combined with application of the flowering inductor paclobutrazol.

The combined use of these technologies (AIP and indoor breeding orchards) allows large-scale controlled crosses and enables the multiplication of highly superior full-sib families, a task formerly considered technically and economically impossible. This is very important to improve difficult-to-root species, since the superiority of pure or hybrid full-sib families can be captured and multiplied on a large scale. This technique is widely disseminated in forestry companies and now accounts for most controlled crosses performed (Assis and Mafia, 2007).

Cloning techniques The first rooted eucalyptus cutting was obtained in Australia in the 1940s (Eldridge *et al.*, 1993). During the next decade, studies carried out by French researchers in the Congo, Tunisia, and Morocco played an important role in understanding physiological phenomena involved in the rooting of *Eucalyptus*, such as juvenility and maturation, as well as aspects of rejuvenation in *Eucalyptus* and their importance in cloning adult trees. This knowledge was important to establish the concept of *Eucalyptus* clonal forests, which occurred in the 1970s in Tunisia (Chaperon, 1987) and in Aracruz in Brazil (Campinhos and Ikemori, 1980). However, the first large-scale cloning system was established in Brazil, by Aracruz, and it served as a model for the development of clonal forests, whose use was extended to other Brazilian companies and to several parts of the world. After the success achieved by Aracruz, it was quickly disseminated, and now it is the main system for the reproduction of *Eucalyptus* species and clones.

Until the 1990s, the development of cloning techniques for *Eucalyptus* species evolved relatively slowly. For over a decade, the commercial production of vegetative propagules continued to be done by the original system, in clonal banks, before being replaced by the clonal hedges system. On the other hand, the techniques initially used for the stem cutting rooting of *Eucalyptus*, as well as the rooting facilities, remained practically the same until the early 1990s. In 1992, the first great change in the rooting technique occurred when microcutting (Assis *et al.*, 1992; Xavier and

Comério, 1996) and later mini-cutting were created (Assis, 1996; Higashi *et al.*, 2000).

Microcutting is a rooting method where the propagules are obtained from shoot apices originating from micropropagated plants and in mini-cutting originating from auxiliary sprouts of plants cloned by stem cuttings. After rooting of the first shoot, the two techniques are identical, varying only in the origin of the initial propagule source. In some clones using mini-cutting, some propagation cycles (serial propagation) are required to reactivate before acquiring full rooting capacity potential. In microcuttings such propagation cycles come naturally by monthly *in vitro* subculturing of explants. Micropropagation is unnecessary for easy-to-root species because high levels of juvenility can be obtained easily by inducing basal shoots, therefore in such cases, mini-cutting is technically and economically feasible.

When plants are established in the mini-clonal hedges to form the mini-stumps by whatever means, multiplication is performed using the mini-cutting technique (Assis and Mafia, 2007). Microcutting and mini-cutting are the most modern concepts for cloning *Eucalyptus* species in large scale and they are currently the most widely used techniques in Brazil. The creation of these technologies was the watershed in the evolution of *Eucalyptus* cloning systems and they resulted in profound changes in the main technical segments that constitute this activity. It marked the beginning of a new cycle in the propagation of woody species.

A major consequence of creating microcutting and mini-cutting was the great change in the methods to produce vegetative propagules in large scale. The clonal hedges that had replaced the original clonal banks system at the beginning of the 1980s were replaced by the mini-clonal hedges. Propagule production began to be performed in a super-intensive manner, supported by hydroponic systems in controlled environments (indoor mini-clonal hedges). Currently, two hydroponic systems are used: sand bed with drip irrigation (Higashi *et al.*, 2000) and intermittent flooding (Campinhos *et al.*, 2000).

The development of these technologies also influenced changes in the conception of rooting facilities that were important for their evolution and improvement. When *Eucalyptus* cloning

attained industrial proportions, most of the physical structures used to root stem cuttings were extremely simple, consisting of partial shading and a misting system. When the mini-cutting technology began to be used, greenhouses had to be used to achieve greater control over the rooting environment. Their importance in rooting became clear and led to their widespread use. Therefore, at the same time as rooting methods were developed, a major evolution occurred in the propagation structures. On the other hand, the development of super-intensive vegetative propagule production systems in hydroponic mini-clonal hedges, with better nutritional and phytosanitary control of the mother plants, is technically and economically advantageous in the commercial production of *Eucalyptus* plantlets. Currently, mini-cutting is the cloning technique most widely used by the large Brazilian forestry companies.

In easy-to-root hybrid clones, micropropagation is not commonly used as a rejuvenation method or simply as a method to rapidly broaden the clonal base of recently selected clones. Except in the systems that use microcutting, where it is a mandatory phase, mini-cutting from sprouts of rooted stem cuttings has worked well for these species and the tendency is that micropropagation will no longer be used for this purpose, especially due to its high costs. Nevertheless, there are technical advantages in using micropropagated plants to form mini-clonal hedges, even in easy-to-root clones. In clones that are difficult to root, micropropagation is still a technique that can potentially increase predisposition to rooting. This class includes *E. globulus* hybrid clones that are increasingly used in plantations for pulp production.

1.1.1.2 Importance of hybridization and cloning in Brazil

In recent years, the growing integration between the different segments of industrial production based on planting forests has been a major factor in the development of technologies dealing with forest production for industrial purposes. The greater proximity between forestry, industry, and commerce has enabled an integrated view of the segments: forest × industrial process × product quality, which is the base for acquiring

competitive differentials in industries. In this way, new prospects arose in the development of technologies that could promote a positive impact on the industrial production chain and, consequently, on industry performance. On the other hand, the perception of the great economic gains by industry as a result of the increased quality of raw materials has significantly enhanced the genetic improvement programs, above all due to their capacity to promote quantitative and qualitative changes in the raw material.

In this context, increasing forest productivity and improvement of wood properties for industrial use are important demands on Eucalyptus breeding programs in Brazil. The main objectives of these demands are the reduction of operational costs, improving raw material performance in industrial processing, and rendering it appropriate for the manufacture of high quality products for different market segments (Assis, 2000). The main contribution of genetic improvement for the forestry-based industry is the generation of superior individuals that can lead to gains in forest productivity, industrial process, and product quality. The greatest challenges for tree breeders have been to use effective strategies to obtain individuals that are superior in both growth and wood quality. The main obstacles to overcome these challenges are that the species traditionally planted in Brazil present certain limitations concerning the wood quality, compared with other *Eucalyptus* species. In this context, interspecific hybridization is a very useful tool, especially since it allows transfer of genes from species with high wood quality but slow growth, to species that have an inferior wood quality but are well adapted to the planting sites and with excellent growth. The main advantage of hybridization, besides the capacity to allow the combination of differentiated characteristics in distinct species, is the possibility of producing trees with superior growth as a result of heterosis or hybrid vigor, a common phenomenon in Eucalyptus (Martin, 1989; Denison and Kietzka, 1992; Nikles, 1992).

The differences between the various species of Eucalyptus adapted to this country, with respect to wood properties, tolerance to biotic and abiotic stresses, as well as to the manifestation of heterosis has been the main factor to produce individuals with superior growth, adaptation, and wood quality, through hybridization. Thus, the

shortest path between industrial demands and their fulfillment by genetic improvement programs is to seek complementarity among species, by increasing the number of desirable characteristics of the wood, combined with the commercial utilization of heterosis. Enhancement of the forest quality by the commercial use of heterosis, through the vegetative propagation of Eucalyptus hybrids in Brazil is clear and there is evidence that it is a very useful phenomenon for a faster gain in forest productivity. In this sense, the contribution of Eucalyptus hybridization to forest development in Brazil is very significant. Most benefits from hybrid use, especially in forest productivity, are accredited to the manifestation of heterosis for growth and to the complementarity of certain species concerning characteristics that combine to produce more appropriate genotypes for the different planting environments (Assis, 2000).

Currently, the vast majority of Eucalyptus improvement programs in Brazil are based on hybrids. In 2005, 84.5% of the clonal plantings of Eucalyptus in Brazil were hybrids, where 66.5% of which were hybrids from controlled crosses. The hybrid *E. grandis* × *E. urophylla* is most often used, with 65% of the planted area. Fifteen percent of the remainder is still natural and spontaneous *E. urophylla* hybrids, 3% Rio Claro hybrids, 0.5% *E. urophylla* × *E. camaldulensis*, 0.4% *E. urophylla* × *E. globulus* and 0.6% *E. camaldulensis* × *E. grandis* (Assis, 2004).

Wood properties are very important for industry because they have an impact on processing costs, production gains, and the technological qualification of the products and are therefore essential in production processes of raw material for industrial use. In this sense, hybridization tends to play an increasingly important role in the Eucalyptus planting programs in Brazil. Because of the success achieved in southern Brazil concerning the incorporation of *E. globulus* genes into site-adapted species and clones, mainly due to the significant enhancement of the wood quality to make pulp, there is a tendency to seek the source of wood quality in this crossing, by pulp companies. A major breakthrough is expected in wood quality to make short-fiber pulp using *E. globulus* hybrids.

From the perspective of industrial processes, *E. globulus* is the most appreciated *Eucalyptus* species by the pulp mills, because its wood presents technological properties that are especially important

for the pulping process. Either because it provides greater production gains, or due to the lower manufacturing costs, its wood tends to be a major competitive differential point for the companies. Evaluations performed on *E. globulus* hybrids show that it is possible to maintain the same forest productivity levels obtained in *E. grandis*, *E. saligna*, *E. dunnii*, *E. urophylla* and several of their hybrids, but with positive changes in wood and pulp quality. Compared to the traditional genetic materials, these hybrids allow increasing wood density; reduction of 4% in lignin content; a 3% increase in pulp yield; 3% increase in hemicellulose content; 1 m³ reduction of wood per ton of pulp in specific consumption and a 5 kg reduction of the chlorine dioxide per ton of cellulose in bleaching, among others.

The forests that are being planted using the new genetic materials from this new type of crossing will provide major gains for the industries, significantly improving their competitive capacity on the short-fiber pulp market. Together with the gains in production and cost reduction, the variability found in the selected individuals also allows the obtention of fibers with anatomical characteristics that are appropriate for different market segments, allowing the qualification of pulp for each specific product.

The efficiency of hybridization to produce genetic gains is fully acknowledged, being used by most forestry companies in Brazil. However, since the hybrid offspring are heterogeneous, transforming these gains quickly and effectively into benefits for the industry depends on functional, large-scale cloning methods. Thus, if on the one hand, interspecific hybridization in Eucalyptus has been the fastest way to achieve genetic gains, cloning on the other hand is the most efficient way of incorporate these gains into the industrial production processes. Cloning as a tool for the implementation of clonal forests derived from high-quality hybrids, is still the ideal technical complement to maximize the benefits of hybridization in the context of forest production for industrial purposes. Currently its maximum potential has been achieved in establishing clonal forests derived from controlled interspecific hybrids, which produce better quality wood, greater volume, growth, and higher resistance to biotic and abiotic factors. In this way, cloning plays a major role and for this reason it has received much

attention in forestry research programs in Brazil. The consolidation of cloning as a method for the commercial reproduction of superior Eucalyptus trees had a positive effect on productivity, costs of industrial processes, and product quality. Cloning Eucalyptus in large scale is currently one of the most important factors in promoting increased productivity, improvement, and homogenization of wood technological properties as an industrial raw material (Assis *et al.*, 2004).

Almost 30 years after the introduction of the concept of clonal forests in Brazil, cloning is definitely a part of the raw material production processes used in various sectors of industrial activity, above all pulp and paper and charcoal, which account for 78% and 21% of the area planted with clones, respectively. In 2004, the area planted with clones surpassed 1 000 000 ha. The current annual rate of new clonal planting is over 250 000 ha, with a tendency for further growth.

Brazilian clonal forestry was initially developed from spontaneous hybrids and natural hybrids presenting heterosis for growth, besides resistance to diseases, above all Eucalyptus canker. Although these hybrids were produced empirically, they were responsible for the great breakthrough in forest productivity that occurred in recent years. Forest productivity was increased threefold by cloning them. Currently the clonal forests are derived from individuals generated in genetic improvement programs, whose main strategy is based on the production of controlled interspecific hybrids. The cellulose increment (AMI cell) was around 6 t ha⁻¹ year⁻¹ in the 1970s. This productivity is now around 12 t ha⁻¹ year⁻¹ for the leading companies in this industry. Using *E. globulus* in the composition of hybrids, it is expected that trees planted from 2010 onward will achieve productivities of 16 t ha⁻¹ year⁻¹.

1.1.1.3 Future perspectives

Due to the current importance of hybridization and cloning for industrial forests, their use has been consolidated and they are increasingly becoming part of the forest-based industrial production processes. The progress achieved in the different techniques involving these activities will ensure new levels of quality and constitute new platforms for the development of Brazilian forestry.

The advances that have occurred in controlled pollination techniques, for instance, will allow the intense use of hybrids in breeding programs of the industries that use *Eucalyptus* as raw material. On the other hand, hybrid production will be increasingly oriented to crosses with *E. globulus* and its subspecies, mainly because of the quality of this wood for pulp production. The use of adapted species and clones, combined with this species, has allowed a significant improvement in the wood quality, in environments where the pure species is not well adapted. This will allow the use of *E. globulus* hybrids both in subtropical and tropical areas, with significant gains in wood quality.

Although there has been significant progress in techniques and *Eucalyptus* rooting methods culminating in a robust, functional, well-established system, several studies on cloning are being performed to develop new systems that could be technically and economically more appropriate than the current ones. Propagule production methods evolved from an extensive system, with low propagule production per square meter, passing through an intensive system in clonal gardens until they reached the present super-intensive systems, with high propagule productivity per square meter in the mini-clonal hedges supported by hydroponics. The search for new advances in this field is directly related to establishing production systems for mini-propagules in bioreactors, using minimum space and with a significantly higher propagule production per square meter. A further advantage of these technologies is that they use highly rejuvenated tissues, which may provide better technical results both for plant production and for the quality of the clonal forests.

1.2 Factors Affecting Expected Genetic Gains in Forest Breeding Programs Using Seeds

Genetic improvement has the main objective of obtaining genotypes, through selection, that present adaptations to environmental conditions, resistance to disease and insects, characteristics desirable for productivity, and the multiple uses of wood. Many species of the genera *Eucalyptus* and *Pinus* have been intensely used in reforestation programs in Brazil and many other countries. The importance of *Eucalyptus* seeds of the

best quality, from the best origins and the establishment of test species, seeds and progeny allowed the selection of genetic material with the potential for adaptation and productivity under the edaphoclimatic conditions found in different regions of Brazil.

The beginning of any breeding program is the selection of the species and populations to be used, by species tests and the origin of the desired characteristics (Resende *et al.*, 2005). The strategies for genetic improvement of exotic forest species has been based on the selection of species/origins and the selection of individuals from the base population, exploring the natural genetic variability existing amongst populations and individuals (Ferreira, 1992). Vencovsky and Barriga (1992) concluded that the knowledge of the type of genetic action that predominates in the genetic basis of a character is an important ingredient in an efficient breeding program. Gene effects are classified into two basic types, allelic interactions (dominance interactions between alleles at an interlocus level) and nonallelic (epistatic actions that arise when alleles or genotypes are influenced by the genetic constitution of other loci).

According to Resende *et al.* (2005), based on experimental evaluation, the selection should be based on means and variance, recommending that the selected material should have an elevated mean and ample genetic variability, allowing continuous gains with selection through various generations. A way to estimate the progress of the selection process is very important when using quantitative genetics in plant improvement (Vencovsky and Barriga, 1992). By the general expression of gain (ΔG) " $\Delta G = ds \times h^2$ ", it can be observed that the progress is a direct function of the magnitude of the differential selection (ds = difference between the mean of the selected group and the mean of the original population) and of the heritability coefficient (h^2) (Wright, 1976; Zobel and Talbert, 1984). The expected progress varies in accordance with the selection scheme and is detailed by Shelbourne (1969), Vencovsky (1978), Vencovsky and Barriga (1992), and Cruz and Regazzi (2001).

Ferreira (1985) stated that the gains obtained depend on the standard used for comparison (selected material or not), the effect of age on the evaluation, and environmental variation at the

sites where the progeny were tested. Sometimes the expected genetic gain at a determined phase of the improvement program cannot be achieved. Factors related to the management of the improved populations, factors related to the production of seeds and saplings, genotype \times environment interaction and others, can contribute negatively and these factors are discussed individually below.

1.2.1 Selection of genetic material

The selection based on indices has been used in forest improvement, offering larger gains when compared to other selection methods (Cotterill, 1986). The utilization of selection indices in *Eucalyptus* spp. improvement requires the estimation of a series of parameters, indices, and progress with selection, to permit the breeder to choose an index adequate for each situation (Resende *et al.*, 1990). Resende *et al.* (2005) states that selection methods applicable to the perennial species can be classified in accordance with the selection units and the procedure used to predict the genetic values. According to the author, the procedure currently used to estimate the variation components and predict the genetic values is AM-BLUP (animal model, evaluation of genetic variation) associated with DFREML (derivative free maximum likelihood).

Oda *et al.* (1989) described the problems in classic genetic improvement with *Eucalyptus* due to high selection intensity, affirming that the risks of intense individual selection in genetic improvement can be reduced if the species used are pure and adapted, the matrices were selected under normal environmental condition (without stress), the size of the effective population is maintained high, and used for short-term programs. In asexual improvement, the authors suggest that better results could be obtained if hybrid species are used, the matrices were selected under stress, heterosis could eventually appear, and high intensity selection was used. The authors present results from experiments with *E. grandis* obtained under high selection intensities. A total of 51 selected trees, as well as the half-sibling progeny, were evaluated with respect to cloning. The selection of the trees was made using populations originally from Coff's Harbour (NSW, Austrália), under the intensity of 1:5000 and populations from

Zimbabwe, at 1:2000. Based on the evaluations of the materials from the different experiments, the following relationships between the characters were established:

- (a) Relationship between the flowering period, the typical qualities of the trees, and the behavior of the respective progenies (material from Coff's Harbour). It was observed that the same trees that originated from inferior progenies presented early flowering and atypical fruits, demonstrating the occurrence of hybrids in the selected material and a loss in productivity due to the heterogeneity in the trees.
- (b) Correlation of the clonal behavior with the behavior of the progenies using diameter at-breast-height (DBH) and height data and comparisons with the inheritance estimates of the progenies (material from Zimbabwe). A clone-progeny correlation for DBH ($r = 0.020$) was very weak (Figure 1) and low for height ($r = 0.33$) (Figure 2), this shows that clones with good performance do not always present good progenies. Gene dominance could favor the selection of heterozygotic trees and heterogenic descendents with low productivity.

This study shows that intense selection can reduce the effective size of the population, leading to the presence of different degrees of endogamy, demonstrating the loss of vigor in the saplings originating for endogamous crosses. In relation to the concept of the effective population size, Vencovsky (1987) discussed the genetic representativity contained within a sample in relation to immediately previous generation. Thus, the effective size is related to the genetic size of the population and not the number of individuals within the population (Resende and Vencovsky, 1990).

1.2.2 Correlations and correlated responses between characteristics

The study of the correlations between characteristics is important in genetic improvement, because the general preoccupation is to improve the material, not only for isolated characteristics but simultaneously for a group of characteristics (Vencovsky, 1978). According to the author,

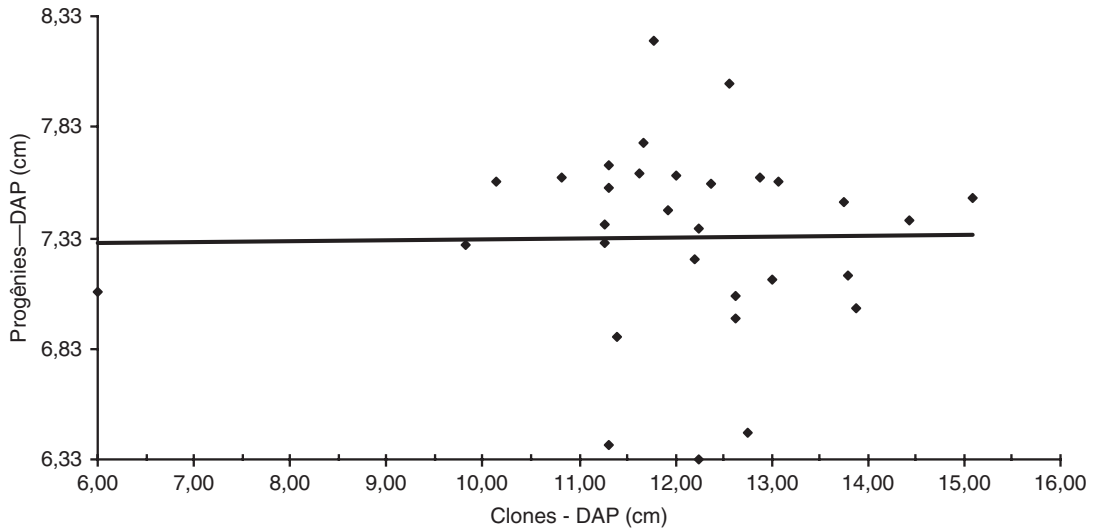


Figure 1 Correlation between the DBH values (cm) for progenies (2 years) × clones (3.5 years) [Source: Oda *et al.*, 1989]

the expected alteration in the mean of a determined characteristic Y, when the selection is for characteristic X is a response correlated with the system of selection considered. The correlation visualized directly in an experiment is phenotypic in nature, provoked by two factors: genetic and environmental (Vencovsky and Barriga, 1992). In genetic improvement it is important to know the association between characteristics, especially when the selection of one of them presents

difficulties, such as low heritability or in the measurement or identification (Cruz and Regazzi, 2001). According to the authors, in genetic studies it is important to differentiate and quantify the degree of genetic and environmental association between the characteristics, such that the genetic correlations are of a heritable nature and as such usable in breeding programs.

Cruz and Regazzi (2001) discussing simultaneous selection of characteristics stated that the

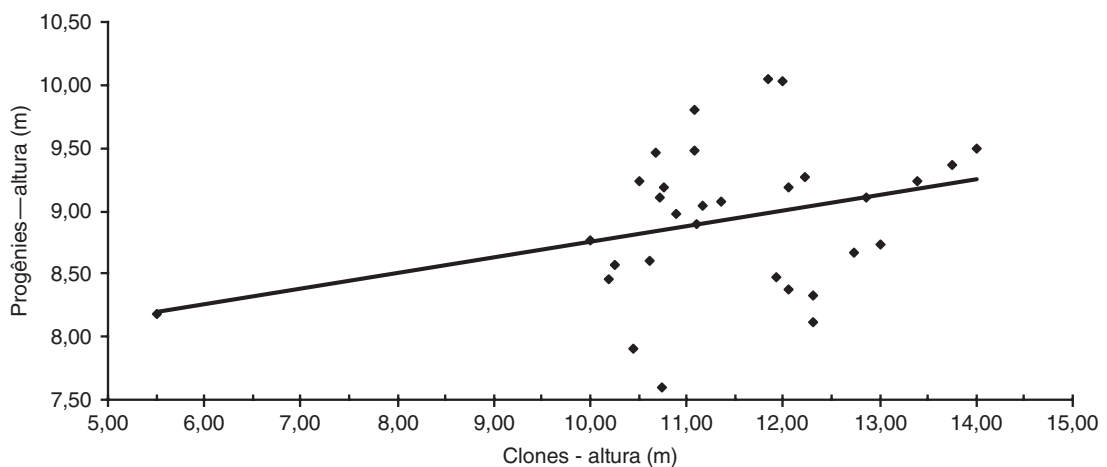


Figure 2 Correlation between the height (m) for progenies (2 years) × clones (3.5 years) [Source: Oda *et al.*, 1989]

use of the index theory of selection is an efficient alternative, permitting the combination of multiple information contained within the experimental unit and making possible selection based on a number of variables that unite various attributes of economic importance. The authors reviewed various proposals to obtain selection indices (classic index proposed by Smith, 1936 and Hazel, 1943; index based on desired gains; base index; index based on the sum the ranks; “weight free” and “parameter free” index).

In *Pinus radiata*, Dean *et al.* (1983) studied 30 families of half-siblings and detected negative correlations between volume and wood basic density. The gain estimates for these characteristics, combined into one index, revealed that it is not possible to improve wood density and volume simultaneously. Moraes (1987) detected a low negative genetic correlation between growth characteristics and wood basic density in open-pollinated *E. grandis* progeny. However, this correlation was not sufficient to seriously weaken the possibility of gains in wood basic density.

Reviewing other genetic and phenotypic correlations between wood basic density and growth characteristics, Moraes (1987) observed the existence of high correlations between height, DBH, and volume, however a correlation between these characteristics and basic wood density was of different magnitudes, both positive and negative. For Sturion (1993) these contrasting results demonstrate that generalization could provoke losses in a selection process, depending on the adopted strategy.

1.2.3 Endogamy

According to Wright (1976), endogamy signifies the crossing of related individuals or descendents of the same individual. The most extreme case of endogamy is self-fertilization; however, there are various degrees of endogamy before this, such as crossing between siblings and members of small isolated populations. The author further states that endogamy does not always cause a reduction in vigor or depression by endogamy, which is caused by the accumulation of recessive deleterious genes and not solely because of self-fertilization.

Eldridge (1978), working with the proportion of self-fertilization and open-crossing in Eucalypts,

confirmed predominance for open-crosses, although this proportion differed between species. Reddy and Rockwood (1989) reported that the quantity of self-fertilization in Eucalypts is higher than 7%. Estimates for the degree of natural self-fertilization vary among the species of *Eucalyptus*: 24% in *E. obliqua* (Brown *et al.*, 1975), 18% in *E. stoatei* (Hopper and Moran, 1981) and 10–38% in *E. grandis* (Hodgson, 1976a).

Hodgson (1976b) studied some aspects of flowering and the reproductive system in *E. grandis* and observed that progenies from self-fertilization were subject to various forms of endogamic depression, including deformation of the leaves and stem, reduction in vigor in comparison with progenies from open-crosses. The author concluded that the product of a seed producing plantation suffers a degree of reduction because of the quantity of self-fertilization, estimated at 30% (Van Wyk, 1981).

Various studies were carried out in order to verify the effects of different degrees of endogamy in Eucalypts, such as Van Wyk (1981), Eldridge and Griffin (1983), Maêda (1987), Griffin and Cotterill (1988), and Hardner and Potts (1995). Eldridge (1976) stated that endogamic depression in Eucalyptus is expressed as low growth of the saplings in the field and low viability of their seeds. Eldridge and Griffin (1983) working with *Eucalyptus regnans*, observed that the number of seeds varied from 42 per 100 in self-fertilized to 71 seeds in open-pollinated and 90 seeds in cross-pollinated flowers.

Irregular flowering and a deficiency in pollination are factors that can provoke endogamy. The presence of endogamy causes various prejudicial effects in Eucalypts, including deformed leaves and stems, reduction in vigor of the saplings, reduced height, reduced survival of saplings in the field, and reduction in the production of viable seeds.

1.2.4 Progeny tests

Individuals selected using an estimation of genetic parameters are then analyzed using progeny tests to ascertain their reproductive value as seed producers and later used to form seed producing plantations (Kageyama, 1980). Shimizu *et al.* (1982) presented the procedures and

recommendations for the installation of progeny tests.

The best way to evaluate the genotypic value of the selected trees is through their progenies, permitting the estimation of their value as progenitors (Weir, 1977). This procedure permits the separation of trees with phenotypic superiority caused by the good planting conditions from those that present genotypic superiority (Allard, 1971; Weir, 1977). Hodge *et al.* (1996) compared the genetic parameters of progenies by open-pollination and controlled pollination, using *E. globulus* and *E. nitens*, and evaluated using two types of populations resulting from open-pollination (native population and a seed producing plantation) and two types of populations resulting from controlled pollination (using families of siblings from self-pollination and pollination between individuals). For *E. nitens* two populations were evaluated, families from the seed producing plantation using open-pollination and self-pollinated sibling families. The progeny tests were established at various sites and after 2 years height, DBH, and cylindrical volume were measured. The authors observed that early estimates of heritability for the open-pollinated progenies derived from native populations could be exaggerated, probably due to the high degree of endogamous depression. It was concluded that genetic gain estimates of open-pollinated populations can be overestimated because of (1) inflated heritability estimates and (2) flaws in the evaluation genotype \times environment interaction.

One point to be discussed during the installation of the progeny test is sapling preparation. Generally the selection of saplings is made in order to make the forest more productive and homogenous. The appearance of abnormal saplings could be linked to endogamous effects; however Nogueira (2005) observed that a controlled cross carried out using two unrelated *E. grandis* trees, resulted in progeny with a 3:1 proportion of normal and abnormal individuals, respectively. The study identified two random amplified polymorphic DNA (RAPD) markers linked to the principal gene that causes the abnormal phenotype.

The preparation of saplings for the installation of progeny tests for *E. grandis* was studied by Mello *et al.* (1993). The authors studied the influence of two systems for the utilization of seeds on the behavior of 14 progenies and three controls, using

two methods of sapling preparation: (a) seeded directly into tubes, thinned and selected and (b) seeded into a germinator, planted and utilize all the seedlings. At 2.7 years of age, the results indicated that significant differences exist between the two systems. The progenies presented superior DBH, basal area, and cylindrical volume when submitted to sapling selection in the nursery. It was also observed that there was a significant interaction between the progenies and the sapling production system.

1.2.5 Seed production

When the seed production plantation is established, it is expected that the seeds produced represent the desired crosses, and it is important that the pollination is effective between the genetic material present in the plantation. Mora *et al.* (1981) reviewed the aspects of seed production in forest species, citing various authors who emphasize that abundant flowering and the production of good quality seeds depend on various factors related to the processes of pollination, pollen germination, pollen tube growth, fertilization, and embryo development. Thus, the success of the plantation depends, among other things, on the quality of the pollen produced and germination efficiency. Contrasting differences in the germination efficiency of the pollen between the selected genetic materials in the plantation, would suggest that not all the materials will participate equally as the masculine progenitor. Thus, it is important to evaluate pollen viability in the trees selected as part of the breeding matrices and to detect male sterility. It is also important to study tree management techniques that influence pollen quality.

Weir and Zobel (1975) discussed seed production plantations for selected material. According to the authors, the strategy to develop and test material adequate for future generations should be elaborated early, during the first generation of the improvement program. If the program is of long duration, the consequences of a restricted genetic base and associated risk of endogamy should be avoided by the continuous inclusion of new genetic material within the population. Thus, the ideal way to select the maximum number of unrelated individuals for advanced generations is diallelic

or partial diallelic, both give good estimates of the parental value of the clones, and general and specific combining abilities of the parent trees.

Kellison (1969) stated that due to the fact that seed productivity is dependent on environmental variables, the geographic location, plantation area, and clone dispersal should be considered before a seed producing plantation is installed. For the installation of plantations for the production of hybrid seeds by open-pollination, the following points should be considered: (a) that the species have coincident flowering; (b) the ratio of pollen receptors to pollen donors should be adequate to favor hybridization; (c) the pollen receptor species should have a shorter period of flowering in relation to the pollen donor species; (d) the pollen receptor species should be relatively or completely self-incompatible; and (e) the selection of hybrid saplings in the nursery is essential and should be preceded by studies on the architecture and inheritance of the characteristics of interest to assess its operational viability. The hybrid seeds produced by controlled pollination and the progeny tests are the base of this study.

Basic studies such as the phenology of the selected clones, determination of the general and specific combining abilities and self-compatibility studies are necessary to conduct a prudent improvement program in order to achieve the pre-established objectives.

1.2.6 Pollen viability

Studies on Eucalyptus pollen, in general, aim at developing techniques adequate for pollen management, including genetic conservation, controlled production of hybrids, and the determination of pollen quality/viability. Sousa and Pinto Jr. (1993) reported the existence of intra- and interspecific differences in pollen germination; these could be genetic or physiological in origin, suggesting the need to develop specific culture media that imitate natural conditions. Studies on the *in vitro* germination of Eucalyptus pollen were carried out by Boden (1958), Gabrielli *et al.* (1965), Borges *et al.* (1973), Griffin *et al.* (1982), Cangiani (1988), Sousa (1988), Menck *et al.* (1990), and Sousa and Pinto Jr. (1993).

Sousa (1988) stated that the nutritional state of the plant during pollen development could

affect longevity. Due to the general opinion that boron is necessary and germination efficiency is poor in media with only sucrose, Sousa (1988) studied the effect of boron and calcium on *E. urophylla* and *E. tereticornis* pollen germination. The authors concluded that boron was necessary although they were unable to define the exact concentration. Therefore, the nutritional status of the seed producing trees, with particular respect to boron, is very important. On the other hand, calcium doses of 220 ppm improved the germination efficiency of the two species tested. It would be interesting to observe the effect of boron supplements in the seed production plantations with respect to pollen viability and seed quality.

Suzano Company evaluated 55 *E. grandis* matrices of a second-generation clonal seed production plantation, from different origins, that are part of Suzano's breeding program. Branches containing flowers were collected, the open flowers and the green buds were removed, and the branch was placed in a recipient with water and left in the greenhouse. The buds in anthesis and the open flowers were cut from the branches and the stamens were removed for pollen extraction. A large variation in the efficiency of pollen germination was observed between the trees analyzed, the amplitude of the variation was from 0.15 to 93.96%, with a mean of 44.77%. This variation in the efficiency of pollen germination was also observed by Boden (1958). As these trees are part of the Suzano's clonal seed orchard, second generation, this data is important to evaluate the pollination among the selected genetic material. Thus, variations in pollen germination efficiency will influence the pollination within the plantation and as such influence the genetic quality of the seed. To investigate pollen germination efficiency between locations, the variation in five different locations was determined: Location 1 = 2.56–92.45%, Location 2 = 10.78–69.92%, Location 3 = 25.03–79.17%, Location 4 = 0.15–82.74%, and Location 5 = 1.22–93.96%. From these data, it can be observed that there is an ample variation in germination efficiency within the progenies used, with the range of efficiencies varying with location probably due to physiological/environmental factors. Thus, any variation detected at the same location should be due to genetic effect.

According to Eldridge (1976) there are only a few examples of male sterility in Eucalypts. The detection of these individuals is important since these trees will not participate as pollen donors.

1.2.7 Pollination

In natural populations, Eucalypts exhibit a mixed crossing system, but predominantly allogamy (Griffin, 1989). The pollination of the genus *Eucalyptus* is mainly entomophilic with the principal pollinator being the bee, *Apis mellifera* L. (Pacheco, 1982). Novelli *et al.* (1982) investigated the influence of bee pollination on seed production in a clonal plantation of *Eucalyptus citriodora* and demonstrated an increase in the number of viable seeds per kilogram and the number of capsules per panicle. Pacheco (1982) studied the effect of bee hives (*A. mellifera* L.) in a seed producing plantation of *E. saligna* on production and quality of the seeds. The pollen from the trees in the center of the plantation were labeled using P³² and it was shown that the number of flowers containing labeled pollen decreased with the increase in distance from the source up to a distance of 300 m. Other results demonstrated a gradual reduction in the number of seeds per fruit with an increase in distance from the hive. At 0–50 m, the number of seeds was 40% higher than that from 300 to 350 m from the hive, with the authors demonstrating pollination activity of the bees up to a distance of 350 m from the hive.

Maêda (1987) estimated for an *E. grandis* seed producing plantation, the fertilization rate for the characteristic that produces albino plantlets. The observed rate was 5.57, which represented a coefficient of endogamy of 0.03. The author considered this rate to be low and the variability of the plantation was maintained by efficient insect pollination. Although there appeared to be a large number of insects present on the plants, only a few effectively participated in the movement of pollen between flowers. When the quantity of pollen transported, visiting frequency and movement among the flowers was observed, *A. mellifera* was confirmed as the most important insect in *E. grandis* pollination.

According to Griffin (1989), pollen is frequently transported to larger distances than those inferred by direct observation of the insect flight behavior. The pollen collected from one flower is not

necessarily deposited in the next to be visited. The biological aspects of the Eucalyptus flower suggests that (a) feeding behavior is not strongly conditioned by flower structure, therefore sticky pollen could be distributed over the whole insect, not solely the part that enters into contact with the stigmas and (b) pollen can maintain its viability for approximately 8 days on the insect's body, indicating that the transported pollen remains functional during this time.

Barbour *et al.* (2006) evaluated the phenology of flowering in species of the subgenus *Symphyomyrtus* native to Tasmania and *E. nitens*, introduced species. The difference in the flowering period of the native and an introduced species was highlighted as the principal barrier to gene flow by pollen. The authors state that the results show the importance of knowing the factors that affect gene flow, with the objective of identifying species and populations, native and exotic, with the highest risk of gene flow.

Griffin and Hand (1979) stated that knowledge about the receptiveness of the stigma, besides the processes of floral development and pollen release, is fundamental to the study of reproductive biology. Sousa and Pinto Jr. (1993) studied stigma receptiveness in *E. dunnii* in order to suggest ways to improve the efficiency of controlled pollination. The authors observed that for maximum efficiency, consequently better seed production, pollination should be carried out on the 6th day after anthesis. The receptive period for Eucalyptus stigma can vary within the subgenus (Pryor, 1951), between species of the same subgenus (Griffin and Hand, 1979) and within the same species. Griffin and Hand (1979) highlighted that there could be differences in floral receptiveness within the same tree. The similarity in floral development rates of different species within the same subgenus *Symphyomyrtus*, but growing in different hemispheres, suggests to the authors that this process is under strong genetic control.

According to Eldridge (1976), the flowers of Eucalyptus are hermaphrodite (male and female in the one flower), because protandry has been observed in some species, with examples of self-incompatibility and male sterility; however, allogamy can be considered as predominant in the genus. Although protandry occurs, this does not eliminate the possibility of self-fertilization, since flowering in the canopy lasts longer than the receptive period of a flower.

Kageyama (1979) studied the isolation of seed production areas, trying to minimize undesirable pollination problems because of the facility in intercrossing between large numbers of *Eucalyptus* species. The fact that pollination is predominantly entomophilic within the genus highlights the need to take care in the minimal distance between plantations to prevent possible contamination. A minimum distance of 200 m between seed production areas is recommended. The authors also suggested the use of barriers consisting of other genera or unimportant *Eucalyptus* species to effectively isolate the seed production areas.

1.2.8 Floral development, phenology of flowering, and fruit formation in *Eucalypts*

The period and intensity of flowering varies within and between species, especially where they grow as exotic plants (Griffin, 1989). This could cause two sorts of problems: firstly, when the populations selected for improvement contain material from different origins they could function as a series of subpopulations partially isolated because of the different flowering periods, decreasing the effective size of the population and increasing the risk of endogamy; secondly, variations in the interaction between the species and the insect vector could affect the pollination efficiency between members of the population.

Studies on floral development and flowering/fruiting intensities in *Eucalyptus* populations have been carried out by Moggi (1959) with *E. camaldulensis*, *E. resinifera*, *E. rudis*, *E. tereticornis*, *E. botryoides*, and *E. gomphocephala*; Hodgson (1976a, b) with *E. grandis*; Ashton (1975) with *E. regnans*; Ferreira (1977) with *E. grandis*; Mora and Ferreira (1978) with *E. urophylla*; Aguiar and Kageyama (1987) with *E. grandis*; Graça (1987) with *E. dunnii*; and Souza (1996) with *E. camaldulensis*. Mora and Ferreira (1978) studied the flowering of *E. urophylla* clones in Piracicaba (SP, Brazil). For each clone a branch was chosen to represent flowering in order to follow floral development in accordance with the following stages: (a) inflorescence formation, without individualization of the floral buds until the first operculum falls; (b) from the fall of the first operculum to the fall of the second; (c) flowering (opening of the floral bud); (d)

Table 1 Comparison of the number of viable seeds per kilogram in populations of *E. grandis* with different degrees of genetic improvement

Degree of genetic improvement	Number of viable seeds (kg ⁻¹)
SCA (seed collection area)—Austrália	400 000 to 800 000
SCA—Brasil	800 000 to 1 000 000
SSO (seed orchard by seeds)	80 000 to 400 000
CSO (clonal seed orchard)	500 000 to 1 000 000

fruit development; and (e) natural seed release. Proposing a vision more quantitative, percentages were calculated related to the production and development of the flower buds, flowers, and fruits. The authors observed that, in general, from the initial development of the buds to flowering takes 4–5 months and fruit formation to natural seed release 7–8 months. The natural loss of floral buds during flowering, fertilization, and fruit formation was 54.85%. The flowering period varied among the clones and could be put into the following categories: clones that did not flower during the experimental period; clones with an extremely short flowering period; clones that flowered almost the whole year; clones with a flowering peak from November to February; clones that flower from April to August; and clones with incompatible flowering characteristics.

Mori *et al.* (1988b) suggested that to obtain maximum production, it is necessary to know the factors that affect productivity and use management techniques to alleviate these problems. Thus, basic knowledge about the biological process involved in seed production is important to predict and study the factors that could reduce productivity (Mora *et al.*, 1981).

The variations in the number of viable seeds per kilogram could be related to the pollination efficiency, frequently weakened by the high variability in flowering or restricted genetic bases are shown in Table 1.

1.2.9 Seed vigor and size, germination and initial development of the seedlings

The level of physiological quality of the seeds should be evaluated through their capacity to

germinate and seedling vigor (Popinigis, 1977; Aguiar, 1984). Popinigis (1977) suggested an index for the speed of emergence (SEI) in the determination of relative vigor between lots of seeds and an index for the speed of germination (SGI).

Valeri *et al.* (1984) commented that previously published papers demonstrate that the size of the seed is one of the factors that affect germination, with larger seeds producing more vigorous plantlets that develop faster. Thus, a classification by size would reduce losses associated with the less vigorous plantlets produced by small seeds that are quickly dominated by the plantlets from larger seeds (Aguiar *et al.*, 1979). The authors studied the influence of *E. saligna* seed size on germination and initial plantlet development. The results demonstrated that plantlets from larger seeds reached the size for thinning and transplanting quicker than those from smaller seeds. Similar results were obtained by Pereira and Garrido (1975) with *E. grandis*, observing that larger seeds presented a higher SEI than the smaller seeds.

The occurrence of self-pollination contributes to the loss of seed quality, provoking the appearance of albino and abnormal plantlets (Kageyama, 1981; Maêda, 1987). Oda *et al.* (1991) studied the influence of the number of viable seeds per kilogram and the size of the seeds on the percentage of albino and abnormal plantlets. This study used *E. grandis* seeds collected at different times of the year and the seeds were classified into three groups (<0.50 , $0.59\text{--}0.71$, >0.71 mm). The results demonstrated that larger seeds were less likely to produce albino or abnormal plantlets and suggested that the production of small seeds could be linked to self-pollination.

Suzano (1994a) evaluated the behavior of different progenies of *E. grandis* with respect to seed vigor and initial plantlet growth, looking for possible links between these variables and the quality of the sapling and the future forest. The seeds of six progenies from the clonal seed orchard (second generation) were divided into two groups, >0.59 mm but smaller than 0.71 mm and >0.71 mm. The germination efficiency, SEI and the height of the plantlets at 32 and 40 days after seeding were evaluated. Differences between the progenies were demonstrated, thus during thinning the plantlets that have higher SEIs and faster initial growth will be preferentially

represented in the final product from the nursery. In this way, when breeding populations and commercial plantations are formed, not all of the progenies are equally represented, thus resulting in lower genetic variability, due to the reduction in the effective population number, and failure to achieve the expected results.

1.2.10 Quality of the seedling for planting

The selection of seedlings in the nursery by height is one of the parameters used to assess quality. The advantage of this selection is to form more uniform lots with a better use of the seedlings in the field (Coelho, 1984). According to the author, the practice of sapling selection in the nursery is a controversial question. Some authors state that a certain time after planting, the forest presented a more homogenous development even though the seedlings from the nursery were not selected. However, other studies report that the selection of taller seedlings give rise to taller trees. The removal of defective seedlings or seedlings with low vigor is, however, necessary. For example, of the total number of seedlings produced in the nursery by Companhia Suzano de Papel e Celulose, 20% were considered as abnormal, those presenting defects such as bifurcations and loss of vigor. Part of these abnormalities could be explained by different degrees of endogamy in the seed production plantation.

Coelho (1984) evaluated the silvicultural behavior of *E. grandis* seedlings up to 1-year old, based on seed, plantlet, and sapling size. The author observed that selection by size of the seedling had highly significant effects on height and diameter, suggesting that the selection for this species by seed or seedling should be encouraged in order to obtain trees with better growth qualities. Various studies demonstrate that the selection of seedlings can enable an initial gain in height, but with time this difference is reduced or disappears (Donald, 1976; Balloni *et al.*, 1978; Morais and Brune, 1983; Suzano, 1994b).

In this way, the selection of seedlings has an effect on the formation of commercial plantations where all the progenies do not participate in equal quantities, thus the genetic variability could be reduced due to the reduction in the size of the effective population.

1.2.11 Interaction genotype \times environment (G \times E)

Zobel and Talbert (1984) stated that when the tests are carried out at the same location, any effect is limited only by the genotype. This fact has two consequences: (a) the heritability could be overestimated, resulting in overestimation of the expected gains when the material is planted in an untested environment and (b) the genotypes selected in one environment may not be the best in other environments resulting in a reduction in the genetic gain. The authors also suggested that the G \times E interaction affects the strategy in forestry breeding programs as seen by tree mortality and/or reductions in growth.

Various studies have reported that stability is genetically controlled (Barriga, 1980; Torres, 1988). Kageyama (1980) evaluated the genetic variation in *E. grandis* (origin Coff's Harbour, Australia) generated by open-pollination in five locations: Agudos, Anhembi, Brotas, Lençóis Paulista, and Resende (SP, Brazil). The evaluation characteristics were as follows: form of the tree trunk, DBH, and height at 2-years-old. The effect of the location, significant on all of the characteristics, reflected in the reduction in heritability (at the level of the location, individual plants, mean of the families, and within the families) and, therefore, in the selection for the locations as a group. The same tendency was observed by Pinto Jr. (1984) in progenies of *E. urophylla* S.T. Blake from Island of Flowers (Indonésia), using conjugated origin testes and locations in four Brazilian states, Aracruz (ES), Anhembi (SP), Bom Despacho (MG), and Planaltina (DF).

Mori *et al.* (1986) estimated the genetic parameter for *E. saligna* from three locations (Itatinga (ACS); Areia Branca (PSC); and Salto (PS)) and planted at three test sites: General Câmara (RS), Brotas (SP), and Bom Despacho (MG). The characteristics evaluated were DBH, height, and cylindrical volume at 3-years-old. The coefficient of heritability when analyzed together was inferior to the majority of the coefficients obtained by test location, demonstrating losses in heritability because of the progeny \times location interaction. The losses in the selection due to this interaction were estimates at up to 88.3% for cylindrical volume.

Mori *et al.* (1988a) studied the progeny \times location interaction in 30 seven-years-old progenies of *E. urophylla* generated by open-pollination from the Island of Flowers (Indonesia) at four locations: Aracruz (ES), Belo Oriente (MG), Bom Despacho (MG), and Grão Mogol (MG). The values of the mean heritability (heritability coefficients at the plant level, progeny mean, and within the progenies) for the four locations were superior to the values obtained when the data was analyzed together, demonstrating the great effect of progeny \times location interaction. The losses in genetic gain due to the progenies \times location interaction for the cylindrical volume, DBH, and height, were 26.73%, 15.74%, and 8.14%, respectively. The authors compared the genetic gains (%), using the progeny mean of the cylindrical tree volume and three improvement strategies: multiple populations with selection of progeny for each location; the selection of individuals with a greater capacity to adapt to the study location, stable selection; traditional selection not considering environmental difference and the progenies \times location interaction. For this comparison, a selection proportion of 1:6 between progenies was used, which made possible the selection of progenies predictably more productive (selection of 5 progenies from 30). The genetic gains in percentage are shown in Table 2.

Carvalho (1989) evaluated the G \times E interaction in 50 clones of *E. grandis*, in the region of São Mateus (ES) in three traditionally reforested areas, using the following genetic parameters: heritability, standard deviation from the estimate of heritability, coefficient of genetic variation, and the quotient between genotypic variation and the coefficient of experimental variation, calculated using the measurable parameters DBH, height, cylindrical volume, and wood basic density. It was observed that the estimated heritability values were relatively high for all the measured characteristics when considered at each location. When the data was analyzed together, there was a general decrease for all the characteristics involved in growth (DBH, height, and volume), and less pronounced for basic density. Van Wyk *et al.* (1989) tested the stability of 31 hybrid Eucalyptus clones at 10 locations in South Africa, observing that many clones were stable for tree height. According to the authors, the results indicated that the selection of clones

Table 2 The genetic gains expressed in percentage (%) for the three strategies and the four locations

Strategy	Aracruz (ES)	Belo oriente (MG)	Bom despacho (MG)	Grão mogol (MG)	Mean
Multipopulation	18.81	10.18	14.18	26.10	17.32
Stable	5.09	0.79	10.72	13.25	7.46
Traditional	1.15	1.93	1.09	1.94	1.53

should be cautious since the behavior of each clone is location specific.

Cruz and Regazzi (1994, 2001) discussed the effect of the $G \times E$ interaction on the prediction of selection gains obtained using a particular improvement strategy. The authors presented situations in which it had been desirable to make gain predictions. These studies demonstrated that the effects of the $G \times E$ interaction should be considered in breeding programs, since it is possible that losses in the expected gains could occur in the selection of genotypes. According to Mori *et al.* (1988a) the $G \times E$ interaction, when badly administered, could result in a reduction of expected gains making it difficult for the breeding program to achieve its goals. The authors suggested, in order to minimize losses, a more adequate multipopulation strategy is necessary that considers the specificity of the genetic material for particular environmental conditions, through the selection of individuals more adapted and productive for each ecological region.

A genetic improvement program should have well-defined strategies, planned for short- and the long-term objectives, in order to obtain genetic material improved for the desirable characteristics. This material should guarantee the sustainability of commercial plantations and the continuity of the improvement program through the selection of advanced generations of trees and superior clones. The breeder should be acquainted with the factors that could reduce the expected genetic gains and how to intervene in the improvement strategies to minimize these losses.

1.3 Application of Molecular Markers in Eucalypt Breeding

The success of a breeding program depends on the genetic resources, which are being explored, the selection procedures, and the strategy used. There are numerous examples of successful Eucalypt

breeding programs throughout the world. The average Eucalypt productivity has been improved in three decades from $12 \text{ m}^3 \text{ ha}^{-1} \text{ year}^{-1}$ in the 1960s, to a current value of $60 \text{ m}^3 \text{ ha}^{-1} \text{ year}^{-1}$. The current performance has been reached because of applied silvicultural research and especially the breeding programs using high performance clones with fast growth, excellent wood quality, rusticity on poor soil, and resistance to diseases and pests.

The success of the breeding programs depends on the ability to distinguish heritable and environmental factors. Genetic markers are heritable when associated with the characters of interest and can increase the efficiency of selection. The segregation analysis of molecular markers permits the construction of genomic or linkage maps, to estimate gene frequency, especially useful to characterize the population gene diversity and germplasm in breeding programs for the genetic conservation of Eucalypts (Mori, 1993). Paternity tests and the study of mating system of different Eucalypt species have contributed to obtain superior clones in breeding programs (Camargo, 2001).

A genetic marker is a character capable of detecting differences between two or more individuals or organisms, be able to distinguish progenitors and progenies, and should present a series of attributes such as high level of polymorphism, stability in different environments, detection of high numbers of unlinked loci and of simple inheritance. In the following sections will be present the main types of molecular markers as well as their main applications in Eucalypt breeding programs.

1.3.1 Application of molecular markers in breeding programs

The selection of Eucalypt phenotypic characters for commercial purposes is time consuming because of the time taken to make field evaluations. One way to shorten this time is the use of molecular

markers. Various types are appropriate for use in Eucalypt breeding programs and some examples are given below.

1.3.1.1 Characterization of genetic diversity

The characterization of gene diversity in breeding programs is very important for researchers. Molecular markers are very useful to study the structure of populations, families, clones, cultivars, and also to discriminate gene pools from different populations.

Polymorphism based upon isoenzyme loci was studied in populations of *E. urophylla*, *E. grandis*, and *E. saligna* to develop tests for discrimination of interspecific hybrids from contaminants. Mori *et al.* (1996) concluded that the *Est-1*, *Est-2*, and *Idh-1* loci were useful for the purpose because some alleles were present in a population and absent in others and vice versa. Clones and progenies of five *E. grandis* subpopulations were analyzed by isoenzyme loci observing that the average inbreeding (*f*) was 0.08 and outcrossing rate (*t*) was 0.88. Much of the genetic diversity was within the subpopulations and there was practically no diversity between subpopulations ($F_{ST} = 0.011$) (Mori and Kageyama, 2001).

Sixty-nine progenies were analyzed representing one open-pollinated family of *E. urophylla* trees. RAPD markers allowed the identification of 72 loci that were analyzed using Jaccard's coefficient, generating matrices of genetic distances. The genetic distances between individuals were 0.40 through 12 groups. The progenies also showed different bark patterns, allowing the establishment of distinct groups. However, the groups based on genetic distances using DNA analysis did not correspond of those based on bark pattern (Pigato and Lopes, 2001).

A total of 44 natural hybrids of Eucalyptus, cultivated in central Brazil were analyzed. The RAPD markers presented efficient discriminating power, determining a mean genetic distance of 54% among them and genetic divergence from 24% to 73%. This demonstrated that there is a wide genetic base among individuals, which is desirable in breeding programs. Clustering analysis established by UPGMA (Unweighted Pair Group Method with Arithmetic mean) method, using 80% as a cut off criterion for the total genetic

distance, established nine distinct groups with average genetic divergence above 60% (Caixeta *et al.*, 2003).

The study of genetic diversity among trees is an important stage of any breeding program that targets the exploration of heterozygosis. The prediction of heterozygosis has been extensively used in Eucalyptus to obtain better hybrid combinations. In a study using 40 *E. grandis* and *E. urophylla* trees (two 10 × 10 circulate partial diallel crosses) were analyzed for their genetic diversity. The crosses were produced in a previous study based on the tree diversity using RAPD markers. Seventeen microsatellites amplified 75 allelic forms and gave a heterozygosis value of 26.1%. The genetic distance varied from 10% to 100%, whereas the mean genetic distance was 64.6%. The cluster analysis for the 40 trees showed high diversity and formed two groups (one for each species), although some genotypes were outside their species-specific group. In comparison with groups formed by the RAPD markers, microsatellite markers were more efficient in discriminating species. However, the correlation values among RAPD and microsatellite markers were low and negative. Microsatellite markers were efficient to discriminate trees of *E. grandis* and *E. urophylla* (Muro-Abad *et al.*, 2005).

1.3.1.2 Fingerprinting

The characterization and identification of individuals are important procedures in plant breeding. Rocha *et al.* (2002) used RAPD and simple sequence repeat (SSR) markers to obtain an exclusive fingerprint for 15 genotypes of Eucalypt hybrids with high potential for vegetative propagation used in breeding programs. Those two molecular techniques produced a set of markers that allowed an accurate identification of all genotypes. The RAPD procedures were also used by Pimenta *et al.* (2001) to obtain information on origin, genetic distance, and relationship among clones.

Hybridization between three species of *Eucalyptus* in the Series *Curviptera*, *E. macrocarpa*, *E. pyriformis*, and *E. youngiana* was investigated using RAPD markers. The dendrogram based on genetic similarities showed the relative proximity and distance among the individuals. Two clusters were identified by the UPGMA dendrogram.

One of them included all of the *E. macrocarpa* genotypes and also one of *E. macrocarpa* hybrid. The other included all of the *E. youngiana* and *E. pyriformis* genotypes and their hybrids (Neaylon *et al.*, 2001).

1.3.1.3 Quantitative trait loci (QTL) mapping

Most Eucalyptus breeding programs have used interspecific hybridization, making it possible to capture nonadditive genetic variance. Propagating genotypes via vegetative propagation greatly enhanced the possibility to use genetic linkage maps for accelerating breeding programs by marker-assisted selection (MAS) and recombination (Grattapaglia and Sederoff, 1994).

Linkage maps were constructed mainly by RAPD dominant markers (Verhaegen *et al.*, 1997), amplified fragment length polymorphism (AFLP) dominant markers (Gaiotto and Grattapaglia, 1997), and SSR co-dominant markers (Brondani *et al.*, 1998). The maps could identify genomic regions in eucalypts with significant effects on expression of economically important characters. Various characters were studied by QTL analysis, such as, characters associated to eucalypt rooting (Marques *et al.*, 2002), insect resistance (Shepherd *et al.*, 1995), plant growth (Squilassi and Grattapaglia, 1998), and *Puccinia psidii* rust resistance in *E. grandis* (Junghans *et al.*, 2001). There are also studies for different eucalypt species and cultures in which few loci with major effects controlling a relatively large proportion (from 10% to 40%) of total phenotypic variation for quantitative characters of silvicultural importance, such as, wood volume and wood density were detected.

Squilassi and Grattapaglia (1998) mapped QTLs in eucalypts for wood volume by RAPD markers, using linkage disequilibrium on selected progenies. The study showed that the MAS was efficient to select through the family mean level; however, it was not efficient at the individual level.

Gion *et al.* (2001) studied 201 full-sib families from interspecific hybrids between *E. urophylla* and *E. grandis* for eight genes involved in the lignin biosynthesis (PAL (phenylalanine ammonia-lyase), COMT1 (caffeate/5-hydroxyconiferaldehyde O-methyltransferase 1),

COMT2, 4CL (4-coumarate:coenzyme A ligase), C4H (cinnamate 4-hydroxylase), CCoAOMT (caffeoyl coenzyme A O-methyltransferase), CAD2 (cinnamyl alcohol dehydrogenase 2), and CCR (cinnamoyl-coenzyme A reductase)) and three Myb transcription factors. The genes were mapped using the single strand conformation polymorphism (SSCP) technique. These genes were located on the two parental genetic maps constructed with polymerase chain reaction (PCR)-based markers. The lignin monomeric compositions (S/G ratio) were obtained using the thioacyolysis method and the QTL analysis for S/G ratio was performed using the interval mapping procedure. Several regions controlling part of the variation were identified, showing that multipoint estimates of the total variation explained through the QTL were 38.0% and 18.5% for *E. urophylla* and *E. grandis*, respectively. The study shows that it should be possible to follow the manipulation of lignin quality in breeding programs using molecular markers.

1.3.1.4 MAS

Some studies were carried out using isoenzyme markers to allow earlier selection of superior genotypes. Menck (1996) studying the acid phosphatase isoenzyme in clones of *E. grandis* cultivated *in vitro* found a correlation between superior genotypes for growth characters and those for efficiency by the use of phosphorous. The author also observed correlations between superior genotypes on the field and higher activities of acid phosphatase enzyme.

The transfer of disease resistance alleles in plants can be expedited by the use of DNA markers. If the markers are tightly linked to the resistance alleles they can be used for MAS. One effective use of MAS is found in the process of pyramiding resistance alleles. The use of MAS is based upon the principle that a gene or a block of genes is associated with a molecular marker of easier identification, making the selection for that marker more efficient than the selection for the phenotypic character. MAS in eucalypt has focused mainly on wood growth, which is an important commercial character, easily measured but of low heritability.

Experimental full-sib families of *E. grandis* and *E. urophylla* were used to evaluate the comparative

efficiency of selection based on molecular markers associated with a favorable QTL and the regular phenotypic selection for diameter at breast height (Squillasi and Grattapaglia, 1998). The authors did not study all possible crosses, evaluating only the maternal QTL allelic contribution, because an effective assessment of MAS in eucalypt depends on experiments where the phenotypic data are gathered at adequate ages and QTL information is preferentially available for all progenitors involved in the pedigree (Squillasi and Grattapaglia, 1998).

Using the BSA (bulked segregant analysis) procedure, Bortoloto (2006) developed a SCAR (sequenced characterized amplified region) marker to detect the flowering time region on *E. grandis* map. The marker presented linkage to the EMBRA 7 (Brondani *et al.*, 1998) microsatellite marker on a paternal map. The author also detected linkage of the *EgLFY* flowering gene to the EMBRA 6 (Brondani *et al.*, 1998) microsatellite marker indicating the region for flowering control.

1.3.1.5 Recombination of genotypes and seed orchard

Eucalyptus species are widely used for planting through tropical and subtropical regions of the world and they are predominantly outcrossing, highly heterogeneous, and genetically diverse (Moran and Bell, 1983). Using microsatellites and AFLP markers, Zelener *et al.* (2005) studied six provenances and 37 selected half-sib families of *E. dunnii* to make a selection to establish a seed orchard. The estimated genetic differentiation showed low values among provenances ($\theta_P = 0.026$) and high values among families ($\theta_S = 0.174$). A high proportion of the total variation observed within families suggested that the orchard design should be based upon individual or family selection rather than provenance selection.

A *E. dunnii* breeding population of 46 provenances from Australia and selected for fitness through subtropical and cold environments was screened by AFLP and microsatellite markers to estimate the genetic diversity. The markers presented no significant differences between the original breeding population and the selected genotypes of seed orchard confirming that the seed orchard can be established with a limited number

of individuals without problems of inbreeding (Poltri *et al.*, 2005).

Microsatellite markers were used to genotype potential pollen donor and maternal progenies from a *E. globulus* seed orchard in Chile and an *E. grandis* seed orchard in Uruguay. The resulting data were used to infer the most likely pollen parents for the seed from each seed orchard. The estimated distances from maternal tree to the most likely male parent served as the data set basis to model the pattern of gene flow in both orchards. The results indicate that the pollen dispersal may reach distances as far as 300 m or more (Russell *et al.*, 2001).

1.3.1.6 Mating system

Eucalypts present hermaphrodite flowers making possible selfing and outcrossing rates at different levels. Different authors have observed that eucalypts present a model of mixed mating, predominantly by outcrossing, however inbreeding can occur by selfing and crossing amongst relatives.

Moran and Brown (1980) studying a population of *Eucalyptus delegatensis* by isoenzymes, observed an average outcrossing rate of 0.77, with differences occurring between young ($r = 0.66$) and adult ($r = 0.85$) plants. Through time, the populations change and usually there is a tendency for endogamous individuals to die, increasing the level of heterozygosity in the population. Moran *et al.* (1989) studying a seed orchard of *E. regnans* observed an outcrossing rate ($r = 0.91$) higher than that of the natural population ($r = 0.74$). The lower rate of outcrossing in natural populations could be due to inbreeding between physically close relatives. In a seed orchard there are no relatives and the inbreeding occurs via selfing. Mori (1993) investigating a seed orchard of *E. grandis*, also using isoenzymes found an outcrossing rate of 0.88.

Patterson *et al.* (2001) determined the outcrossing rates at two positions on the tree: the top and the bottom of the canopy in *E. globulus*, in a remnant of a native stand in southern Tasmania using isoenzyme markers. In trees previously determined to have high levels of self-incompatibility, outcrossing rates were high at both, the top and bottom of the canopy (from 0.87 to 0.99). In contrast, the outcrossing rates

in self-compatible trees were significantly different in both positions of the canopy: lower at the bottom (from 0.27 to 0.66) than the top (from 0.74 to 0.90). These results were of particular significance to collection of open-pollinated seed for breeding or deployment, where unfortunately, the most accessible seed may also be the most inbred.

Mori *et al.* (2004) studying *E. grandis* progenies by microsatellite markers observed an outcrossing rate of 0.67 indicating that the species shows crossing among relatives and selfing.

1.3.1.7 Diagnosis of disease resistance

Eucalypt rust, caused by *P. psidii*, is one of the most serious diseases in Brazil and considered to be the most serious threat to eucalypt plantations worldwide. Junghans *et al.* (2001) analyzed the number of sorus and pustule size to characterize rust severity on *E. grandis* seedlings which were further screened with RAPD markers. One of the markers (AT/917) showed complete cosegregation with *Ppr-1* (*P. psidii* resistance, gene 1). All the resistant plants showed the marker, while no susceptible plants had this marker. The AT9/917 sequence was used to obtain specific primers (SCAR), which will be used for screening clones in an *E. grandis* genomic library.

Using microsatellite markers, Mori *et al.* (2004) also studied rust resistance character in progenies of *E. grandis*. Nei's genetic distance varied up to 0.400 and the index of gene diversity varied from 0.383 to 0.713, having an average of 0.587.

1.3.1.8 Analysis of paternity

It is very common to use molecular markers to check the paternity of a superior elite tree obtained by open-pollination. The analysis of paternity is especially important to determine the other parent contributing to the genotype with superior gene combination.

Estimates of the level of multiple paternities correlated with outcrossing within and between fruits in a pre outbred population of the bird-pollinated mallee, *Eucalyptus rameliana*, were made by Sampson (1998) using six isoenzyme loci. The correlation of outcrossing paternity (r_p) was

positive and significant within fruits (0.26) and the effective number of mates for a single fruit was estimated to be 3.85. The specialization of floral structure and phenology in *E. rameliana* for bird pollination has probably contributed to correlation of paternity within fruits because there are fewer male parents available at any one time when compared to the mass-flowering species.

Paternity analysis using microsatellite markers was conducted on a *E. nitens* seed orchard in Victoria, Australia. An average of 40 seeds per clone were germinated and screened for paternity using the four loci. Paternal contribution varied among clones, suggesting that panmictic pollination was not occurring, probably due to differences in flowering time and flower numbers between the clones such that each clone was subjected to a different pollen pool. Distance between clones was another important factor influencing paternal contribution (Grosser *et al.*, 2001).

1.3.1.9 Comparison of breeding generations of *Eucalyptus*

Pigato and Lopes (2001) studied the diversity and the genetic distances in four generations of *E. urophylla*, which provided data to help guide the breeding program. The initial base population was introduced by seeds collected in Indonesia (P_0 generation). In the subsequent segregating generations originated by open-pollination, recombinations were designated as P_1 , P_2 , and P_3 . One hundred and seventy four individual trees representing the four generations were analyzed. The RAPD technique allowed the identification of 86 positions analyzed using the Jaccard Coefficient. The genetic distance from P_0 generation was 0.33, 0.34 from P_1 , 0.40 from P_2 , and 0.38 from the P_3 generation. The genetic distances between individuals increased in relation to the base population, being 0.15% from P_1 generation, 18.93% from P_2 , and 13.31% from P_3 , showing an increase in genetic diversity in the advanced generations, despite selective processes. Genetic diversity of 14 populations of *E. grandis* were studied by Mezzena (2003) utilizing microsatellite markers. The populations were at different levels and breeding generations. The inbreeding coefficients within the populations

were very similar ($f = 0.16$). The author observed a little loss of heterozygosity from F_1 ($H_o = 0.64$) to F_2 ($H_o = 0.60$) breeding generations.

2. DEVELOPMENT OF TRANSGENIC EUCALYPTUS

2.1 *In Vitro* Culture in Eucalypts as a Prelude to Genetic Transformation

From the agribusiness point of view, eucalypts are important in many aspects. Essential oils can be extracted from the leaves; tannins from the bark; and wood, methanol, charcoal, and cellulose for paper production can be extracted from the trunk. Hence, it is not strange that millions of hectares are planted in the world. Currently, Brazil has the biggest collections of *Eucalyptus* spp., even bigger than Australia and Indonesia (Ferreira, 1992). In addition, this genus has species with various interesting attributes such as fast growth, adaptation to poor soils, fertile hybrid formation, and capacity to produce roots from stump sprouts (coppiced).

Commercially, *Eucalyptus* propagation has been traditionally done by seed, but, the cellulose and paper industry has stimulated research to attempt clonal multiplication (Xavier and Comério, 1996; Rosse *et al.*, 1996), to optimize field production, reduce costs, and to achieve better genetic gain (Thorpe and Harry, 1990). Ikemori *et al.* (1994) showed that pulp and cellulose production was significantly higher in cutting cloned forests ($10.9 \text{ t ha}^{-1} \text{ year}^{-1}$) than in unimproved seedling forest ($5.9 \text{ t ha}^{-1} \text{ year}^{-1}$). However, the propagation via rooted cuttings offers difficulties such as loss of rooting capacity in the adult trees (Brune, 1982; Burger and Lee, 1987) and plagiotropic, a bushlike growth pattern (Durand-Cresswell *et al.*, 1985; Flynn *et al.*, 1990). *Eucalyptus* spp., despite the fact that many species can propagate by cutting due to the presence of an epicormic bud at the base of adult trees others, such as *E. regnans*, *E. nitens*, *E. fraxinoides*, and *E. deglupta*, are recalcitrant (Hartney, 1980).

In cloning, tissue culture has been used for several years as a tool to propagate uniform and selected material with major commercial value (Karnosky, 1981; Haissig *et al.*, 1987; Hartmann *et al.*, 1990; Kozai and Kubota, 2001). On

the other hand, the development of breeding programs with woody plants for additive or nonadditive genetic variation has been too slow. Therefore, tissue culture has been effective in these programs, aiming to diminish the time to obtain intra- and interspecific hybrids. Recently, *in vitro* culture has contributed efficaciously to the research on biolistic or *Agrobacterium tumefaciens* transformation with the objective to reach new genetic commercial arrangement for forestry at short, medium, and long terms (Rochange *et al.*, 1995; Machado *et al.*, 1997; González *et al.*, 2002).

There are many reviews reporting *in vitro* techniques showing morphogenesis either via organogenesis or embryogenesis to obtain whole plants in *Eucalyptus* spp. (Hartney, 1982; Durand-Cresswell *et al.*, 1985; LeRoux and van Staden, 1991a; Sita, 1993). In this context, problems with genotype, contamination, and phenol oxidation have been reported as serious challenges to be overcome. For the time being, problems with somaclonal variation and genetic stability (Tibok *et al.*, 1995; Azmi *et al.*, 1997; Rani and Raina, 1998) or vitrification (Bunn, 2005; LeRoux and van Staden, 1991b) have not been cited as important drawbacks.

Tissue culture is a strong biotechnological tool for micropropagation with potential large-scale propagation of *Eucalyptus*. Having elite material with determined degrees of genetic gain so as to replace seeds by cloned propagation, with low cost and high quality products for wood and cellulose and competitiveness is the basic premise of any company. Here we attempt to review the use of *in vitro* tissue and organ cultures to produce *Eucalyptus* clonal forestry, showing different experimental procedures according to the literature available by the author.

2.1.1 The explants

Table 3 shows that different explants that have been utilized to initiate *Eucalyptus* tissue culture and that the use of nodal segments from elite adult plants is more frequent. Although contamination has been recorded (Fossard *et al.*, 1977; Durand-Cresswell *et al.*, 1985), this has not been described as a problem in *Eucalyptus*, even if explants from field or simply surface-sterilized by HgCl_2 or NaOCl was used (Figure 3) (Rao

Table 3 Summary of some protocols for *Eucalyptus* micropropagation

Species	Explant	Basal medium	Growth regulators (μM)	Organogenesis (via)	Rooting (μM)	References
<i>Eucalyptus torrelliana</i>	Nodal explants from tree	MS	BAP: 2.2 + KIN: 0.93	Shoot induction	NAA: 11	Gupta <i>et al.</i> , 1983
<i>E. sideroxylon</i>	Nodal explants from coppice regrowth of tree	MS	BAP: 2–4 + NAA: 0.5–1	Shoot induction	IBA: 10	Burger, 1987
<i>E. citriodora</i>	Mature seed	B5; B5M B5R	NAA: 16.0 NAA: 27 —	Directly somatic embryogenesis		Muralidharan <i>et al.</i> , 1989
<i>E. macarthurii</i> ; <i>E. smithii</i> Hybrid MG25	Nodal explants from 1-year-old plant	MS	BAP: 0.88 + NAA: 0.05	Shoot induction	IBA: 10	LeRoux and van Staden, 1991a
<i>E. saligna</i> <i>E. globulus</i>	Nodal explants from coppice stumps of tree	MS	BAP: 2.5 + NAA: 1.25	Shoot induction	IBA: 10	Bennett <i>et al.</i> , 1994
<i>E. grandis</i>	Leaf explants from <i>in vitro</i> plants	KG, G22, R5, GBA	BAP: 0.2; BAP: 2.0 + NAA: 2.5; Zeatin: 8.0 + NAA: 0.5; BAP: 5.0 + NAA: 0.5;	Callus	IBA: 1.25	Lainé and David, 1994
<i>E. urophylla</i>	Hypocotyl explants from 14-day-old	MS	BAP: 0.9 + NAA: 1.1; Zeatin: 4.6	Callus	NAA: 5.4 BAP: 0.04	Tibok <i>et al.</i> , 1995
<i>E. dunnii</i>	Seedlings—3 days old	B5	NAA: 16.5	Callus—somatic embryogenesis		Termignoni <i>et al.</i> , 1996
<i>E. grandis</i> \times <i>E. urophylla</i>	Hypocotyls, cotyledonary-node, from 14-day-old seedlings Primary leaves with different ages from <i>in vitro</i> plants	SP	TDZ: 2; BA/NAA: 2.5/0.5, 5/0.5, 10/0.5; Zeatin/NAA: 2.5/0.5, 5/0.5, 10/0.5	Callus	IBA: 2.5	Barrueto <i>et al.</i> , 1999
<i>E. impensa</i>	Shoot segments from trees	MS	BAP: 0.25 + Kin: 2.5	Shoot induction	IBA: 5 + NAA: 0.5	Bunn, 2005

and Venkateswara, 1985; Gupta and Mascarenhas, 1987; Bennett *et al.*, 1994). However, it seems that this cannot be generalized since 70% of *in vitro*-inoculated nodal explants from forest trees show contamination, while greenhouse nodal fragments remained virtually contamination-free (Fossard *et al.*, 1977). On the other hand, with respect to surface sterilization of explants, most reports show use of NaOCl or HgCl₂ with varying periods from 10–20 min for NaOCl, and from 5–15 min for HgCl₂. Concentrations varied from 0.12% to 9.0% of NaOCl and from 0.02% to 0.1 % for HgCl₂. Prior to the treatments, fungicide has sometimes been used (LeRoux and van Staden, 1991a, b).

Also, the material sometimes was thoroughly rinsed with tap water and detergent (Gupta and Mascarenhas, 1987; Das and Mitra, 1990), before sterilizing with HgCl₂ or commercial bleach.

Ca(OCl)₂ is an energetic oxidant, with low phytotoxicity, similar to NaOCl. However, due to its powder formulation and the need to be filtered, few studies have used it as a surface sterilizer for the inoculation material. In these cases, concentrations varied from 5% to 7% for 5–20 min (Cresswell and Nitsch, 1975; Fossard *et al.*, 1977; Trindade and Pais, 1997).

Other chemical substances against bacteria and fungi have rarely been mentioned in *Eucalyptus*



Figure 3 Seedlings of *E. grandis* × *E. urophylla* approximately 14-day-old in SP medium, seeds surface disinfected with NaOCl (9%) for 10 min, showing hypocotyls, cotyledons, roots, and contamination free. Growth conditions: $24 \pm 2^\circ\text{C}$, 16 h photoperiod, and photosynthetic photon flux density at $50 \mu\text{m}^{-2} \text{s}^{-1}$

studies, besides ethanol and H_2O_2 . Alkydimethylbenzalkonium chloride, ADBAC for example, was a product rarely cited (Bunn, 2005), and biocide-type isothiazolones such as PPM (Niedz and Bausher, 2002) were not used in Eucalyptus micropropagation. The later is heat stable, with a broad spectrum against plant contaminants (Compton and Koch, 2001). Physical methods such as the use of hot water and UV light have practically not been mentioned in explant sterilization procedures for Eucalyptus.

In general, phenolic oxidation is mostly inconvenient in *in vitro* cultures (Prieto *et al.*, 2005) often demanding the use of various alternative substances to overcome it (Ziv and Halevy, 1983). Changes in peroxidase activity and level of phenolic compounds were showed by Arezki *et al.* (2001) in *E. camaldulensis*.

In Eucalyptus, browning has hindered the establishment of some protocols such as those related to protoplasts (Teulières and Boudet, 1991), cell suspension (Teulières *et al.*, 1989; Barrueto *et al.*, 1997), nodal explants from adult trees (Rao, 1988; Das and Mitra, 1990), or floral origin (Warrag *et al.*, 1991), being necessary the use of antioxidant substances such as polyvinylpyrrolidone and ascorbic acid in several cases, or adsorbents of toxic substances such as

activated charcoal. Other methods reported the use of periodic subcultures, initial dark period, or using explants from established shoot cultures (LeRoux and van Staden, 1991a, b).

2.1.2 Shoot multiplication and hormone treatment

Once the explants have been established in a basal medium, multiplication follows via organogenesis or embryogenesis. Usually the basal medium used in this phase is an extension of the medium used previously, frequently a Murashige and Skoog (MS) medium (Table 3). Salt and vitamins from the MS nutrient medium have been used in their original form (Rao, 1988; MacRae and van Staden, 1990; Bennett *et al.*, 1994; Yang *et al.*, 1995) or with modifications (Hartney, 1982; Barrueto *et al.*, 1999) depending on the purpose (induction of organogenesis or somatic embryogenesis) and the genotype used.

Auxin and cytokinin have been useful to obtain *in vitro* plants from basal medium at different levels. Calli have been obtained with $2 \mu\text{M}$ thidiazuron (TDZ) using juvenile material from a *E. grandis* × *E. urograndis* hybrid (using cotyledon, hypocotyls, and cotyledonary

node), and plants were obtained when these calli were submitted to different concentrations of 6-benzylaminopurine/ α -naphthalene acetic acid (BAP/NAA) or Zeatin/NAA. The best results were obtained with Zeatin: 5.0 μ M and NAA 0.5 μ M, respectively, using cotyledonary node explant (Barrueto *et al.*, 1999).

Calli were also obtained with hypocotyl juvenile material from *E. grandis* using a combination of 21.5 μ M NAA and 4.6 μ M kinetin (Kin) and organogenesis was observed when the material was transferred to a hormone-free medium (Warrag *et al.*, 1991). Callus induction was also observed in *E. citriodora*, from cotyledons and hypocotyls submitted to different auxins: IAA (indole-3-acetic acid), NAA, 2,4-D (2,4-dichlorophenoxyacetic acid) (range: 0.1–5.0 mg l^{-1}) plus coconut milk (15%); however, organogenesis occurred when calli were placed in 2.7 μ M Zeatin and 1.1 μ M IAA (Sita, 1979). According to the same author, calli from leaves originated from mature plants induced with 27 μ M NAA and 28 μ M IAA, but no organogenesis was observed. Organogenesis was observed in *E. urophylla*, from hypocotyl explants when MS was supplemented with several NAA/BAP concentrations, showing better results with NAA at 1.1 μ M and BAP at 0.88 μ M (Tibok *et al.*, 1995); however, shoot production of *E. grandis* \times *urophylla* was also effective, at 13.7 per explant with NAA/BAP at 0.54 μ M and 0.44 μ M on apical shoots and auxiliary bud, respectively (Yang *et al.*, 1995).

At low concentrations (NAA 0.54 μ M and BAP 0.9 μ M), shoot multiplication from nodal explants of juvenile *Eucalyptus* of many species (*E. smithii*, *E. saligna*, etc.) showed good results (LeRoux and van Staden, 1991a, b). Using auxiliary meristems, shoots were also obtained with different genotypes of *E. tereticornis*, inoculated onto MS basal medium. Among the growth regulators tested, NAA/BAP/IAA at 5.3, 4.4, 1.1 (μ M), respectively, was more effective giving 32–45 shoots per explant. Repeated subculture in MS plus IAA 0.1 μ M and BAP 0.44 μ M, produced around 200 shoots per explant (Rao, 1988). With nodal segments from an 8–10-year-old *E. tereticornis*, 18–22 shoots per explant were achieved with modified MS supplemented with NAA 0.54 μ M and BAP 4.44 μ M (Das and Mitra, 1990). Low concentrations of growth regulators were also used with *Eucalyptus impensa* to obtain

shoot multiplication in half-strength MS medium plus BAP 2.5 μ M and Kin 2.5 μ M from shoot segments as explants (Bunn, 2005).

Regeneration competency from auxiliary and apical buds was observed in 20-year-old *E. citriodora*. This was achieved by incubating the explants at 15 °C for 72 h, and then incubating them in a liquid MS-2 media (120 rev min^{-1}). Five to eight shoots per bud developed. After this, individual shoots were transferred to solid MS-2. In this new condition, 10–15 shoots were obtained per explant (Gupta *et al.*, 1981). The MS-2 medium was supplemented with Kin 0.93 μ M, BAP 1.33 μ M, calcium pantothenate, and biotin (0.1 mg l^{-1} of each). According to the authors, it could be possible to get 100 000 viable plants per year from a single bud using this protocol.

Somatic embryogenesis was obtained with *E. citriodora* using NAA 16.13 μ M and zygotic embryos as a starting point. The basal medium (B5) was supplemented with sucrose 50 g (Muralidharan *et al.*, 1989). In a peculiar protocol, somatic embryogenesis was also described for *E. grandis*. This was obtained with leaves from *in vitro* seedlings using 2,4-D (2.3 μ M) for callus induction. Callus proliferation was reached in MS medium with 4 g l^{-1} of activated charcoal containing (μ M): 0.05 NAA, 0.44 BAP, and 0.27 gibberellin. However, additional study was mentioned for implanting large-scale production (Watt *et al.*, 1991). In conclusion, low concentrations of BAP and NAA seem to be more effective for *Eucalyptus* shoot multiplication under a wide range of experimental conditions (Table 3); however, these wide experimental conditions, basically, included *Eucalyptus* solid cultures and not liquid cultures, probably because liquid medium can cause vitrification—hyperhydricity (Monsalud *et al.*, 1995).

2.1.3 Shoot elongation

Usually, shoot elongation has been obtained using a half-strength medium. In *E. camaldulensis*, regenerated shoots were transferred to quarter strength B5 basal medium plus NAA 0.05 μ M (Kawazu *et al.*, 1996). In *E. grandis* \times *E. urophylla* shoot elongation was more efficiently obtained with MS medium containing half-strength potassium nitrate and sucrose (Yang *et al.*,

1995). On the other hand, the elongation of shoot clusters may be mediated with gelling agents. In *E. grandis*, the MS medium containing Gelrite plus a combination of NAA/BAP/GA (μM): 0.05, 0.44, and 0.27, respectively, improved shoot-cluster elongation (MacRae and van Staden, 1990). In contrast, optimum shoot elongation of *Eucalyptus torelliana* and *E. camaldulensis* was achieved with solid and liquid MS-2 media, respectively, using Kin/BAP: 0.23 μM and 0.44 μM , respectively (Gupta *et al.*, 1983). In addition, *E. grandis* shoot elongation was promoted in MS plus gibberellic acid 2.7 μM prior to rooting (Sita and Rani, 1985).

2.1.4 Rooting

The formation of roots from excised shoots in one-fourth strength MS containing IAA 10 μM with no cytokinin was reported by Hartney (1982). In addition, shoots of *Eucalyptus sideroxylon* from modified MS (half-strength salts, full-strength organic constituents) rooted with 10 μM IBA (indole-3-butyric acid). IBA was more effective than NAA in adult or coppice tissues (Burger, 1987). Differences in rooting between juvenile and old tree material were also noted in *E. citriodora* (Gupta *et al.*, 1981). In general, this is a physiological characteristic of woody species that *in vitro* regeneration and rooting ability decrease with age. For *E. tereticornis*, the presence of IBA in the basal medium was essential for rooting, as well as the use of a dark period before the transfer of shoots to light (Das and Mitra, 1990). A period of 72 h in darkness, prior to a 16 h photoperiod along with hormonal treatment, was used to root four *Eucalyptus* genotypes, giving different rooting ability, inclusive, deficient rooting was observed in *Eucalyptus macarthurii* and *E. smithii*. In addition, these species also showed poor rooting from coppice cuttings (LeRoux and van Staden, 1991a). *Eucalyptus globules* showed better results when low concentrations of IBA (1.0–2.5 μM) were used and when NH_4NO_3 was removed from the root-induction medium (Bennett *et al.*, 2003).

Rooting of different clones of *E. globulus* was verified after different periods of rejuvenation using subculturing routines (Trindade and Pais, 1997). Root induction improved when riboflavin and choline chloride were included in the medium and boron was removed from the rooting medium.

The authors also reported that IBA dipping before transfer to the rooting medium improved the appearance of adventitious roots. Experiments with *E. grandis* \times *E. urophylla* using modified SP supplemented with IBA 2.5 μM for 5 days and transfer of shoots to a similar medium with activated charcoal (1 g l^{-1}) greatly improved rooting but resulted in little callus formation at the base of some shoots (Barrueto *et al.*, 1999).

Despite auxin being an important factor in root induction, care must be taken to avoid callus induction at the base of the shoots (Mehra-Palta, 1982; Rao, 1988; Cheng *et al.*, 1992). On the other hand, cytokinin is a critical component of the multiplication media, but inhibitory for the rooting media. However, subsequent adventitious root production in rooting medium was better when Kin was used during the last multiplication (Bennett *et al.*, 1994). As seen above, rooting is a highly clone-dependent characteristic, hence, one must be careful when testing or improving rooting conditions, especially with shoots from a mature tree, older the tree, smaller the rooting ability.

2.1.5 Temperature and light

In general, the culture conditions for *in vitro* Eucalyptus plant establishment were a constant temperature of $25 \pm 2^\circ\text{C}$ and a 16 h photoperiod, involving different ranges of photosynthetic photon flux density (PPFD) from cool fluorescent light bulbs (Rao and Venkateswara, 1985; Subbaiah and Minocha, 1990; Termignoni *et al.*, 1996; Sartoretto *et al.*, 2002; Bennett *et al.*, 2003).

2.1.6 Hardening plantlets

The transfer from tube to soil under greenhouse condition is an important step in the micropropagation process. Usually, intermittent mist or a plastic cover is required as well as plastic pots with sterile vermiculite and adequate natural or artificial light and temperature. When established, plantlets are transferred to polyethylene bags with a mixture of soil and sand. Survival is variable, but, usually high. Generally, a hardening protocol contains steps similar to those previously described with variation in the composition of the rooting substrate (inclusion of fertilizer, fungicide, etc).

Finally, the plants are taken to the field and their performance is evaluated in terms of survival, growth rates, and biomass production. On the whole, micropropagated *Eucalyptus* plants have the same appearance as their sources (Mehra-Palta, 1982; Gupta and Mascarenhas, 1987; Warrag *et al.*, 1989; LeRoux and van Staden, 1991a). Consequently, tissue culture techniques have been observed with great interest in agronomy, forestry, and ornamental plant production, due to the economic benefits of clonal propagation on a commercial level; however, the productivity of commercial clones can be reduced by viral infections, but culture of meristem tips is a good way to combat this (Rutledge and Douglas, 1988).

2.2 Genetic Transformation of Eucalypts

To maintain and sustain forest vegetation, conventional approaches have been exploited for propagation and improvement, but tree breeding efforts are restricted to the most valuable and fast growing species. However, such methods are limited with several inherent bottlenecks because trees are generally slow growing, long-lived, sexually self-incompatible, and highly heterozygous plants. Due to the prevalence of high heterozygosity in these species, a number of recessive deleterious alleles are retained within populations, resulting in high genetic load and inbreeding depression. This limits the use of traditional breeding methods such as selfing and backcrossing, and makes it difficult to fix desirable alleles in a particular genetic background (Williams and Savolainen, 1996). Thus, conventional breeding is rather slow and less productive and cannot be used efficiently for the genetic improvement of trees. To circumvent these impediments clonal or vegetative propagation has been deployed for recovering dominant, additive, and epistatic genetic effects to select superior genotypes. Plant tissue culture and genetic transformation methods offer an important option for effective multiplication and improvement of trees within a limited time frame. Biotechnological approaches for *in vitro* regeneration, mass micropropagation techniques, and gene transfer studies in tree species have been encouraging, particularly in the last decade. With these techniques, genetic engineering assumes additional significance, allowing introduction of

desired gene(s) in a simple step, for precision breeding of forest trees (Giri *et al.*, 2004).

Until recently, trees were considered to be recalcitrant for genetic transformation studies involving molecular techniques. The main obstacle for genetic transformation of trees is the regeneration of transformed plantlets. *Agrobacterium*-based genetic transformation is normally the main method used for developing transgenic trees. Further regeneration of plants from single cells is a requisite for *Agrobacterium*-mediated gene transfer to achieve homogenetically transformed plants (Giri *et al.*, 2004).

Choice of explants having competence for transformation and regeneration is a crucial factor. At this point in time efficient tissue culture techniques become the foundation for genetic transformation studies. In addition to the regeneration through organogenesis, somatic embryogenesis definitely offers the advantage of single cell regeneration and currently appears to be the most promising approach to introduce new genes into woody tree species (Giri *et al.*, 2004).

Despite the progress in recent years, challenges remain for the transformation of hardwood trees. Although elite individual of hardwoods such as poplar, eucalypts, and sweet gum can be maintained by vegetative propagation methods, genotypic variation in regeneration prohibits the inclusion of many genotypes in tree improvement programs. Even for the elite genotypes that are propagated for clonal deployment, transformation can be limited by *Agrobacterium* susceptibility or *in vitro* regeneration. At the moment hardwood transformation is largely limited to either the clones that are easy to transform and regenerate or juvenile materials that have higher regeneration and transformation potential. Maturation is another common problem. While seedling or juvenile tree explants are relatively easy to regenerate and transform, explants of mature trees tend to lose regeneration potential. Unfortunately, elite clones are selected from older trees with years of field performance data (Nehra *et al.*, 2005).

A limitation to the use of this technique lies in the general recalcitrance of eucalypts to transformation and poor regeneration capacity (Poke *et al.*, 2005). At this time, the stable transformation has only been successful in a small number of species including *E. camaldulensis*, *E. globulus*, *E. urophylla*, *E. grandis*, and *E. urophylla* ×

E. grandis hybrids (Mullins *et al.*, 1997; Ho *et al.*, 1998; Moralejo *et al.*, 1998; González *et al.*, 2002; Tournier *et al.*, 2003; Valerio *et al.*, 2003). These are predominantly laboratory studies. Field studies of transgenic eucalypts have been reported in the United Kingdom and Spain in 1995, in Portugal and South Africa in 1997, and Uruguay and Chile in 1997/1998 (Potts *et al.*, 2001) including field testing of glyphosate-resistant transgenic *E. grandis* (Llewellyn, 2000).

Despite the clear potential of genetic engineering for improving woody plants during the last 10 years, progress has been slow on *Eucalyptus*, which is still considered recalcitrant. In particular, regeneration and tissue culture is often very poor, probably due to the high concentration of phenolic compounds in the cells (Tournier *et al.*, 2003) or to low endogenous cytokinin content as shown by Azmi (1999). Regeneration capacity is even lower on a selective antibiotic-containing medium making it often impossible to recover transgenic shoots even when stable transformation is achieved (Serrano *et al.*, 1996; Sartoretto *et al.*, 2002).

2.2.1 Gene transfer

From a scientific point of view, the transfer of specific genes with a known function is very similar to classical breeding, but due to a restricted approach, the efficiency is increased. The prerequisites for gene transfer aiming at cultivar improvement are as follows:

- Availability of the trait to be transferred as cloned-DNA;
- Availability of a powerful transfer system; and
- Availability of a reliable regeneration system predominantly from a single transformed cell.

The last two points are solved in principle, although regeneration is still a problem. Regeneration is more an art than a science, particularly in transformation of *Eucalyptus* clones. However, recipes are available and, with sufficient trials and the use of a range of different genotypes, success should be achievable. Transfer studies are predominantly via *Agrobacterium*, as such transformations are more stable than microbombardment techniques during subsequent meiosis. Microbombardment has, however, the advantage that it can transform meristematic cells,

therefore the regeneration of plants is relatively easy to achieve. The most critical problem is still gene isolation. Most of these isolated genes are used in transformation experiments for basic research to elucidate biochemical pathways and add knowledge particularly in the field of metabolomics (Wenzel, 2006). Most of the patented genes available in the market, which are expected to give a determinant trait, have not been sufficiently tested in *Eucalyptus*. However, patented genes for specific traits in engineered plants have not generated improved commercial *Eucalyptus* clones, yet.

2.2.2 The use of biolistics in the genetic transformation of *Eucalyptus*

Microprojectile bombardment, which bypasses the problems associated with host specificity of *Agrobacterium* and regeneration systems, offers an alternative approach for the delivery of DNA into cells that are also competent for regeneration. Because the particle gun concept involves physical introduction of DNA into the cell, it potentially allows the transformation of any living cell, independent of its type or localization in the tissue (Sartoretto *et al.*, 2002).

Serrano *et al.* (1996) obtained stable transformation of *E. globulus* cells for the first time, using biolistic DNA delivery in zygotic embryos as the target material; however, no transgenic plants were regenerated. Sartoretto *et al.* (2002) reported a procedure for genetic transformation of calli from a *E. grandis* × *E. urophylla* hybrid using particle bombardment of calli derived from cotyledon and hypocotyl. However, the calli were unable to regenerate transgenic shoots, suggesting that the conditions suitable for regeneration are unsuitable for transformation and *vice versa*. In spite of some interesting advances using the particle gun delivery system, no transgenic plants of any *Eucalyptus* species have been reported using the biolistic strategy.

2.2.3 *Eucalyptus* transformation using *A. tumefaciens*

Mullins *et al.* (1997) obtained stable transformation of one clone of *E. camaldulensis* using leaf explants inoculated with *A. tumefaciens*. In this

work, the reporter gene β -glucuronidase and the *NPTII* gene were used for selecting transgenic tissues. Ho *et al.* (1998) reported an efficient system for transformation of seedling material from *E. camaldulensis* by the inoculation of hypocotyls segments with *A. tumefaciens*. The work used the Ti-plasmid vector harboring chimeric neomycin phosphotransferase and β -glucuronidase genes.

Moralejo *et al.* (1998) regenerated transgenic *E. globulus* plants using hypocotyls and cotyledons from young seedlings. The explants were inoculated with *A. tumefaciens* and the genes introduced were *GUS* and *NPTII*. Due to confidentiality problems, no precise transformation protocol has been published for most of the eucalypt species. Maunders *et al.* (1997), even though the protocol is not described, reported a reliable transformation system for several species of *Eucalyptus* including commercially important *E. grandis* and *E. globulus*, starting from either mature elite clones or improved seed material.

Transformation of forest tree species remains difficult, particularly within the genus *Eucalyptus*. Harcourt *et al.* (2000) produced transgenic plants of *E. camaldulensis* containing both the insecticidal *cryA3* gene and the *bar* gene by *A. tumefaciens*-mediated transformation of seedling explants. The transgenic lines exhibited tolerance to the broad-spectrum herbicide and resistance to the first instars of chrysomelid beetles. These plants are likely to provide better insect and weed control options in plantations, particularly during the vulnerable establishment phase, mainly in elite genetic background.

Chen *et al.* (2001) regenerated transgenic *E. camaldulensis* plants of elite clones. The genes introduced were cinnamate 4-hydroxylase (*C4H*) and *NPTII*. The main objective of this work was to alter the lignin content of the wood. The transgenic plants were obtained but the wood modification was not evaluated. For *Eucalyptus* species, transformation and plantlet regeneration are generally more efficient with juvenile materials, such as hypocotyls, cotyledons, and leaf disks from *in vitro*-germinated seedlings, than with clonally derived material from field-grown trees that have poor regenerability in tissue culture (MacRae and van Staden, 1999). For forest tree species, the true value of genetic engineering lies in its integration into conventional breeding programs to improve economically important traits that

cannot be modified by conventional means within a reasonable time frame. González *et al.* (2002) obtained transgenic *E. grandis* \times *E. urophylla* plants using seedling material and *A. tumefaciens*. The chimeric construct contained the *nptII* gene and the *Lhcb1*2* gene, coding the 28 kDa chlorophyll *a/b* binding pea protein from LHCII antenna. The transformation system used the inoculation of seeds with *A. tumefaciens* followed by sonication for a few seconds. The objective of the work was to develop a transformation protocol and evaluate the alteration in the process of photosynthesis.

Tournier *et al.* (2003) regenerated *E. grandis* \times *E. urophylla* juvenile clones with the antisense *CAD* gene and *NPTII* like selective gene. The transgene expression was demonstrated with high inhibition of endogenous *CAD* gene, but physiological and biochemical wood modifications were not evaluated.

Valerio *et al.* (2003) developed a procedure for *A. tumefaciens*-mediated genetic transformation of a juvenile *E. camaldulensis* clone using antisense *CAD* gene. Some transgenic lines exhibited a strong inhibition of *CAD* activity, associated with a decrease in transcription. The wood chemical analysis showed no differences in lignin quantity, composition or pulp yield, compared to control trees. These results underline the problems of extrapolating genetic engineering results from a model to a genetically distant target plant species. Other techniques such as RNA interference-type procedures may be used for more complete gene suppression.

Recent developments in transgenic trees can have multidirectional benefits. The benefits range from manipulating generation time, plant protection, wood quality, production of compounds of pharmaceutical value, and recovery of polluted soils.

These successes have opened up avenues to include agronomically more useful genes for transfer into tree species as has been demonstrated in crop plants (Giri and Vijaya, 2000). Following the global trend in forestry biotechnology there are now 24 genetically modified tree species that have been approved for field trials. Recently, it has been emphasized that genetically modified trees can be excellent tools for physiological research (Herschbach and Kopriva, 2002). The research completed so far demonstrates the potential of

these techniques in the improvement of forest tree species. One of the most important aspects of transgenic trees is integration of the introduced gene into the genome and its expression. For long-lived tree species, new questions arise regarding the stability of the integration and expression of foreign genes. Biosafety considerations, including the impact of transgene dispersion through pollen and unexpected effects on nontargeted organisms, are now receiving attention. With recent research developments, molecular genetics provide tools that may allow genetic improvement to make up lost ground. If current progress in tissue culture and genetic transformation combined with biotechnological applications continues, the future may witness super tree species tailored for special agronomic and economic characteristics (Giri *et al.*, 2004).

Private companies claim to possess routine transformation protocols for different Eucalyptus species and hybrids; however, because of the confidentiality issues, these methods are not widely available (MacRae and van Staden, 1999).

3. IMPORTANT AREAS FOR IMPROVEMENT USING GENETIC TRANSFORMATION

3.1 Eucalyptus Diseases, Control Strategies, and Genomic Approaches to Identify Genetic Resistance

3.1.1 Eucalyptus diseases and control strategies

Eucalyptus cultivation in Brazil was limited only to a few thousand hectares until the 1970s, when eucalypts were considered to be practically immune to diseases. The increasing demand for forest products and the need to conserve the native forests pushed the expansion of Eucalyptus plantations to about 3.4 million hectares. About 1 million hectares of this are planted with 362 different clones, of both pure species and hybrids, distributed in areas varying from 10 to 34 000 ha/clone. It is expected that within this year an additional 250 000 ha of eucalypt forests comprising 55 clones will be planted, occupying areas varying from 10 to 9000 ha/clone/company. The expansion of plantations to warmer and

more humid regions conducive to infection by plant pathogens, the regional peculiarities of climate and soil, combined with the possible introduction of diseases from other countries and adaptation of some local pathogens from native Myrtaceae to Eucalyptus species have resulted in the incidence of various fungal and bacterial diseases that cause significant losses in some highly susceptible clones. In spite of the risks, cloning has proved to be an excellent tool for disease control. The knowledge of Eucalyptus species and their interactions with different potential diseases is important to establish strategies to ensure stability of wood production. This review summarizes the main Eucalyptus field diseases and their control.

3.1.1.1 Rust

The symptoms are the appearance of yellow-colored powdery urediniosporic sporulation over the affected organs is the typical feature for rust diagnosis (Figures 4a–c). In highly susceptible materials, the infection causes deformation, necrosis, hypertrophy, mini-cankers, and death of the apical meristems (Figure 4d). Although the uredinal phase is more common and is the main form of pathogen dispersal, less frequently, teliospores can be produced, during the warmer periods, on fully expanded leaves.

Causal agent: *Puccinia psidii* Winter.

Control: The existence of high inter- and intraspecific genetic variability for resistance to rust allows for disease control by planting resistant clones, progenies, or species. *Corymbia citriodora*, *C. torelliana*, *E. camaldulensis*, *E. microcorys*, *E. pellita*, *E. pilularis*, *E. propinqua*, *E. resinifera*, *E. robusta*, *E. saligna*, *E. tereticornis*, and *E. urophylla* are important sources of resistance. In regions, favorable to rust infection, planting of *E. grandis* seeds (provenances: South Africa and Coff's Harbour 9583), *E. phaeotricha*, *E. cloeziana*, *E. globulus*, and *E. nitens* should be avoided. There is, however, ample intraspecific variability, which permits selection and cloning of resistant genotypes for planting. In *E. grandis*, resistance might be dominant and controlled by a major gene (*Ppr-1*) (Junghans *et al.*, 2003). Thus, rust can be controlled through use of resistant progenies, whose seeds are harvested from resistant homozygous mother plants, as practiced by some Brazilian forest industries in São Paulo and



Figure 4 Eucalyptus rust (*Puccinia psidii*). (a) Infected apical shoots of *Eucalyptus urophylla*; (b) pathogen esporulation on *E. globulus* leaf; (c) rust incidence on stem and leaves of *Syzygium jambos*; and (d) necrosis and death of apical shoots of *E. grandis*

southern Bahia. It is also possible to select trees with rapid growth characteristics, which rapidly escape the disease by virtue of the microclimate in the upper canopy being unfavorable for disease development. Similarly, selection can be made from clones of *E. globulus* and related species that rapidly pass through the susceptible juvenile leaf stage to produce resistant adult leaves. In the case of highly susceptible genetic materials of high commercial value, the disease can be controlled by systemic fungicide spray such as triadimenol (Bayfidan 25 PM ou 25 CE) (0.5 g.i.a.l^{-1}) and azoxystrobin (Amistar 500 WG) (0.1 g.i.a.l^{-1}) at 2 weeks intervals. In nurseries, especially in clonal hedges and mini hedges, the disease is controlled by fortnightly spraying of triadimenol or azoxystrobin, using the above concentrations.

3.1.1.2 Cylindrocladium leaf blight

The lesions can develop at the base, at the apex, or at the leaf margins, and can cover a large portion of the leaf area, inducing severe premature defoliation of the lower, middle, and apical thirds of the tree canopy during the 1st and 2nd years after planting (Figure 5a). It is believed that, when only the basal or middle third of the canopy is affected, the trees tend to recover. However, under disease favorable conditions, the apical portion is also defoliated, especially in highly susceptible materials, leading to reduced plant growth. Defoliation also allows for high light intensity penetration into the stand leading to growth of competing weeds (Figure 5a). Disease symptoms can vary depending upon the species of *Cylindrocladium* and *Eucalyptus*. The



Figure 5 *Cylindrocladium* leaf blight. (a) Defoliation in commercial plantation of eucalyptus hybrid (*E. grandis* × *E. urophylla*), the arrows indicate growth of weeds due to light penetration increase; (b) smaller leaf spot, typical of *C. pteridis*; and (c) larger leaf spot, typical of *Cylindrocladium* spp.

leaf spots of *C. pteridis* are smaller, circular, or elongated and light gray progressing to light brown in color (Ferreira *et al.*, 1995) (Figure 5b), while those of *C. candelabrum*, *C. floridanum*, *C. ilicicola*, *C. parasiticum*, and *C. scoparium* are larger, light to dark brown with a gray green halo (Alfenas *et al.*, 2004) (Figure 5c). In most *Eucalyptus* species, the

lesions are light or pale brown, but in *E. cloeziana* they are dark brown.

Causal agents: *C. candelabrum*, *C. floridanum*, *C. ilicicola*, *C. ovatum*, *C. parasiticum*, *C. pteridis*, and *C. scoparium*.

Control: Considering the natural recovery of infected plants during subsequent periods,

climatically unfavorable to the disease, no control measure is currently used. However, because of high reduction of the photosynthetic area, a significant loss in volume is expected, which may justify adoption of control measures to reduce potential losses. In this case, planting blight-resistant progenies, provenances, species, and clones is the best control strategy. The determination of inheritance model and genetic base is essential for a breeding program to obtain resistant materials.

3.1.1.3 *Rhizoctonia leaf blight*

In field plantations and clonal hedges, the infection starts on the leaves of the lower branches and progresses to the plant apex, causing intense leaf blight and defoliation (Silveira *et al.*, 2000) (Figure 6a). The disease is characterized by large and irregular leaf spots (Figures 6b and c). Depending on the disease intensity, whitish-mycelium-covered branches and leaves can be observed (Figure 6b), with the possible presence of whitish or light to dark-brown sclerotia (Figure 6d). Initially, the affected leaves show irregular light-gray to light-brown lesions of different sizes leading to blight of almost all leaves that become pale in color. Initially, infected leaves remain attached to the plant, but tend to fall with time. Other marked characteristics of the disease are hanging leaves attached by the fungal hyphae, adhering to one another, and connected by hyphae resembling a web (web-blight) (Figure 6c). The pathogen survives in soil, from which it disseminates by water splash to the surface of lower leaves or by growing epiphytically up the trunk reaching the higher portions of the canopy. In general, the fungus does not sporulate, and the most important features are sclerotia formation along the infected organs, right angle branching of hyphae, and presence of a constriction at the first septum in the branched hyphae when observed under the microscope. The sexual phase of some isolates of *Rhizoctonia solani* (*Thanatephorus cucumeris*) can be produced under controlled conditions, but is rarely found in natural infections (Silveira *et al.*, 2000).

Causal agents: *Rhizoctonia solani* (AG1-1B) and binucleate species of *Rhizoctonia* not yet identified.

Control: Although there are no studies about the genetic variability for resistance, it is unlikely that

resistant genotypes will be found within eucalypt species. However, artificial inoculations should be conducted to examine this hypothesis.

3.1.1.4 *Bacterial leaf blight*

At the initial phases of infection, the disease is characterized by water-soaked translucent leaf spots (Figure 7a), resulting from water leakage into the intercellular spaces, and is followed by intense defoliation, girdling, and mortality of the apical portion of highly susceptible materials (Figure 7b). As the disease progresses, the lesions become necrotic and dry with perforations and deformation of the leaf blade (Figure 7c). Its precise diagnosis requires laboratory examination, using exudation tests in which bacterial slime emanates from newly formed lesions.

Causal agents: Species of *Xanthomonas axonopodis*, *X. campestris*, *Pseudomonas cichorii*, and others (Gonçalves, 2003).

Control: Selection and planting of resistant genotypes.

3.1.1.5 *Phaeophleospora leaf blight*

Angular purple brown spots, distributed on both sides of mature leaves (Figures 8a–c), result from exudation of conidial masses (cirri) (Figure 8d), black sporulation resembling black mold. The disease is sometimes confused with the leaf spots caused by *Cylindrocladium*, plant bacterial infection or phosphorus deficiency, especially if observed on the upper leaf surface. However, typical sporulation of the pathogen is the major characteristic for diagnosing the disease. It generally occurs on old leaves of plants in the field or in the nursery.

Causal agent: *Kirramyces epicoccoides* (*Phaeoseptoria epicoccoides* = *Phaeophleospora eucalypti*), teleomorph *Mycosphaerella suttoniae* (Crous, 1998).

Control: No specific control measure has been used, but selection and planting of resistant genotypes may be effective.

3.1.1.6 *Pilidiella leaf spot*

Large, light brown to pale leaf spots, with dark concentric halos (Figure 9) formed by the



Figure 6 *Rhizoctonia* leaf blight. (a) High defoliation; (b) leaves with lesions and pathogen mycelium (arrows); (c) leaves with blight symptoms stuck each other, with the fungal mycelia holding the dead leaf; and (d) dark-brown sclerotia over infected leaves

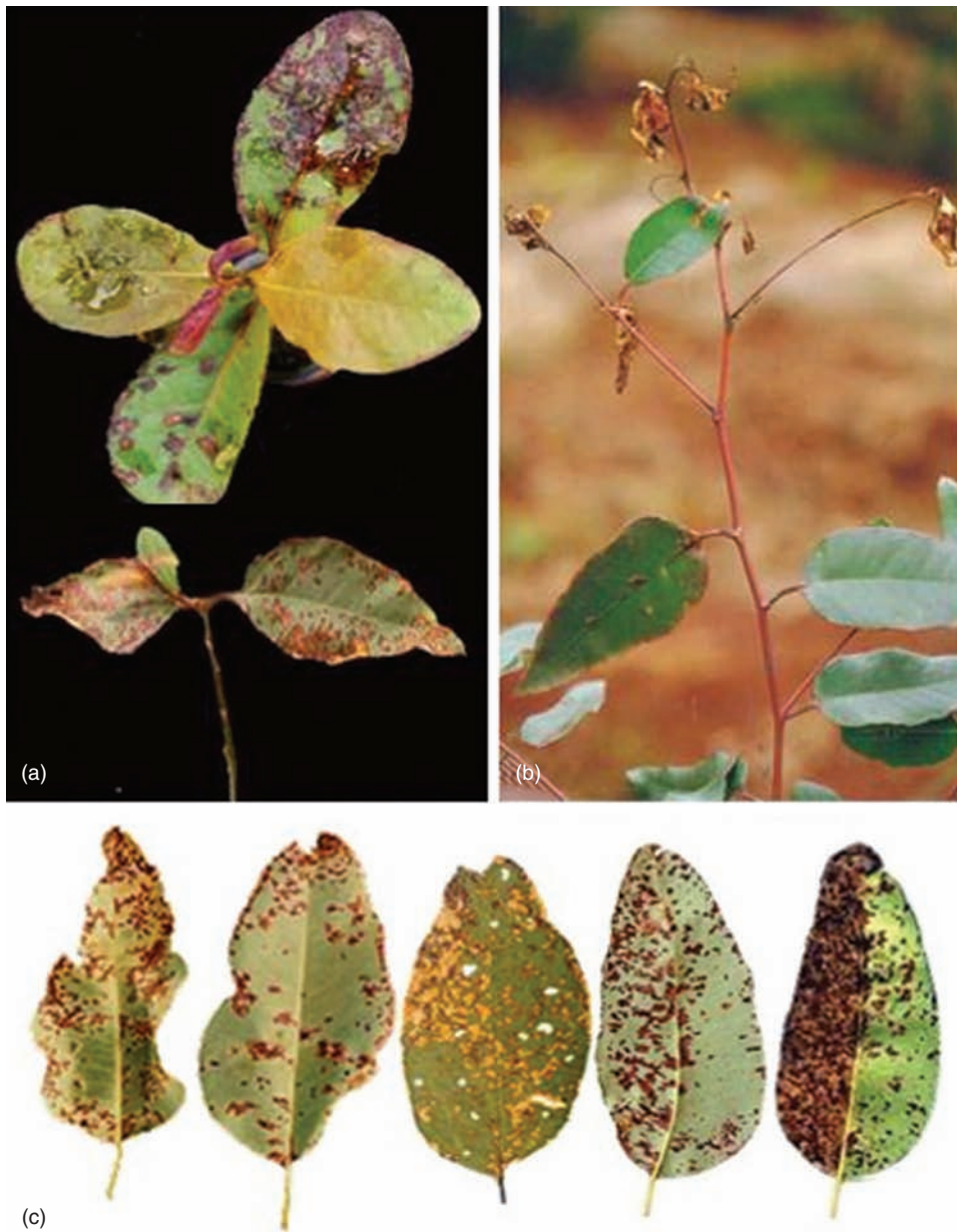


Figure 7 Bacterial leaf blight. (a) Water-soaked translucent leaf spot on *Eucalyptus cloeziana* plants; (b) death of apical portion of *E. urophylla*; and (c) differences in the symptoms

exudation of spore mass. Typical conidiophores and conidia of the pathogen can be observed by microscopic examination of histological sections through the pycnidium. This fungus penetrates host tissues through wounds (leaf friction by the strong winds), insect (thrips, larval, and

aphids), or mite injuries and also through the lesions caused by other leaf pathogens as *P. psidii* and *Cylindrocladium* spp. The photosynthetic area is reduced in case of high disease severity, but defoliation is not usually observed.

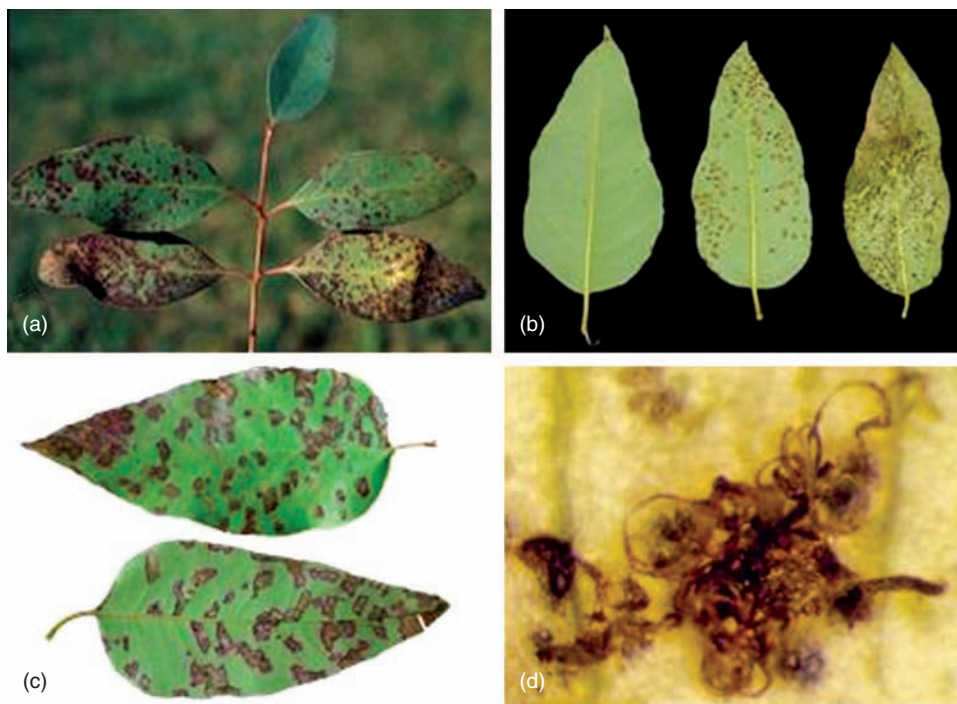


Figure 8 *Phaeophleospora* leaf spot. (a) Angular purple brown spot with fungi sporulation; (b) healthy leaf and leaves with symptoms; (c) detail of angular spots; and (d) exudation of conidial masses (cirri)

Causal agent: *Pilidiella eucaliptorum* (= *Coniella fragariae*).

Control: No specific control measures are employed, but planting of resistant clones is the best strategy of control.

3.1.1.7 *Aulographina* leaf spot

Circular or elongated dark-brown corky spots occur over the main vein, petiole, and twigs (Ferreira, 1989) (Figures 10a and b). Superficial dark-brown to black, elongated, curved, or branched fruiting bodies with a longitudinal slit (hysterothecia) are formed over the lesions (Ferreira, 1989) (Figure 10c). Asci and the ascospores of the fungus can be observed by microscopic examination of histological sections of the ascomata.

Causal agent: *Aulographina eucalypti*.

Control: The disease does not cause important damage, therefore, no specific control measures are being used.

3.1.1.8 *Mycosphaerella* leaf spot

The fungus infects young leaves of *E. globulus*, *E. nitens*, and *E. dunnii* and mature leaves of many other species, including *E. grandis*, *E. saligna*, *E. urophylla*, and their hybrids. The spots vary from circular to irregular circular shape and are light to pale brown, being darker on the lower leaf surface (Figures 11a–d). Dark ascomata, asci, and ascospores are formed on the lesions.

Causal agents: The disease is caused by various species of *Mycosphaerella*. Although it is not well known, it is believed that *M. parkii* (anamorph = *Stenella parkii*), *M. suberosa* (anamorph = not determined), and *M. suttoniae* (anamorph = *Phaeophleospora epicoccoides*) are the most common species on Eucalyptus in Brazil.

Control: No control measures have been adopted in Brazil, but for species such as *E. globulus*, *E. maidenii*, *E. dunnii*, and *E. nitens* it is possible to select faster growing clones, which rapidly pass to the less susceptible adult stage and, thus escape the disease.



Figure 9 *Pilidiella* leaf spot. Leaf spot showing pathogen penetration by wounds or other pathogens lesions

3.1.1.9 *Cryptosporiopsis* leaf spot

The pathogen infects leaves on the basal third branches without causing significant defoliation. The lesions are light to grayish-brown of varying sizes, semicircular or circular in shape (Figure 12a). They are encircled by a dark halo and the center has a dark rust colored spot of up to 6 mm diameter (Ferreira *et al.*, 1998) (Figure 12b). Typical conidiophores and conidia can be observed microscopically in the histological sections of conidiomata. Like *Pilidiella eucalyptorum* and *Hainesia lythri*, the fungus penetrates the host through wounds.

Causal agent: *Cryptosporiopsis eucalypti*.

Control: Since the disease does not cause significant economical losses, no specific control measures have been adopted. However, it is believed that planting of resistant genotypes may be the best strategy.

3.1.1.10 *Ralstonia* wilt

The first symptoms appear on 4- to 8-month-old plants. Initially, the leaves show wilting, becoming reddish, yellowish (Figure 13a), and latter pale to dark-brown in recently dead plants (Figure 13b). Stem section of wilted plants, exudates bacterial pus as cream-colored drops (Figures 13c and d).



Figure 10 *Aulographina* leaf spot. (a) Typical symptoms; (b) detail of corky spots over the main vein; and (c) typical hysterothecia

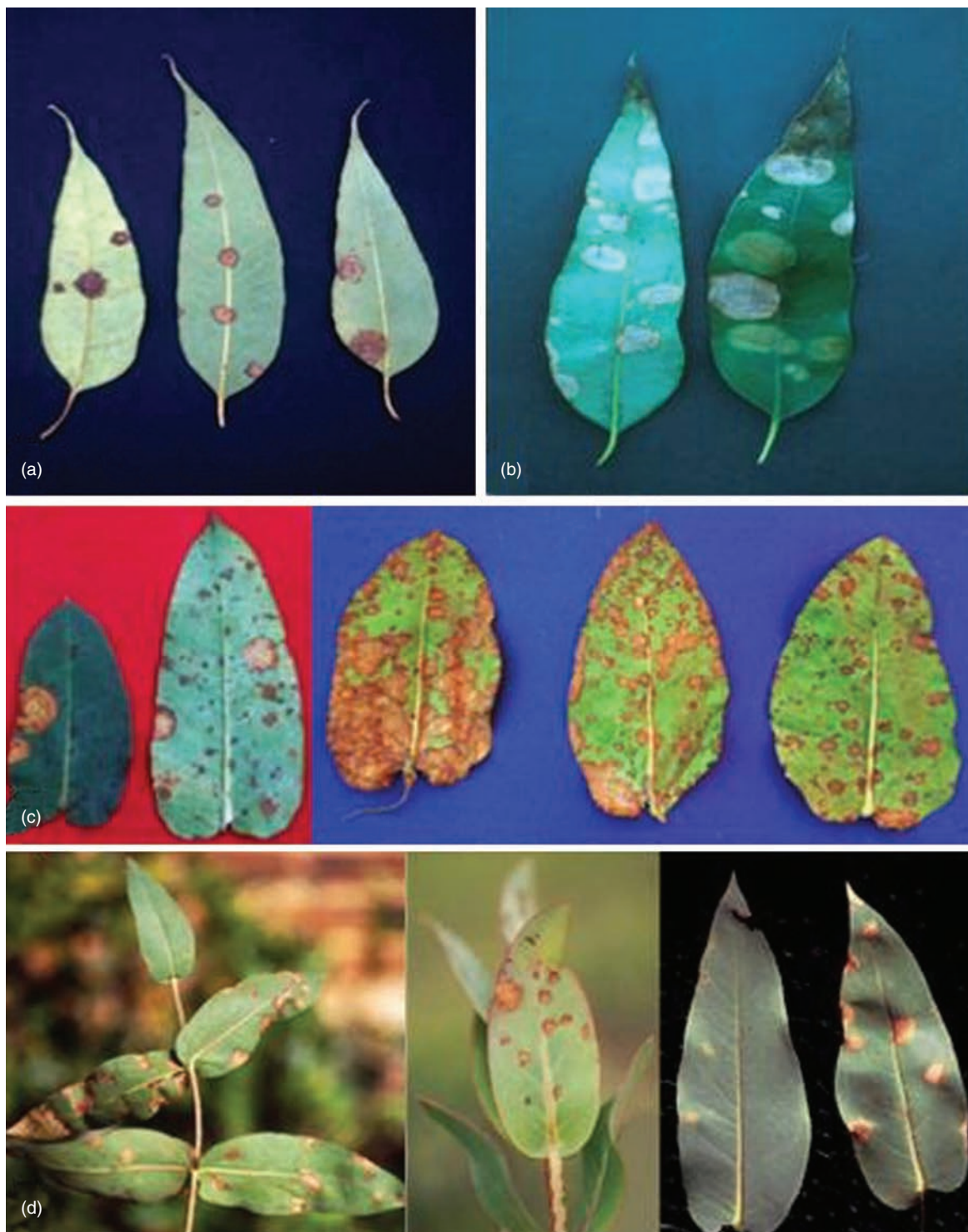


Figure 11 *Mycosphaerella* leaf spot. (a) Typical spot of *Mycosphaerella suberosa*; (b) typical spot of *M. parkii*; (c) lesions caused by *M. marksii*; and (d) typical spot of *M. juvenis*



Figure 12 *Cryptosporiopsis* leaf spot. (a) Lesions on the lower (left) and upper side (right) of leaf and (b) detail of spot lesions

Causal agent: *Ralstonia solanacearum* (= *Pseudomonas solanacearum*).

Control: Production of plantlets exempt of contamination with the pathogen.

3.1.1.11 *Ceratocystis* wilt, dieback, and canker

Initially are observed epicormic shoots along the trunk, and depression in the bark, then dieback, canker, wood discoloration, and wilt, leading to plant death (Figures 14a–d, Figures 15e–g).

Causal agents: *Ceratocystis fimbriata*.

Control: Planting of resistant genotypes.

3.1.1.12 *Eucalyptus* canker

Infection may be observed on 6-month-old plants (Figure 16a). When it occurs on young or adult plants of small stem diameter or on mini-stumps in clonal hedges, usually causes death by stem girdling. The canker can occur at any height of the stem, but usually occurs at the tree base (basal canker), causing superficial or deep lesions on the bark surrounded by callus (Figures 16b–d). A typical canker is formed if the lesion is deep and localized at a specific point of the trunk, while in superficial lesions, not reaching the cambium, the plant responds by producing new tissues resulting in the trunk swelling and bark cracking at the



Figure 13 *Ralstonia* wilt. (a) Plant with wilt symptoms and basal defoliation; (b) dead plants; (c) symptoms in the stem section immediately after cut; and (d) exudates bacterial pus (arrows)

infection point (Figure 16e). The weakened trunk at this point can break (Figure 16f), especially in regions of strong winds. Dark pycnidia and or perithecia produced on dead bark are the signs of the disease, essential for unequivocal diagnosis.

Causal agent: *Chrysophorte cubensis* (= *Cryphonectria cubensis* = *Diaporthe cubensis* = *Endothia eugeniae*).

Control: Planting of resistant species, provenances, families, or clones. *C. citriodora*,



Figure 14 Dieback, canker, and wilt caused by *Ceratocystis fimbriata*. (a) Development of basal shoots due to veins colonization by the fungus; (b) natural infection showing dieback; (c) dead plant; and (d) canker



Figure 15 Continue from Figure 14. Transversal (e) and radial (f) xylem discoloration symptoms; and (g) inoculated plant with wilt symptoms (left), and detail of stem discoloration (right)

C. torelliana, *E. cloeziana*, *E. pilularis*, *E. paniculata*, *E. pellita*, *E. urophylla*, *E. robusta*, *E. resinifera*, and *E. microcorys* are the more resistant species, while provenances of *E. grandis* and *E. saligna* are the most susceptible. However, there is a high intraspecific variability as to

resistance, which allows for selection and cloning of resistant genotypes for planting. In clonal hedges, canker can be controlled by selective shoot harvesting that reduces the stress on the mini-stumps, avoiding predisposition to the disease.



Figure 16 Eucalyptus canker caused by *Chrysophorte cubensis*. (a) Scattered death of *Eucalyptus saligna* plants in the field; (b) typical canker without bark removal; (c) typical canker (deep lesion flanked by callus); (d) basal canker; (e) trunk swelling, and bark cracking on the infection point; and (f) plant with canker, broken by wind at the infection point

3.1.1.13 Pink disease or rubellosis

Lesions and girdling are observed on the stem and branches of 1- to 3-year-old plants. A pink colored mycelium grows on young lesions (Figures 17a and b). Epicormic shoots emerge from below the girdled portion of the stem (Figure 17a). Later, the lesions dry out and lose the typical color (Figure 17c), leaving behind cankers (Figure 17d) on the thicker nongirdled stem and branches. The stem may break and lose apical dominance (Figures 17e and f). Pink to salmon colored mycelium, containing basidia and basidiospores may be observed on the lesions.

Causal agent: *Erythricium salmonicolor* (= *Corticium salmonicolor*).

Control: Selection and cloning of resistant genotypes.

3.1.1.14 Coniothyrium canker

Small discrete necrotic lesions with a strong depression in the bark are formed along the trunk (Figures 18a–c). The infection occurs through the younger tissues of the green stem. In highly susceptible genotypes, the lesions coalesce and cause extensive necrosis, generally followed by kinopocket formation (gummosis) (Figure 18d) and reduced plant growth, dieback, and emission of epicormic shoots along the trunk, due to partial cambium death and stem girdling. The fungus



Figure 17 Pink disease or *Eucalyptus rubellosis*, caused by *Erythricium salmonicolor*. (a) Pink mycelium and epicormic shoots below infected area; (b) and (c) stem lesion showing mycelium growth and bark cracking; (d) typical canker; (e) trunk breaks on the infection point; and (f) trunk break and loss of apical dominance

produces globose and substomatal pycnidia, containing conidiospores and conidia.

Causal agent: The disease, first described in South Africa, was attributed to *Coniothyrium zuluense* (Wingfield *et al.*, 1997). In Brazil, the disease was found on *E. grandis* and attributed to *Coniothyrium* sp. (Ferreira, 1977).

Control: In Brazil, it has not caused serious damage to the affected plants, thus no control measures have been adopted.

3.1.1.15 Botryosphaeria canker

The infection occurs in young tissues, resulting in breakage with the forking of the main stem at the infection point, gum exudation, darkening, depression, and bark cracking (Figures 19a–f). Generally, the lesions are superficial and confined

to the bark region, showing fructification of the pathogen. Pseudothecia containing asci and ascospores are formed on the lesions.

Causal agent: *Botryosphaeria ribis*.

Control: No specific control measures have been adopted.

3.1.2 Approaches to identify genetic resistance

3.1.2.1 Sources of disease resistance and breeding strategies

Considering that a relatively small number of clones, possibly with narrow genetic base, are planted in some regions of Brazil, it is imperative to establish an interspecies breeding program to obtain new resistant genotypes. Predicting the



Figure 18 *Coniothyrium* canker. (a), (b), and (c) Necrotic lesions with a depression in the bark; and (d) details of the xylem infection with kinopocket formation



Figure 19 *Botryosphaeria* canker. (a) Canker; (b) depression and bark cracking; (c) gum exudation; (d) break of apical trunk; (e) forking of main stem in the infection point; and (f) details of superficial infection in the bark region

eventual dry periods and predominance of high temperatures, drought resistant species, such as *E. camaldulensis*, should be considered for crossings. Other species, such as *E. pellita* (Papua New Guinea provenance) and *E. urophylla*, are excellent source of resistance to canker. The former is also an important source of resistance to leaf blights. On the other hand, *E. globulus* can be used as a gene source for reducing the lignin

and extractive contents, increasing wood density, improving pulp yield, and fiber quality, while *E. grandis* (Atherton or Coff's Harbour) has high adaptability and is suitable for high cellulose yield. Introgression of genes of *E. globulus* should be carried out using pollen obtained from elite mother plants from the southern part of Brazil, Uruguay, Spain, Chile, Portugal, or Australia, where it is possible to cultivate these plants as a pure

species. In the final composition, hybrids should contain 50–75% genes of *E. grandis*, obtained by a series of crossing and back crossings, and selfings if necessary. However, *E. deglupta*, *E. resinifera*, and *E. robusta* can also be tested, although little is known about their resistance to diseases, fiber quality, and adaptability. Since *E. globulus* and *E. pellita* have contrasting characteristics, for example, disease resistance, site adaptability and extractives, and lignin content, they should contribute a maximum of 12.5% of genes in constituting interspecific hybrids. Since wood lignin and extractives have essential functions in defense mechanisms, reduction of their concentrations can potentially result in higher susceptibility. Thus, in each generation of crosses, the resistant genotypes should be selected through artificial inoculation with specific pathogens of interest for each region. It is also essential to determine the resistance of commercial clones and its genetic basis to different diseases such as stem canker, rust, leaf blights (fungal and bacterial), ceratocystis wilt, and bacterial wilt through artificial inoculations of controlled crossing progenies. When resistance is dominant, resistant homozygous mother plants should be used for seed harvesting, since irrespective of the pollen origin, the progeny should be resistant.

3.1.2.2 Genomic approaches to identify genes for rust resistance

Currently, one of the biggest threats to Eucalyptus plantations is neotropical rust caused by the biotrophic fungus *P. psidii*. This disease attacks young trees, normally younger than 2 years old, thus it principally affects nurseries and recently planted areas, and depending on the severity of the infestation can result in a significant to almost total loss of production. In order to investigate the genes differentially expressed during *P. psidii* infection, Moon *et al.* (2007) constructed two SAGE (serial analysis of gene expression, Velculescu *et al.*, 1995) libraries representing susceptible and resistant material. Susceptible and resistant individuals were selected from a segregating population of half-siblings of *E. grandis* (Suzano Papel e Celulose), after being naturally infected with *P. psidii* Winter under field conditions. Bulks representing each phenotype

Table 4 The percentage of transcripts for the identified genes within each functional category^(a)

Category	Susceptible library (%)	Resistant library (%)
Metabolism and energy	4.4	20.1
Processes	32.3	31.4
Transport	1.1	2.3
Structural and organization of structure	4.8	16.5
Information pathways	19.3	1.7
Unknown function	38.1	28.0

^(a)Reproduce from Rison *et al.* (2000). © Springer

were formed using ten susceptible and ten resistant individuals selected from an *E. grandis* segregating population of half-siblings (Suzano Papel e Celulose). 31 645 and 39 964 tags, representing a cumulative gene count of 4095 and 5213, were generated from the susceptible and resistant bulks, respectively. The Z-test indicated that 239 were preferentially expressed in the susceptible library and 232 in the resistant. Using the National Center for Biotechnology Information (NCBI) public database (<http://www.ncbi.nlm.nih.gov>), which contains approximately 15 000 Eucalyptus ESTs and complementary DNAs (cDNAs), the authors were able to associate nucleotide sequences to 40 and 72 tags, susceptible and resistant, respectively.

Table 4 shows the differences in the number of transcripts within each functional category for the differentially expressed genes for the susceptible and resistant libraries. It can be seen that in the susceptible library the expression of genes involved in metabolism and the production of energy is considerably reduced showing about 25% of the level observed in the resistant material, probably reflecting the generally debilitated state of the infected susceptible plants. Genes preferentially expressed in the resistant library in this category were associated with the metabolism of sugar nucleotides and carbohydrates, indicating a higher biosynthetic activity directed toward the production of structural components.

Another interesting category was structural and organization of structure, which represents the genes involved in cell wall synthesis and cytoskeletal organization. This category was reduced in the susceptible material, approximately 33% of the level observed in the resistant library. Genes involved in the synthesis of the cell wall were

preferentially expressed in the resistant library, including those associated with cellulose and lignin biosynthesis. We also observed genes involved in the formation of the cytoskeleton, the tubulins, preferentially expressed in the resistant material.

Although the number of transcripts produced for the processes category was almost the same in the two libraries, the genes that they represent show differing pictures in the presence of the pathogen. In the susceptible library, the preferentially expressed genes demonstrated a response to oxidative stress and general stress response proteins. However, the resistant material showed the induction of genes more specifically for defense responses, including a catalase and a kinase.

The category representing the information pathways was almost ten times more expressed in the susceptible than the resistant material. These genes were associated with ubiquitin regulated protein turnover and proteases were mainly expressed in the susceptible library indicating a very active protein degradation system.

From these preliminary results Moon *et al.* (2007) suggest that two completely different processes are occurring in the susceptible and resistant plants. Firstly, from the generalized lesions observed in the susceptible material, it is obvious that plant is fighting a losing battle against the pathogen and is trying to limit the oxidative damage within the infected leaves and active proteolysis is occurring within the areas surrounding the necrotic lesions. Secondly, in the resistant plants various distinct processes are occurring simultaneously contributing to the expression of the resistant phenotype. Primary metabolism shows an increase in the expression of genes involved in the production of the raw materials for cell wall formation, sugar nucleotides, and carbohydrates. Coupled to this is the preferential expression of the genes associated with cellulose biosynthesis and the principal genes involved in lignin biosynthesis and polymerization, most probably re-enforcing the cell wall structure making fungal penetration more difficult. The preferential expression of genes involved in the formation of the cytoskeleton and vesicular transport factors would also indicate that the process of cellular polarization is occurring (reviewed by Schmelzer, 2002). Cellular polarization is defined as the process of cytoskeletal organization that allows the translocation of the cell nucleus

and transport of vesicles containing cell wall matrix material or specific defense related proteins directly to the site of infection. This process is documented as being an important process during the host cell response to pathogens. The authors also observed catalase and a kinase, preferentially expressed in the resistant material, probably involved in signal transduction during the initial infection and later in the process of systemic acquired resistance. Unfortunately, they were unable to identify any gene specifically involved in the destruction of the fungal pathogen, such as chitinases, thaumatin, or R genes, probably due to the lack of publicly available Eucalyptus ESTs that represent these genes. The authors suggest that sustainable resistance to *P. psidii* in *E. grandis* is the result of more than one predominant gene.

3.2 Wood Quality and the Transcriptome Involved in Eucalyptus Wood Formation

3.2.1 Wood quality requirements for pulp and paper, charcoal biomass fuel end-uses

Eucalyptus wood has gained a respectable position as raw material for several utilizations. Thanks to an excellent forestry technology, which was developed in countries like Brazil, Portugal, South Africa, Spain, Chile, Uruguay, and Australia, Eucalyptus has reached the status of “super-tree”. In a historical first moment, the efforts coming from tree breeding and silvicultural techniques were directed to the production of volume and/or weight in the trees. The acquired productivity would provide the desired competitiveness to the wood-based businesses. Wood-specific unit costs and forest operation costs were dramatically reduced by these technologies. Thanks to the high productivity of the planted forests and to the low production costs, Eucalyptus wood soon gained importance with several industries, such as pulp and paper, charcoal for steel manufacturing, lumber, and furniture. This raw material could easily supply fibers and biomass fuel, in unbeatable conditions. In a short period of time, the world quickly became surprised and enchanted with Eucalyptus for its fast growth rates and wood quality. The quality targets are becoming more and more sophisticated for each of the wood

utilizations. In the beginning, the selection of the raw materials was based on productivity (volume or dry weight), yields in the manufacturing conversion processes, and low wood costs. Today, the needs for product differentiation and for adding value in the production chain have oriented wood and trees to new requirements. Besides the traditional needs for productivity, yields, and costs, there are two new and important wood requirements: tree and wood uniformities. The control of forest variability may be seen as a simple task, but it is tremendously difficult. The anticipation using the cloning techniques was very high with the aim of controlling variability. However, the dream of having very similar trees has not been realized. Although a lot more uniform, the cloned trees show a considerable level of variability. This happens in the tree characteristics and in the wood quality (anatomical, physical, and chemical properties).

Currently, the foresters have placed attention on the production of trees having good shape, straight trunks, higher shape factors, and low percentage of bark. Additionally, the foresters also consider the resistance of the trees to pests and diseases, and tolerance to detrimental weather conditions (frost, winds, water deficits in the soil, flooding, etc). All these issues are demanding a lot of attention and research. The target for high quality *Eucalyptus* planted forests is not that simple. Even being healthy, with good shape and fast growth, the trees may not be suitable to some end-uses. For example, *E. grandis* trees, with fantastic growth rates, and tree shapes, may not be well suitable to the production of charcoal. The wood density may be too low for this purpose, the logs become very cracked when dried and the charcoal is poor in density, in calorific value per volume, very bulky, and generates more fines during manufacturing and handling. On the other hand, trees of *Eucalyptus robusta* may give low pulp yield in the conversion to kraft pulp due to the excess of extractives and/or lignin.

In short, now it is just the right time not only to have forests and trees, but also with end-use oriented qualities. A very good forest oriented for the production of charcoal will not be so desirable for kraft pulping, since both utilizations have opposite requirements in terms of wood lignin content. Whereas for charcoal, the best wood quality is the one with very high lignin content

and wood density but for kraft pulping, lower the lignin content the better. The wood density for pulping cannot be very high due to the problems with liquor impregnation.

Although the knowledge for the required wood characteristics is well understood by most researchers, the truth is that we are still finding many mistakes in the way breeding programs are being performed. The major mistake is the precarious sampling procedure being used in the majority of the tree and wood quality improvement programs. As far as the variability remains high for many qualitative parameters, even for cloned forests, the size of sampling should be larger than is generally used. Additionally, the sampling should be more representative, collecting samples from all segments of the population according to their frequency. Currently, there is a trend to take few trees with the average volume. Average sized trees are not synonymous of trees with average basic density, or average lignin content, etc. The first recommendation has to be made related to trees and wood sampling. Definitively, the size and the representativeness of these samples need to be improved. The second recommendation is to increase the number of analyses, the number of replications for each required quality parameters being evaluated. In general, the analyses are made with a single measurement and the mean value is used in the tree and wood improvement program. Depending on the statistical error that the forest breeder is willing to allow, the numbers of replications have to be considerably higher. There is a chance that the breeder is accepting what is accepted as a good genome but is definitively not good for a given property of the tree or wood. This is something that needs to be immediately reevaluated.

3.2.1.1 *Eucalyptus* wood quality requirements for the production of kraft pulp

Independent of the pulp mill, the pulp sector has fundamental issues including high productivity, high operational efficiency (no losses, no problems, no breaks, and no stops), low production costs, and uniform quality of the process and products. For achieving these targets, the raw material must be as uniform as possible so as not to cause

strong impacts on the pulping process and pulp qualities. To reduce this variability, the pulp mill engineers blend woods. Blending very different wood is acceptable if the result has a reasonable average quality. This is not an ideal situation, but it is the most usual.

When the pulp maker asks for uniform wood he is not only referring to wood basic density, but also to a series of wood quality parameters that are very important in the conversion process: proportion of bark in the wood chips, wood chip dimensions, decay level of the wood, wood moisture, wood chip bulk density, etc. The objective is to have a cooking and bleaching operation with the minimum variability, without undesirable surprises. The final quality must be uniform and within the specification limits and the process losses should be minimal. When the mill manager standardizes the wood intake, the first thing is to guarantee an appropriate quantity of dry wood being fed to the digesters. It is important to keep chip bulk density as uniform as possible for continuous addition of the same dry weight of wood to the digesters. The mill manager is carrying out "the management of the quantity of dry wood taken into the mill." When a uniform flow of wood dry weight is guaranteed, the liquor, steam, and chemical flows do not sharply change and the process runs smoothly, and the process and product quality is more easily achieved.

The second type of management is "management of wood variability" that tries to guarantee a low variability of wood parameter such as wood basic density, lignin content, extractives content, and active alkali consumption during kraft cooking, bleaching chemical consumption, yields in the conversion of the wood to bleached pulp, etc. There are other associated goals, such as to minimize the overload of dry solids to the recovery boiler turnover; reduce the specific wood consumption in cubic meters of wood by air-drying metric tons of pulp; guarantee stable quality and to reduce the production costs.

Management of dry quantities and management of wood variability are the basic requirements in any pulp mill. Having fulfilled these needs, the next type of management is "management for product differentiation" or "tailor-made orientation in the manufacture of products." This type of management requires substantial changes and offers important challenges to the mill personnel.

The changes may happen in wood quality (for example, low and high basic density woods), process conditions (for example, ECF or ECF-Light bleaching sequences), or even other recently added qualities (certified or noncertified wood). Differentiation of products is more easily achieved in mills with more than one fiberline. This means that the mill may run each fiberline with a differentiated product, without experiencing the usual troubles in making the transition from one product to another using a single fiberline. Anyhow, the tailor-made concept will only be a winner when the pulp maker has guaranteed the two previous types of management: dry quantity and variability. It is very simple to say, but very difficult to understand and to implement. Conflicts and misunderstandings are frequent between commercial, production, and product innovation areas in a pulp mill. Each of these areas has their own needs, product uniformity, product uniqueness, and product differentiation. As a result, few Eucalyptus pulp mills have products that may be said to be completely differentiated in their products portfolio. Most of the pulp manufacturers aim to have a single product, as uniform as possible, with the minimum cost, and maximum in productivity and in operational efficiency.

It is relatively difficult to say what is the single most important wood characteristic for a given pulp mill. The reason is that there is not a universal wood property to be managed. Depending on the pulp mill bottleneck, wood quality is defined to guarantee the maximum performance of a particular mill. The most common bottlenecks are the capacities of digester, recovery boiler, drying machine, lime kiln and alkanization, pulp washing, pulp bleaching chemicals. As a conclusion, it may be said that the type of mill bottlenecks will define the most desirable wood quality. This is the case for existing mills. For new greenfield mills, the quality may be previously built at the forest. However, soon the mill starts up, and the bottlenecks will appear to define the new wood quality standards. This is the reality, no doubt about it. This is also the cause of domestic conflicts within the company.

A list of important properties of Eucalyptus wood that influence the kraft pulp production process is presented.

- Wood cleanliness. This is an item not clearly understood by many forest managers, who try to mechanize their operations to reduce costs and do not realize the impact on wood cleanliness, which in turn affects pulp mill efficiency. The wood must be as clean as possible in terms of bark, soil, leaves, stones, decayed wood, etc.
- Wood basic density. To the pulp maker, the uniformity of wood density is very important. The more uniform is the wood, the better and simpler is the management of the quantity and variability. In many mills the design capacity allows the use of light woods, which are easier to be impregnated with cooking liquor and demand a lower alkali charge. However, when the digester is the bottleneck, denser wood is used to raise mill production in the digester. Again, the kind of bottleneck is the source of specifications for the wood quality. When the mill solves the bottleneck using wood quality, normally it is the forest breeders who need to produce wood with the desired specification, sometimes simply by changing clones or searching for new clones that fit the specifications. Pulp mills are short-term oriented and forest breeders are long term. The conclusions and behaviors are very different in both cases. Depending on the bottleneck experienced, some mills prefer to use low-density wood ($0.40\text{--}0.45\text{ g cm}^{-3}$), other medium ($0.50\text{--}0.55\text{ g cm}^{-3}$), and others high density ($0.55\text{--}0.60\text{ g cm}^{-3}$).
- Active alkali consumption and pulp yield. These variables are a consequence of the wood characteristics such as lignin, extractives, ash, density, *Eucalyptus* species, tree age, etc. The pulp maker wants to manufacture more and better, without overloading the production process. For this reason, the wood quality improvement programs need to be oriented by processing qualities in pulp manufacturing.
- Specific wood consumption ($\text{m}^3/\text{adt} = \text{cubic meters of wood by air-dry ton of pulp}$). This consumption is the result of many interconnected wood and kraft process variables: wood basic density, pulp yield, decay of wood, process losses of fibers, wood chipping operations, etc. The specific consumption of the wood is responsible for important fraction of the pulp cost. Wood is the main component in the pulp production cost, even in low-cost wood countries. For this reason, it is one of the most vital indicators to the mill manager. All qualitative wood characteristics that may impact the specific wood consumption should be optimized.
- Lignin content and lignin type. Lignin is abundant in *Eucalyptus* wood, especially those planted in Brazil. The total lignin content in Brazilian *Eucalyptus* wood varies from 24% to 32%, relatively high for hardwoods. This affects the pulp yield in the conversion to kraft pulp, as well as the consumption of active alkali and the generation of dry by the recovery boiler. There are *Eucalyptus* species with lower lignin contents and as such are more appropriate for pulping operations. *E. globulus* and *E. dunnii* when compared to *E. urograndis*, *E. urophylla*, *E. grandis*, and *E. saligna*, offer woods of 2–8% less total lignin (based on dry wood). For this reason, tree breeders are trying to combine in hybrids for cloning these wood characteristics in association with the fast growth rates from other species. Thus, gains in wood-specific consumption, alkali charges, and pulp yield should be significant. As a rule, for 1.2–1.5% reduction in lignin, the kraft pulp yield increases 1% based on dry wood. The active alkali consumption reduces around 0.2–0.3% for the same lignin reduction. Therefore, the production of low lignin clones is a new challenge for hybridization and cloning, without the need for genetic modification to insert genes for low lignin in the wood. At the same time, in Portugal, the need to maintain the purity of *E. globulus* and to improve the forest productivity should be possible through genetics and silviculture. With this in mind, the Portuguese foresters are willing to improve the productivity of *E. globulus* in equivalent tons of pulp per hectare per year. In Brazil, *Eucalyptus* commercial forests produce from 9 to 15 $\text{adt ha}^{-1}\text{ year}^{-1}$, while in Portugal the *E. globulus* forests produce 6–8 $\text{adt ha}^{-1}\text{ year}^{-1}$. Even with lower growth rates, the *E. globulus* trees are offering a competitive raw material thanks to the better wood basic density, lignin content, and pulp yield. However, not only the quantity of lignin in the wood is important, but also its quality. Lignin with high syringyl/guaiacyl ratio offers easy cooking and

pulp bleaching because guaiacyl type lignin is more difficult to be removed during the chemical reactions. This ratio in Brazilian *Eucalyptus* varies from 2 to 3, but in Portuguese *E. globulus* it may vary from 2.5 to 6.

- Extractives content. Extractives are undesirable in the pulping process because they have an impact on pulp yield and contaminate the process and the final product with pitch. The total extractives content in the *Eucalyptus* wood varies as a function of species, age, silvicultural stresses, pests, and diseases, etc. Values are variable from 1.5% to 6%. Extractive can be removed in several ways, normally extracting them with a solvent (water, caustic soda, ethanol, toluene, dichloromethane, etc.). In all cases, the lower the extractives, the better is the pulping, bleaching, and cleanliness of the final product.
- Ash content. Wood is rich in mineral elements and these are absorbed by the trees as nutrients. When the wood is harvested, the minerals are exported from the soil through the trees to the mills. Minerals are measured in the wood as ash content, after burning the sawdust of the sampled wood. Ash content in *Eucalyptus* wood varies from 0.3% to 1.0%, the most important minerals being calcium, potassium, and magnesium. With the trend to use closed water cycles in the pulp mills, these minerals are able to build up in these systems, bringing enormous problems with incrustations, pitch formation, formation of “stones” in the recovery boiler, etc. Ash content is very variable among the *Eucalyptus* species and for this reason it is an important parameter for tree breeding. When low ash woods are selected, the exportation of nutrients from the soil is minimized. The trees are more efficient in wood formation using or immobilizing fewer minerals in their tissues. These minerals, also known as nonprocess elements, leave the pulp mills as pollution (solid residues, air pollution particulate or dust, and dissolved ions in liquid effluents). This means that a fantastic natural resource is transformed into pollution, in mills not oriented to prevent or to develop clean production techniques for controlling and recycling these minerals. For this reason, the emphasis today is to prevent the intake of minerals into the mills, both those in the wood

composition, or as contaminants (soil, stones, etc.). Bark is also very rich in ash (about 5–10 times richer in ash content than wood), thus the contamination of logs and chips with bark brings additional amounts of minerals into the milling process affecting pulp making.

3.2.1.2 *Eucalyptus* wood quality requirements for the production of paper

All these wood characteristics and related consequences mentioned until now favor the pulp mills in their targets for productivity, costs, and efficiency. However, they are only part of the wood features to be evaluated. *Eucalyptus* pulp is a raw material for the manufacture of several grades of papers. For each paper grade and for each paper mill design, there are different wood and pulp quality requirements. It is important to mention that, independent of the type of paper to be manufactured, all the paper makers have what are known as basic physiological needs. These needs are similar to those for the pulp manufacturer: productivity, operational efficiency, quality, and costs.

Productivity requires a high-speed paper machine, fast drainage at the wet end, high consistency after the wet presses, and minimum number of paper sheet breaks along the machine. Quality implies the maximum percentage of paper in the specification range and minimum generation of broken paper lines. Machine operation efficiency is the dream of any paper manufacturer. He wants his machine working smoothly, at the maximum speed as possible, no breaks, and achieving the required quality in the manufactured products. The consequence of all this is that the specific unit cost is also optimized here. No doubt that a good pulp is the one able to provide good paper machine runnability and appropriate quality in the end product.

Some of the desired pulp properties are closely related to these performances and directly related to wood quality, others depend on the conversion of wood to pulp (cooking, bleaching), and many are a combination of these two factors influencing the pulp quality. For example, some properties that are related to pulping and bleaching are viscosity and degradation of cellulose chains, fiber deformations and individual fiber strengths,

surface charges in fibers, etc. One very important pulp property that is related to wood quality and pulp conversion is the hemicellulose content in the pulp. This parameter depends on the wood content and the ability of the pulping process to preserve it in the fibers.

There are many pulp properties that are dependent both on wood quality and on pulping/bleaching processes. There are also many cases where the exigencies are placed a lot on the wood quality, when the wood is not the only factor to determine the pulp quality for paper. Wood quality affects properties such as WRV—water retention value, WWS—wet web strength, fiber bonding, and individual fiber strength. However, other pulp properties are directly related to pulp manipulation processes, for example, the fine content of the pulp (parenchyma cells and fiber fragments) is generated in operations such as wood chipping, pulp pumping, pulp dynamic mixers, pulp dewatering presses, etc.

As a rule, there are some physiological properties that any pulp has to fulfil to be acceptable to papermakers. They are related to the following:

- Drainage at the wet end section in the paper machine. This behavior is very much affected by the fiber population (number of fibers per gram of pulp), by the initial or refined pulp freeness (drainability of the pulp measured as Canadian Standard Freeness or Schopper Riegler degree), by the Water Retention Value (hydration and swelling ability of the pulp furnish), and by the fine content in the pulp furnish.
- Paper sheet strength along the paper machine, mainly at the wet end and press section. Sheet behavior is very much dependent on the individual fiber strength, fiber bonding, furnish contaminants (shives, sand, solid debris, etc.) and consolidation of the paper web. Individual fiber strength is related to fiber wall thickness, fibril angle, fiber deformations, and micro-fractures and the *Eucalyptus* species used.

As long as the basic physiological needs are achieved it is possible to differentiate the product to be supplied to different markets or customers. One of the most important ways to reach differentiated products is through the production

of wood with different qualities providing very different pulp fibers allowing the manufacture of different products. This is what is known as tailor making the wood to the end product. The following wood and pulp quality parameters are important drivers in the differentiation of paper products:

- Fiber population or the number of fibers per gram of pulp. The fiber population is related to the weight of each individual fiber, to fiber coarseness and to the percentage of fiber wall in the fiber volume. There are a number of fiber properties associated to fiber population and fiber coarseness: fiber wall fraction (ratio between cell wall thickness and fiber ray), Runkel index, fiber flexibility index (ratio between the lumen diameter and fiber diameter), index of fiber collapsibility, ratio fiber wall thickness and fiber perimeter, wood basic density, and fiber length. Pulps with lower fiber population show better drainage in the wet end and the paper sheets are more porous, bulkier, more permeable, and absorbent. They are very much appreciated by paper makers because they allow faster machine speeds, if they furnish enough strength to the wet paper sheet.
- Individual fiber strength. This fiber characteristic is very difficult to be measured in short fibers such as those from *Eucalyptus*. There are tests correlated to this parameter such as the zero spans, very useful for predicting pulp quality and behavior in the paper machines.
- Fiber bonding ability. This test is measured by the wet/dry zero span technique or by other equipment for bonding tests, such as the Scott bond tester. It is also related to the hemicellulose content of the fibers, fiber population, fiber drainability (CSF or SR), fiber fines content, and fiber collapsibility.
- Fiber swelling. This fiber property is affected by the pulping and bleaching operations during pulp manufacture and by the pulp hemicellulose content. Several properties are associated to the swelling of fibers: water retention value, fiber charges, fiber wall microporosity, and fiber wall microfractures.
- Fiber deformations. The deformations in the fibers are measured as curl index, fiber kinks, fiber latency, and fiber microfractures in the

cell wall. They affect fiber strength, but they provide substantial improvements in the paper sheet porosity, bulk, smoothness, and water absorption.

Eucalyptus pulps are special products in the manufacture of bulky and/or opaque papers. Today, Eucalyptus pulps are preferred raw materials in the manufacture of tissue, printing and writing, carton boards, industrial filter, impregnation based, cigarette, and many other papers. Eucalyptus fibers may be the sole fiber in the pulp furnish or to be part of a blend with other short and/or long fibers.

Tissue papers demand softness, smoothness, absorption, bulk, and the exact strength to provide machine runnability and very fast drainage in the wet end. The fibers cannot collapse because this will flatten the paper surface, the paper becomes stronger in tensile, but all the tactile properties are lost. Pulp fines are also undesirable for two reasons: fiber bonding and building up in the paper machine white water system, reflecting in drainability losses. The most indicated Eucalyptus fibers for tissue manufacture are those showing low fiber population and consequently high coarseness, low fine content, low bonding ability, low hemicellulose content, high bulk, and water absorption in the manufactured paper sheets. Fiber deformations are also important, since these deformations improve the bulk, porosity and absorption of the paper. It is important to remember that fiber deformations may be artificially created in the pulp mills. The manufacture of industrial filter papers and impregnation-based papers demand the same properties, but at different levels. This means, to go to these specialty paper markets, the differentiation must be even more pronounced. Thus the simplest way to move between very specialty markets is to work towards very high coarseness (low fiber population, high wood basic density), low hemicellulose content, and to intensify fiber deformations (by high consistency presses, fiber shredding, or pulp flash drying).

For printing and writing papers, the desirable paper properties are formation, paper strength, porosity, dimensional stability, and opacity. A higher fiber population provides improved opacity associated with lower fiber coarseness. Fiber bonding and hemicellulose and pulp fine content is

important to improve strength. However, there are limits depending on each paper machine system and operation. A very high fiber population may improve opacity and formation, but drainage at the wet end and consistency after wet presses may deteriorate and machine speed is reduced. Fiber deformation may not be so important, but may help to balance the pulp properties, since it may be created by machines. A higher hemicellulose content favors refining, bonding, consolidation of the paper web and strength properties (tensile, burst, tear, folding). An ideal pulp should have high strength at the low levels of refining. However pulp refining raises energy costs, reduces the life of refiner discs, reduces machine drainage and machine speed, increases steam consumption and a very important paper property that is dimensional stability. Definitely, the best pulps are those showing good strength at low levels of refining. Paper maker is very sensitive to this. Besides these properties, there is another wood anatomical characteristic that is very important to printing grade paper: vessel element content and vessel dimensions (especially the diameter). Large, wide, and numerous vessels are undesirable for P&W (printing and writing) papers giving a defect known as vessel picking. The paper maker needs to have special conditions to combat the vessel-picking tendency in the paper. Thus, wood with smaller and less numerous vessels is preferred.

There are many other grades of papers manufactured with Eucalyptus pulps, but generally Eucalyptus fibers are used to improve paper formation, opacity, smoothness, dimensional stability, bulk, and porosity. The Eucalyptus fiber population in the pulps is rigid and difficult to collapse an important property for paper making. There is another key driver to paper makers for using fibers: the market pulp prices of this fiber. Thanks to the low production costs, high pulping yield, and lower chemical and wood consumption, Eucalyptus pulps are generally less expensive than softwood pulps. No doubt that the production costs are also key issues for paper makers. The same are to the entire Eucalyptus pulp and paper production chain.

Eucalyptus pulps have today gained the status of the most admired fiber supply. They are growing in an unbeatable rate in the paper business. Eucalyptus pulps are versatile and may be used as the single fiber or blended with others, such

as hardwoods, softwoods or recycled fibers. Wood and fiber quality improvements due to genetic and silvicultural operations and of the conversion process may contribute to further worldwide appreciation of Eucalyptus.

3.2.1.3 *Eucalyptus* wood quality requirements for wood charcoal manufacturing and biomass fuel

Brazil has had enormous success in the utilization of Eucalyptus biomass as firewood and for charcoal production. The first biomass fuel-oriented forest plantations were based on high wood density *Eucalyptus* species, such as *E. paniculata*, *E. camaldulensis*, *E. tereticornis*, *C. citriodora*, and *C. maculata*. Wood basic density is a fundamental wood characteristic because it leads to better quality charcoal and a higher calorific value per volume of wood or charcoal. However, tree growth rate is also important in order to reach the maximum possible production of dry biomass per hectare (trunk, branches and bark). Thus, if an *Eucalyptus* species leads to high-density wood, but its growth rate is poor, the production of total biomass may not economically attractive. For this reason, the wood biomass segment has directed its efforts to the diversification of species, and hybridization. Currently, more species are being explored in the forest breeding: *E. cloeziana*, *E. pellita*, *E. urophylla*, and hybrids, such as *E. urograndis*. These species are adapted to grow in tropical regions of Brazil, where the attacks of pests and diseases are more frequent, due to higher temperature and humidity. In case that charcoal production could migrate to temperate regions, where there are other species very suitable such as *E. dunnii*, *E. viminalis*, *E. benthamii*, *E. saligna*, *E. globulus*, and hybrids.

It is important to mention that wood density is a key property, but there are other wood characteristics that are important when wood is destined for fuel purposes. The trees should be as straight as possible to favor the feeding of the charcoal ovens or biomass furnaces or chippers. The bark content is required to be as low as possible, since the wood for energy is not debarked and Eucalyptus bark has a higher percentage of minerals, lower carbon content, basic density and calorific value. Furthermore,

the high phosphorous content in the bark may make difficult the utilization of this material in the production of some grades of charcoal.

The following wood properties should be included in a forest breeding program when wood is for biomass fuel and charcoal manufacture.

- Basic Wood density and forest growth rate. It is essential to optimize these two key parameters at once. Faster growth and higher wood density are desirable with the goal of maximizing dry biomass production per hectare.
- Lignin content, lignin composition, and carbon content. The lignin calorific value is higher than that obtained from the cellulose and hemicelluloses carbohydrates. The carbon content in lignin is somewhat higher than in the wood carbohydrates. For this reason, the higher the lignin content, the higher the carbon content, and the better the wood is for fuel. The ratio syringyl/guaiacyl is another point to be considered since guaiacyl lignin is richer in carbon content than syringyl. This means that, contrary to the wood for pulping, the wood for energy the syringyl/guaiacyl ratio should be as low as possible.
- Ash content. Wood minerals do not generate heat, but they consume heat during combustion and they also reduce the carbon content of the wood and bark based on dry weight content. Both wood and bark should have minimal ash content.
- Volatile extractives. Charcoal production implies substantial dry weight losses and the usual yields when manufacturing charcoal with Eucalyptus wood varies from 30% to 40%. Many types of wood extractives are very volatile and they are lost in the exhaust flue gases during controlled combustion. Depending on the manufacturing process, the extractive content of wood and bark may affect the charcoal yield.
- Wood moisture. High-density woods have lower moisture content due to the fact that they have less open spaces or porosity to hold water. For this reason, they are easily dried for consumption as fuel raw material. High speed drying is a good quality parameter for wood selection, thus the lower the wood and bark moisture, the better is the raw material is for biomass fuel.

- Anatomical composition of the wood. It has been proven that wood that is rich in vessel elements and parenchyma cells is less recommended for charcoal manufacturing. In general, the lower the density and the quantity of these anatomical elements, the more friable and poorer quality charcoal is produced.
- Fissured and cracked logs. It is very important that the logs have good dimensional stability, with minimal cracks and fissures. These wood defects give a higher fine content in the charcoal and a lower yield as consequence, since fines have to be screened out from the charcoal to improve its quality.

Wood may be engineered and improved by selection, genetics and silvicultural operations, therefore for maximization of results, it is important to know and to understand the parameters that need to be improved. They need to show good heritability and to add value to the conversion processes and to the end-use products. Sound planning, representative sampling, high quality evaluation, and data interpretation are vital to the success of any wood quality improvement program. Many of the current successes can be attributed to the excellent opportunities offered by hybridization and cloning techniques. Wood basic density has been the most important quality parameter to predict wood quality but it is not the unique wood characteristic. Another point is that wood basic density is a very good parameter to compare wood from the same species, or with similar behavior for a given utilization. For example, even with the same wood density, two very different species of *Eucalyptus* may show very different behavior in pulping and papermaking. *E. saligna* wood with 0.5 g cm^{-3} usually shows a completely different performance than *E. robusta* wood with exactly the same wood basic density.

Definitively, there are many roads to walk in the direction to the future. Thus, future improvement programs need to be efficient and efficacious if they are to maintain continuous competitiveness of *Eucalyptus* as source of industrial raw material. These programs must not only face economic and quality issues, but also environmental and social performances. These new roads are demanding new additional challenges, and because of this, science, technology, knowledge, and goodwill are to be soundly matched.

3.2.2 Characterization of the transcriptome involved in the process of wood formation in *Eucalyptus*

Wood is formed from the vascular cambium, which is the secondary meristem, responsible for the diametric growth of the stem. When the cambial initials divide, one cell remains at meristematic status while the other is destined to become a xylem or a phloem mother cell. During differentiation, the xylem mother cells undergo an ordered series of developmental steps that include cell division, cellular expansion, and deposition of secondary cell wall, giving rise to supporting, storage, and conducting cells (Larson, 1994). The xylem transport function is carried out by a specific cell type, the vessel elements, which undergo a differentiation process characterized by successive deposition of secondary cell wall layers, culminating in PCD (programmed cell death) and autolysis.

The genetic factors controlling wood formation in *Eucalyptus* are still not fully understood. In recent years, many genes involved in wood formation have been identified by large-scale genomic approaches in *Poplars*, *Pines*, and *Eucalyptus* (Hertzberg *et al.*, 2001; Lorenz and Dean, 2002; Paux *et al.*, 2004). Hertzberg *et al.* (2001) established a hierarchical pattern of gene expression through different zones of developing xylem in *Populus* by isolating cells at different stages of xylogenesis. By microarray analysis, the authors showed that genes encoding enzymes involved in cellulose and lignin biosynthesis, as well as a large number of transcription factors and potential xylogenesis regulators are under strict control at each xylem differentiation stage. Paux *et al.* (2004) developed a targeted approach of functional genomics by the construction of a xylem-leaves subtractive library to identify genes involved in the control of *Eucalyptus* wood formation. The two main classes of ESTs preferentially expressed in xylem were related to auxin signaling through ubiquitin proteolysis, cell wall biosynthesis, and remodeling. More recently, the induction of tension in wood has been used as a model to study wood formation due to its higher cellulose content and poor lignification (Paux *et al.*, 2005; Andersson-Gunnerås *et al.*, 2006).

In the attempt to investigate the genes expressed during juvenile wood formation in *E. grandis*,

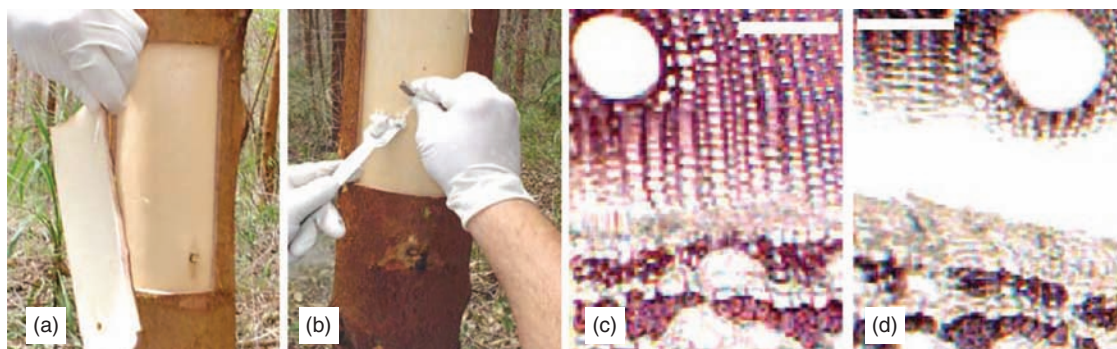


Figure 20 Biological material sampling. (a) Removal of the tree bark. (b) sampling the cambial region by scraping the tissue. (c) transversal section of *Eucalyptus* wood before removal of the bark. (d) transversal section of the *Eucalyptus* wood after removal of the bark. Bars = 100 μ m

when growth is maximized, Carvalho *et al.* (2008) produced two SAGE libraries (Velculescu *et al.*, 1995) from the cambial region of 3- and 6-year-old trees representing juvenile wood. Overall, the authors identified 444 genes involved in cellulose biosynthesis, nucleotide sugar metabolism, and lignin biosynthesis, as well other physiological processes related to wood formation. The tissue samples were collected from a half-sibling population of *E. grandis* originated from a single mother tree of a second generation of clonal seeds orchard, introduced from Coff's Harbour, Australia. A total of 40 3-year-old and 40 6-year-old trees were sampled from stands located in Itapetininga, State of São Paulo, Brazil (23°35'20" S and 48°03'11" W) at an altitude of 656 m. The 3- and 6-year-old trees were spaced at 3 \times 1.5 m and had an average height of 18 and 25 m, respectively. The cambial region of the trees were harvested in the same morning during the summer of 2003, by opening a window (20 \times 15 cm) in the bark at breast height, scraping the stem exposed tissue and the inner surface of the bark, and immediately freezing the sample in liquid nitrogen (Figures 20a and b). The stem tissue was scraped until the fibrous material below the differentiating cells was reached. The inner side of the bark was also scraped to guarantee that all the meristematic material would be represented in the SAGE libraries. This procedure was firstly used by Foucart *et al.* (2006) who showed that the cambial cells get adhered to the removed bark. Figures 20(c) and (d) also shows two transversal sections of *Eucalyptus* wood (xylem, cambial region, and

phloem) where it is possible to observe that the meristematic cells tend to follow the phloem cells when the bark is removed. Two bulks, one representing the 3-year-old trees and the other the 6-year-old trees, were made by grinding and mixing all the samples material.

The 3- and 6-year-old SAGE libraries were produced from the sequencing of 737 and 703 clones generating 22 660 and 22 024 tags, respectively. As the number of tags produced by the two libraries was very similar, the authors combined the sequencing reads from both and analyzed the data as one library representing the cambial region of juvenile *E. grandis* trees. The SAGE 2000 software extracted 43 304 tags from the sequencing data file and produced 26 958 tags with three or more copies representing 3066 unique tags or genes. From this total the authors have assigned ESTs to 657 unique tags (data not shown), 444 and produced 26 958 tags with three or more copies representing 3066 unique tags or genes. From this total the authors have assigned ESTs to 657 unique tags (data not shown), 444 with a defined function, and 213 with unknown functions. For most of the unique tags (79%) it was not possible to associate an EST or a complete cDNA due to the small number of *Eucalyptus* sequences currently available in public databases (Figure 21a).

The 444 tags that presented a tag-gene association were annotated and assigned to functional categories as defined by Rison *et al.* (2000). The most expressed categories were metabolism and energy and structure and organization of structure,

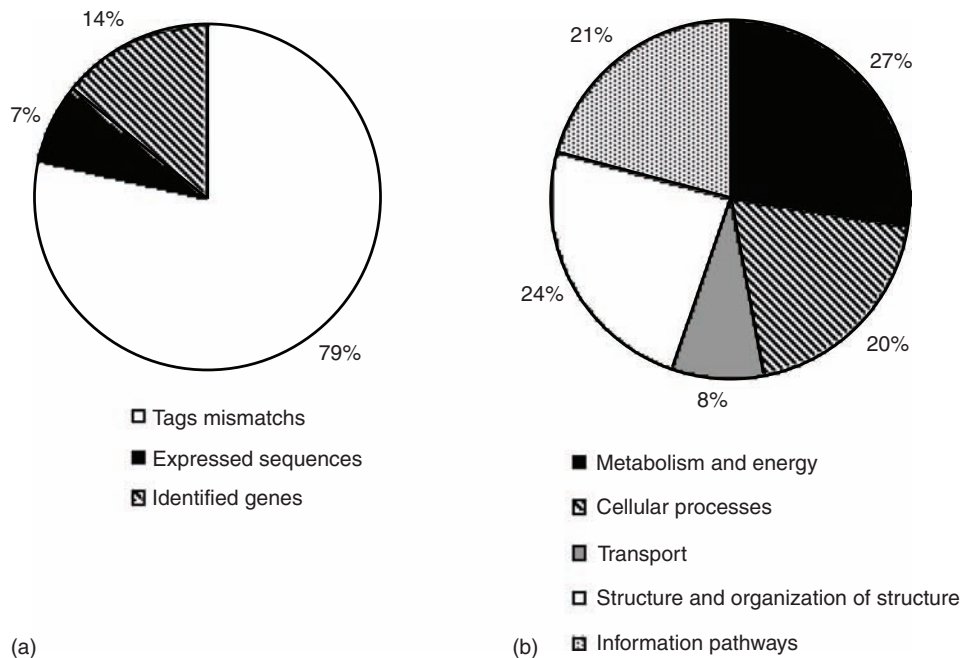


Figure 21 Distribution of the 3066 genes analyzed. (a) Tags mismatches: tags with no homology to the Eucalyptus public EST sequences, Expressed sequences: tags associated to an EST with unknown function in the GenBank, Identified genes: tags associated to an EST with a probable function defined in the GenBank. (b) Functional classification of the 444 identified genes. Functional Categories: Metabolism and energy, Cellular processes, Transport, Structure and organization of structure, and Information pathways

followed by information pathways and processes (Figure 21b).

It is important to note that among the tags in the subcategory autotrophic metabolism, there are genes associated with glycolysis, TCA cycle, alcoholic fermentation, and ATP synthesis. In nonphotosynthetic organs, carbohydrates are consumed through respiration to produce energy and carbon skeletons for cellular metabolism and biosynthesis of structural molecules, including cell wall polymers. However, the quantity of free O_2 in the cambial region might be limited by the physical barrier imposed by the bark and, also due to the O_2 consumption during respiration. Thus, a proportion of the necessary energy to secondary growth could be provided by alcoholic fermentation (Kimmerer and Stringer, 1988). The possible role of alcoholic fermentation as an alternative or principal supply of energy in juvenile *E. grandis* trees is also suggested by the presence of alcohol dehydrogenase and pyruvate decarboxylase transcripts. Other recent studies have reported the presence of transcripts

or proteins associated with anaerobic respiration during xylem formation and secondary growth (Gion *et al.*, 2005; Juan *et al.*, 2006; Ranik *et al.*, 2006).

Another interesting observation in the work of Carvalho *et al.* (2008) was the existence of tags representing light induced proteins and components of the photosystems I and II in wood forming tissues (Carvalho *et al.*, 2008). The presence of functional chloroplasts with active photosystems in the outer peridermal layers (chlorenchyma) and also in deeper stem tissues such as ray cells and pith, have been reported in many woody species (Pfanz *et al.*, 2002), what could explain the presence of transcripts related to photosynthesis in Carvalho's data. Supporting this result Celedon *et al.* (2007) also found rubisco proteins using 2D-LC-MS/MS in the same samples used in the previous study. The outer bark of the stems have a rather low permeability to gaseous diffusion, leading to accumulation of CO_2 in the intercellular air spaces, around 500–800 times higher ambient air (Pfanz *et al.*,

2002). Thus, stem photosynthesis may be an important mechanism to partially reduce the internal anaerobiosis, avoiding a further restriction to dark respiration and ATP production (Pfanz *et al.*, 2002).

3.2.2.1 Sugar-nucleotide metabolism and cell wall biosynthesis

Sugar-nucleotide metabolism provides the precursors for biosynthesis of hemicelluloses and pectins during wood formation. These precursors represent 65% and 26–36% of the primary and secondary cell walls, respectively (Mellerowicz *et al.*, 2001). The UDP-glucose dehydrogenase (UGDH) and UDP-glucuronate decarboxylase (UXS), responsible for UDP-glucuronate and UDP-xylose production, respectively, were represented by highly frequent tags (Figure 22a). However, only one UGDH tag with 22 copies was observed, while the total expression of the three UXS tags accounted for 47 copies. This expression pattern is consistent with previously reported findings pointing to a low activity of UGDH when compared to other enzymes in subsequent reaction steps in the same pathway, suggesting its function as rate limiting in the synthesis of matrix polysaccharides (Dalessandro and Northcote, 1977).

Among the UXS tags, one (TACTCGGTTG) with 27 copies is associated with the *Arabidopsis* *AtUXS3* soluble form. The other two, occurred at a frequency of 20 copies and are associated with the *Arabidopsis* *AtUX4* Golgi membrane form (Harper and Bar-Peled, 2002). A more detailed analysis of the ESTs associated with the *AtUXS4* tags revealed that the tag (TCATTATCAA) was present in both ESTs sequences distant 27 and 146 bp from the poly-A, suggesting that probably two alternative transcripts of *AtUXS4* are expressed in *E. grandis* wood forming tissues.

Besides UDP-xylose production, another important UDP-glucuronate-derived branch leads to pectin metabolism. The pectic polymers are mainly important in the primary cell wall where they comprise approximately 47% of the polysaccharides. Despite its importance in cell wall structure, only a few genes responsible for pectin biosynthesis have been identified to date.

In dicotyledonous plants, the primary cell wall consists basically of a cellulose microfibrils framework embedded in a polysaccharide matrix of pectin and cross-linked glycans (Carpita and Gibeaut, 1993). During cell extension occur modifications in the structure and composition of the cross-linked pectin-xyloglucans (Bourquin *et al.*, 2002). For example, xyloglucan endotransglycosylases (XETs) are responsible for cell wall remodeling during primary cell wall biosynthesis by cutting and rejoining the xyloglucan chains. XETs probably act during secondary cell wall deposition, as well as, by creating and reinforcing the connections between primary and secondary wall layers (Bourquin *et al.*, 2002). Despite its well-known importance in cell wall remodeling, only one XET tag with 19 copies was observed, although this result could be due to the lack of *Eucalyptus* XET ESTs publicly available. Moreover, an increased number of transcripts for pectinesterases and pectate lyases were observed during increased secondary growth in poplar tension wood (Andersson-Gunnerås *et al.*, 2006). However, under normal growth conditions experienced in our study, two pectinesterases tags at a frequency of 35 and 11 copies were observed in the cambial region library, and only one low expressed tag for a polygalacturonase (Figure 22a) in contrast to the 11 highly expressed genes identified in active poplar cambium (Geisler-Lee *et al.*, 2006).

3.2.2.2 Cellulose biosynthesis

Current models of cellulose biosynthesis involve not only CESA proteins but also membrane-associated proteins like KORRIGAN (endo-1,4- β -glucanase) and SUSY (sucrose synthase) (Joshi *et al.*, 2004). Five tags corresponding to *Eucalyptus grandis* cellulose synthase (*EgCesA*) genes were identified, one showing high similarity to the primary cell wall related cellulose synthase gene *EgCesA4*, and the other four showing high similarity to the secondary cell wall related genes *EgCesA1*, *EgCesA2*, and *EgCesA3*. Two tags (AATTGATATG and GAATCAAAAT) represented the *EgCesA1* gene, the first occurred in both ESTs sequences at a distance of 151 and 30 bp from the poly-A tail, indicating a possible alternative transcript (Figure 22a).



According to Ranik and Myburg (2006), some genes implicated in Eucalyptus secondary wall formation (*EgCesA1*, *EgCesA2*, and *EgCesA3*) have higher expression levels than those involved in primary cell wall formation (*EgCesA4*, *EgCesA5*, and *EgCesA6*) in xylem. Carvalho *et al.* (2008) observed the same transcriptional profiles for primary cell wall *EgCesA* genes. The *EgCesA4* tag shows low expression (Figure 22a) and the *EgCesA5* and *EgCesA6* tags are represented only as single-copy transcripts (Carvalho *et al.*, 2008). The higher expression of the secondary cell wall associated *CesA* genes was expected since the

The pool of UDP-glucose destined to cellulose synthesis can be produced either by UDP-glucose pyrophosphorylase or sucrose synthase. In Carvalho's study, the higher frequency of SUSY transcripts compared to UDP-glucose

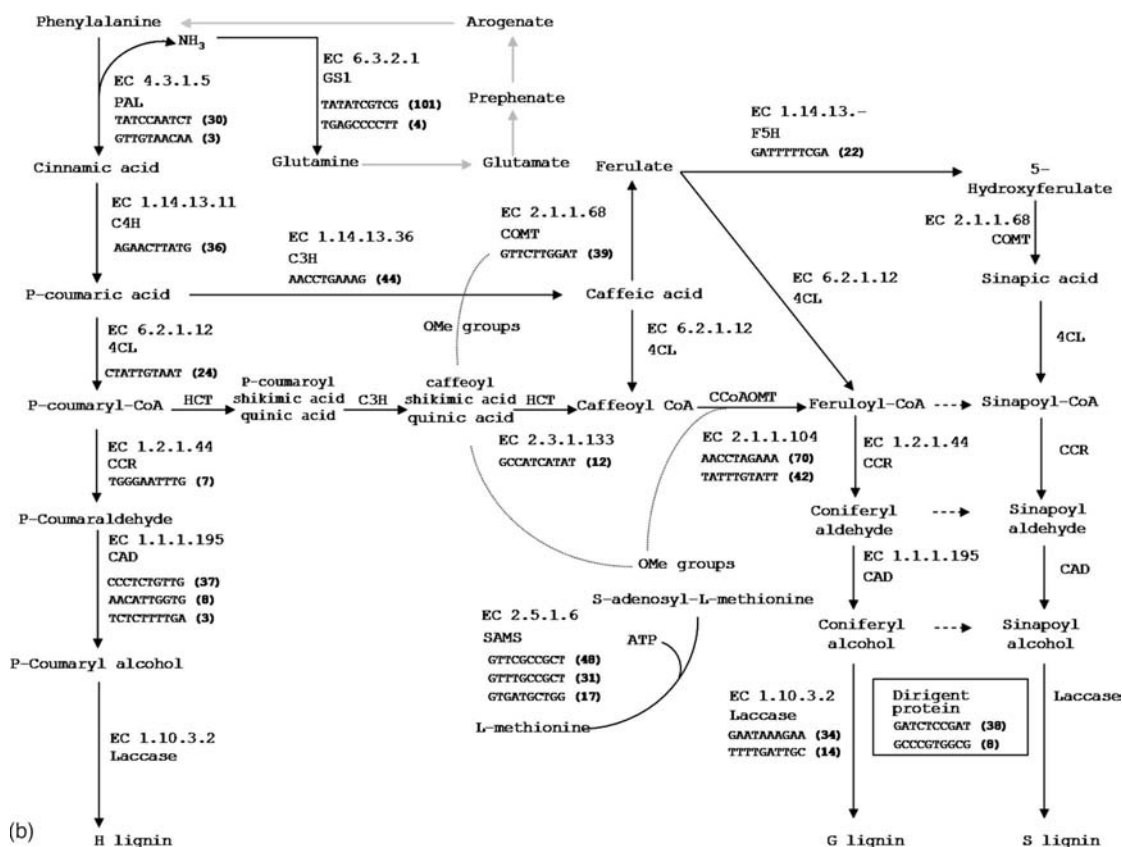


Figure 22 (Continued)

pyrophosphorylase transcripts strongly suggests that SUSY activity is probably the main source of UDP-glucose for cellulose synthesis in *E. grandis* differentiating xylem (Figure 22a). SUSY transcripts were the most abundant carbohydrate-active enzymes (CAZyme) transcripts in poplar and have been shown to be highly expressed during secondary cell wall biosynthesis in tension wood formation (Andersson-Gunnerås *et al.*, 2006). The membrane-associated SUS (SUSY) was detected in developing cotton fibers giving rise to a functional model for cellulose biosynthesis where this enzyme directly channels UDP-glucose to the membrane-bound cellulose synthesis complex avoiding competition from the cellular metabolic pool of UDP-glucose, and providing a more efficient cellulose synthesis (Amor *et al.*, 1995), especially important during active secondary growth.

3.2.2.3 Lignin biosynthesis

The phenylpropanoid pathway starts with the deamination of phenylalanine to cinnamic acid by PAL. Cinnamic acid is then converted to coumaric acid by C4H and diverted to monolignol synthesis (Figure 22b). Both enzymes from the early steps of phenylpropanoid biosynthesis were represented by highly frequent tags (Figure 22b). Both PAL genes (*AtPAL1* and *AtPAL2*) are believed to be the most important players in lignin synthesis during vascular lignification among the four *Arabidopsis* PAL genes (Raes *et al.*, 2003). The C4H tag was observed at a frequency of 36 copies and is associated with the poplar *PtreC4H2* gene, which is more xylem specific than the *PtreC4H1* and more weakly expressed in phloem (Lu *et al.*, 2006).

Only one tag representing the *E. camaldulensis* 4*CL1* gene (Figure 22b) was observed. The enzyme

4CL produces CoA thioesters of hydroxycinnamic acids, which are the precursors for H (*p*-coumaryl alcohol), G (coniferyl alcohol), and S (sinapyl alcohol) lignin subunits. One poplar 4CL gene was reported to be up-regulated in the cambial zone undergoing lignification (Hertzberg *et al.*, 2001) confirming its important role in the initial steps of lignin synthesis. Lignin polymers in angiosperm wood are composed by great amounts of G and S units while only small amounts of H units are added. This is because H units are predominantly deposited into the middle lamella and cell corners followed by G units, which are mainly laid down in the secondary wall, and S units that are deposited at the late stages of lignification (Lewis, 1999). Thus, it is expected that the key enzymes in the biosynthesis of the monolignols G and S show higher expression levels during secondary growth. The tags for COMT, CCoAOMT, and S-adenosylmethionine synthase (SAMS) showed a general higher expression level than those for 4CL and CCR (Figure 22b). These results agree with Paux *et al.* (2005), who suggested that the expression of 4CL, CCR, and CAD is under a common transcriptional control while COMT and CCoAOMT form another co-regulated transcriptional cluster. Similar expression profiles for these two enzymes was also observed by Hertzberg *et al.* (2001) supporting this hypothesis.

The determining step for the diversion of *p*-coumaryl-CoA for G and S monolignols synthesis is its conversion into *p*-coumaroyl shikimic acid/quinic acid by hydroxycinnamoyltransferase (HCT), since *p*-coumarate 3-hydroxylase (C3H) is not able to use *p*-coumaryl-CoA as substrate (Schoch *et al.*, 2001). It has been shown that *p*-coumaroyl shikimate and *p*-coumaroyl quinate are important intermediates in the phenylpropanoid pathway with HCT acting both upstream and downstream of C3H, producing caffeoyl CoA (Hoffmann *et al.*, 2004). Interestingly, the C3H tag, similar to the *Arabidopsis* CYP98A3 class of P450 gene whose expression is more evident in lignifying vascular cells (Nair *et al.*, 2002), was almost four times more expressed than the HCT tag (Figure 22b). Alternatively, C3H can also act on *p*-coumaric acid precursors producing caffeic acid, which in turns can be diverted to ferulate, by COMT, or to caffeoyl CoA, by 4CL (Figure 22b).

Although COMT was first believed to convert caffeic acid into ferulate (Dixon, 2001), it was

subsequently shown that COMT preferentially catalyzes the conversion of 5-hydroxyferulate, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol into sinapic acid, sinapaldehyde, and sinapyl alcohol, respectively, and thus acts preferentially on ferulic acid/coniferaldehyde 5-hydroxylase (F5H) derived products (Parvathi *et al.*, 2001). A differential regulation for F5H and COMT genes is supported by recent findings obtained through a proteomic approach where F5H proteins were not found among the expressed proteins during poplar cambial regeneration, while COMT isoforms were detected at all stages (Juan *et al.*, 2006). Consistent with this previous result, our data showed that COMT transcripts expression was almost twice the F5H expression level (Figure 22b).

According to Carvalho's data, the CCoAOMT transcripts had the highest expression level of all lignin biosynthetic enzymes observed in *E. grandis* wood forming tissue (Figure 22b). This is in agreement with the results of Paux *et al.* (2004) that showed a preferential expression of this gene during Eucalyptus wood formation. CCoAOMT adds a methyl radical to caffeoyl CoA, producing feruloyl CoA in an alternative route for monolignol production (Zhong *et al.*, 1998). The importance of this alternative route was demonstrated by the down-regulation of CCoAOMT in transgenic tobacco and poplar plants leading to an altered S/G ratio and significantly decreased lignin content (Zhong *et al.*, 1998, 2000). As the number of CCoAOMT transcripts is much higher than the number for COMT, we suggest that the synthesis of S and G units is being preferentially carried out directly from caffeoyl CoA in *E. grandis*.

It is interesting to note that CCoAOMT and SAMS tags have similar high expression levels (Figure 22b), even though the presence of a common transcriptional regulatory system for both genes is still unknown. Although SAMS is a housekeeping enzyme involved in methionine metabolism, its activity occurs in xylem tissue undergoing secondary growth (Juan *et al.*, 2006). According to Cantón *et al.* (2005), SAMS might be functioning in a local providing of methyl groups consumed by CCoAOMT and COMT thus ensuring high rates of lignification.

The lignification processes also requires a large nitrogen input to support the first step

in the phenylpropanoid biosynthesis catalyzed by PAL. Extensive amounts of ammonium are liberated through phenylalanine deamination and an efficient system to recycle nitrogen is needed to prevent a severe N deficiency in plants during active lignification (Cantón *et al.*, 2005). Therefore, the authors proposed a mechanism in which the liberated nitrogen is locally recycled and re-incorporated into glutamine by GS1 (glutamine synthetase). Carvalho's results also suggest a possible role for GS1 during lignification as the GS1 tag showed high expression (101 copies), occupying the 17^o position among the 50 most expressed tags in *E. grandis* juvenile wood forming library (Table 4; Figure 22b).

The final step in lignin biosynthesis is the polymerization of monolignols that is conducted by peroxidases and laccases (Baucher *et al.*, 2003). Although the role of laccases in lignin polymerization is still a matter of debate, their importance during wood formation has been reported (Paux *et al.*, 2004). The results observed by Carvalho *et al.* (2008) corroborate a greater role for laccases in lignin polymerization during Eucalyptus wood formation due to the lack of tags representing peroxidases (Figure 22b). Supporting this idea, no significant decrease in peroxidase expression was observed in poplar tension wood (characterized by decreased lignin content), while the laccase gene lac3 is co-regulated with the lignin biosynthesis genes (Andersson-Gunnerås *et al.*, 2006). However, Hertzberg *et al.* (2001) identified two peroxidase genes up-regulated in poplar lignifying tissues, as well as, two laccases with an overlapping expression pattern, indicating specific but different roles in the polymerization process. The authors also demonstrated the induction of a dirigentlike protein coincident with lignification. Dirigent proteins can mediate the monolignols coupling mainly in the S (1) sublayer and middle lamella of lignifying secondary xylem cell walls (Burlat *et al.*, 2001). Carvalho *et al.* (2008) found one low expressed tag similar to the *Forsythia* × *intermedia* dirigent protein and a highly expressed tag similar to the *Picea glauca* dirigent protein (Figure 22b). The authors suggested that probably there are similar roles for laccases and dirigent proteins in monolignol polymerization in *E. grandis*.

The data obtained by Carvalho *et al.* (2008) offers an insight into the expression of functionally

related genes involved in cell wall biosynthesis during the first years of development of Eucalyptus, and this information is just beginning to be combined to produce an overview of the important genes for wood formation. In the near future, the information generated by functional genomics, molecular markers, transgenesis, will provide the breeders with important information to decide in the selection of the best genotypes to improve productivity, resistant to biotic and abiotic stresses, and better wood quality.

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Pines

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1. INTRODUCTION

Pines are a group of evergreen and resinous conifers (Table 1). The bark of the most pines species is thick and scaly, but for some it is thin and flaking. The branches are produced in regular pseudowhorls, thus appearing like a ring of branches arising from the same point (Bannister, 1965; Axelrod, 1986; Ades and Simpson, 1991; Burdon *et al.*, 1992a,b; Bergman *et al.*, 1995; Burdon and Carson, 2000). Many pines are uninodal, producing just one whorl of branches each year from buds residing at the tip of the year's new shoot growth. However, others are multinodal, producing two or more whorls of branches per year (Bannister, 1965; Burdon *et al.*, 1992a, b; Bergman *et al.*, 1995; Burdon and Carson, 2000). The spiral growth of branches, needles, and cone scales are arranged in Fibonacci number ratios. The new spring shoots are sometimes called candles; they are light colored and point upwards at first, then later darken and spread outwards (Bannister, 1965; Bergman *et al.*, 1995). These candles offer foresters a means to evaluate fertility of the soil and vigor of the trees (Burdon *et al.*, 1992a, b; Burdon and Carson, 2000).

1.1 History, Origin, and Distribution

Pines are native to most of the northern hemisphere (Burdon *et al.*, 1992a, b; Burdon and Carson, 2000). In North America, they range from

the Arctic south to Nicaragua and Hispaniola, with the highest diversity in Mexico and California (Bannister, 1965; Bergman *et al.*, 1995). In Eurasia, they range from Portugal and Scotland east to the Russian Far East, Japan, and the Philippines, and south to northernmost Africa, the Himalayas and Southeast Asia, with one species (Sumatran pine) just crossing the equator in Sumatra (Bannister, 1965; Burdon *et al.*, 1992a, b; Bergman *et al.*, 1995; Burdon and Carson, 2000). Pines are also extensively planted in many parts of the southern hemisphere (Burdon *et al.*, 1992a, b). Herein in this chapter, we describe the history, origin, and distribution of Loblolly pine (*Pinus taeda* L.), Scots Pine (*Pinus sylvestris* L.), and Monterey Pine (*Pinus radiata* D. Don).

1.1.1 Loblolly pine

Loblolly pine (*P. taeda* L.) is one of the pines native to the southeastern United States. Loblolly pine is the most important commercial timber in the southeastern United States, comprising over 50% of the standing pine in the southeast (Dvorak *et al.*, 2000). The species name, *taeda*, comes from the Latin word for "torch of resinous wood". Loblolly pine belongs to the yellow pine group and is considered the most commercially valuable forest species in the southern United States (Farjon and Styles, 1997; Dvorak *et al.*, 2000). Its native range extends from southern New Jersey to Florida, and as far west as Texas. In the Chesapeake Bay

Table 1 Pine species distributed over the world

Serial number	Scientific name	Common name	Serial number	Scientific name	Common name
1	<i>Pinus brutia</i>	Turkish pine	52	<i>Pinus contorta</i>	Lodgepole pine
2	<i>Pinus canariensis</i>	Canary Island pine	53	<i>Pinus coulteri</i>	Coulter pine
3	<i>Pinus cembra</i>	Swiss pine	54	<i>Pinus echinata</i>	Shortleaf pine
4	<i>Pinus halepensis</i>	Aleppo pine	55	<i>Pinus edulis</i>	Colorado pine
5	<i>Pinus heldreichii</i>	Bosnian pine	56	<i>Pinus elliotii</i>	Slash pine
6	<i>Pinus mugo</i>	Mountain pine	57	<i>Pinus flexilis</i>	Limber pine
7	<i>Pinus nigra</i>	European Black pine	58	<i>Pinus glabra</i>	Spruce pine
8	<i>Pinus peuce</i>	Macedonian pine	59	<i>Pinus jeffreyi</i>	Jeffrey pine
9	<i>Pinus pinaster</i>	Maritime pine	60	<i>Pinus lambertiana</i>	Sugar pine
10	<i>Pinus pinea</i>	Stone pine	61	<i>Pinus longaeva</i>	Great Basin Bristlecone pine
11	<i>Pinus sylvestris</i>	Scots pine	62	<i>Pinus monophylla</i>	Single-leaf Pinyon
12	<i>Pinus amamiana</i>	Yakushima White pine	63	<i>Pinus monticola</i>	Western White pine
13	<i>Pinus armandii</i>	Chinese White pine	64	<i>Pinus muricata</i>	Bishop pine
14	<i>Pinus bhutanica</i>	Bhutan White pine	65	<i>Pinus palustris</i>	Longleaf pine
15	<i>Pinus bungeana</i>	Lacebark pine	66	<i>Pinus ponderosa</i> (syn. <i>P. washoensis</i>)	Ponderosa pine
16	<i>Pinus dalatensis</i>	Vietnamese White pine	67	<i>Pinus pungens</i>	Table Mountain pine
17	<i>Pinus densata</i>	Sikang pine	68	<i>Pinus radiata</i>	Monterey pine or Radiata pine
18	<i>Pinus densiflora</i>	Japanese Red pine	69	<i>Pinus reflexa</i>	Southwestern White pine
19	<i>Pinus eremitana</i>	North Vietnam White pine	70	<i>Pinus remota</i>	Texas Pinyon or Papershell Pinyon
20	<i>Pinus fenzeliana</i>	Hainan White pine	71	<i>Pinus resinosa</i>	Red pine
21	<i>Pinus fragilissima</i>	Wulu pine	72	<i>Pinus rigida</i>	Pitch pine
22	<i>Pinus gerardiana</i>	Chilgoza pine	73	<i>Pinus sabineana</i>	Gray pine, Foothill pine, or Digger pine
23	<i>Pinus henryi</i>	Henry's pine	74	<i>Pinus serotina</i>	Pond pine
24	<i>Pinus hwangshanensis</i>	Huangshan pine	75	<i>Pinus strobus</i>	Eastern White pine
25	<i>Pinus kesiya</i>	Khasi pine	76	<i>Pinus taeda</i>	Loblolly pine
26	<i>Pinus koraiensis</i>	Korean pine	77	<i>Pinus torreyana</i>	Torrey pine
27	<i>Pinus krempfii</i>	Krempf's pine	78	<i>Pinus virginiana</i>	Virginia pine
28	<i>Pinus latteri</i>	Tenasserim pine	79	<i>Pinus apulcensis</i>	Apulco pine
29	<i>Pinus luchuensis</i>	Luchu pine	80	<i>Pinus arizonica</i>	Arizona pine
30	<i>Pinus massoniana</i>	Masson's pine	81	<i>Pinus ayacahuite</i>	Mexican White pine
31	<i>Pinus merkusii</i>	Sumatran pine	82	<i>Pinus caribaea</i>	Caribbean pine
32	<i>Pinus morrisonicola</i>	Taiwan White pine	83	<i>Pinus cembroides</i>	Mexican pinyon
33	<i>Pinus orthophylla</i>	Wuzhi Shan White pine	84	<i>Pinus chiapensis</i>	Chiapas White pine
34	<i>Pinus parviflora</i>	Japanese White pine	85	<i>Pinus cooperi</i>	Cooper's pine
35	<i>Pinus pumila</i>	Siberian Dwarf pine	86	<i>Pinus cubensis</i>	Cuban pine
36	<i>Pinus roxburghii</i>	Chir pine	87	<i>Pinus culminicola</i>	Potosi pinyon
37	<i>Pinus sibirica</i>	Siberian pine	88	<i>Pinus devoniana</i> (syn. <i>P. michoacana</i>)	Michoacan pine
38	<i>Pinus squamata</i>	Qiaojia pine	89	<i>Pinus durangensis</i>	Durango pine
39	<i>Pinus tabuliformis</i>	Chinese Red pine	90	<i>Pinus engelmannii</i>	Apache pine
40	<i>Pinus taiwanensis</i>	Taiwan Red pine	91	<i>Pinus estevezii</i>	Estevez's pine
41	<i>Pinus thunbergii</i>	Japanese Black pine	92	<i>Pinus gordoniana</i> (syn. <i>P. douglasiana</i>)	Gordon's pine
42	<i>Pinus uyematsui</i>	Uyematsu White pine	93	<i>Pinus greggii</i>	Gregg's pine
43	<i>Pinus wallichiana</i>	Blue Pine or Bhutan pine	94	<i>Pinus hartwegii</i>	Hartweg's pine
44	<i>Pinus wangii</i> (syn. <i>P. kwangtungensis</i>)	Guangdong White pine	95	<i>Pinus herrerae</i>	Herrera's pine
45	<i>Pinus yunnanensis</i>	Yunnan pine	96	<i>Pinus hondurensis</i> (syn. <i>P. caribaea</i> var. <i>hondurensis</i>)	Honduras pine
46	<i>Pinus albicaulis</i>	Whitebark pine			
47	<i>Pinus aristata</i>	Rocky Mountains Bristlecone pine			
48	<i>Pinus attenuata</i>	Knobcone pine			
49	<i>Pinus balfouriana</i>	Foxtail pine			
50	<i>Pinus banksiana</i>	Jack pine			
51	<i>Pinus clausa</i>	Sand pine			

Table 1 Pine species distributed over the world (*continued*)

Serial number	Scientific name	Common name	Serial number	Scientific name	Common name
97	<i>Pinus jaliscana</i>	Jalisco pine	109	<i>Pinus orizabensis</i>	Orizaba Pinyon
98	<i>Pinus johannis</i>	Johann's Pinyon	110	<i>Pinus pinceana</i>	Weeping Pinyon
99	<i>Pinus lawsonii</i>	Lawson's pine	111	<i>Pinus praetermissa</i>	McVaugh's pine
100	<i>Pinus leiophylla</i>	Chihuahua pine	112	<i>Pinus pringlei</i>	Pringle's pine
101	<i>Pinus lumholtzii</i>	Lumholtz's pine	113	<i>Pinus pseudostrobus</i>	Smooth-bark Mexican pine
102	<i>Pinus maximartinezii</i>	Big-cone Pinyon			
103	<i>Pinus maximinoi</i> (syn. <i>P. tenuifolia</i>)	Thinleaf pine	114	<i>Pinus quadrifolia</i>	Parry Pinyon
104	<i>Pinus montezumae</i>	Montezuma pine	115	<i>Pinus rzedowskii</i>	Rzedowski's pine
105	<i>Pinus nelsonii</i>	Nelson's Pinyon	116	<i>Pinus strobiformis</i>	Chihuahua White pine
106	<i>Pinus occidentalis</i>	Hispaniolan pine	117	<i>Pinus tecunumanii</i>	Tecun Uman pine
107	<i>Pinus oocarpa</i>	Egg-cone pine	118	<i>Pinus teocote</i>	Teocote pine
108	<i>Pinus patula</i>	Patula pine	119	<i>Pinus tropicalis</i>	Tropical pine

region this rapidly growing tree thrives in the maritime forest, at the Bay's edge, in a variety of soils, including well-drained upland areas with poor nutrient concentrations to poorly drained lowland areas and abandoned fields (Farjon and Styles, 1997; Dvorak *et al.*, 2000). It prefers the Bay's relatively long, hot, and humid summers and adapts well to tree farm environments.

The name loblolly also means a "low wet place", but these trees are not limited to that specific habitat (Farjon and Styles, 1997). Other common names include: oldfield pine, due to loblolly's status as an early colonizer of abandoned fields; bull pine, due to its size; and rosemary pine, due to loblolly's distinctive fragrance compared to the other southern pines (DeCleene and DeLey, 1976; Devey *et al.*, 1999, 2002). With the advent of fire control, loblolly pines have come to dominate areas in the Deep South that were once populated with greater numbers of Longleaf pine and, especially in Florida, Slash Pine. Loblolly's rate of growth is rapid, even among the generally fast-growing southern pines. The yellowish, resinous wood is highly prized for lumber, but it is also used for pulp fibers (Libby *et al.*, 1968; Libby, 1973, 1995). This tree is commercially grown in extensive plantations, along with Slash pine.

Although Loblolly pine grows primarily in the Deep South, it ranges north along the mid-Atlantic coast to southern New Jersey, in the interior north to eastern Tennessee, and as far west as central Texas (Ledig and Conkle, 1983; Ledig, 1988, 1998). Loblolly pine is the pine of the "Lost Pines" area around Bastrop, Texas, and in McKinney

Roughs along the Texas Colorado river. These are isolated populations on areas of acidic sandy soil, surrounded by alkaline clays that are poor for pine growth.

1.1.2 Scots pine

Scots pine (*P. sylvestris* L.; Family Pinaceae) is a common tree ranging from Great Britain and Spain east to eastern Siberia and the Caucasus Mountains, and as far north as Lapland (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). In the north of its range, it occurs from sea level to 1000 m, while in the south of its range, it is a high altitude mountain tree, growing at 1200–2500 m altitude. In the British Isles it is now native only in Scotland, but historical records indicate that it also occurred in Ireland, Wales, and England as well until about 300–400 years ago, becoming extinct here due to overexploitation; it has been reintroduced in these countries (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). Similar historical extinction and reintroduction applies to Denmark and the Netherlands. Scots pine is the only pine native to northern Europe, forming either pure forests or alongside Norway spruce, silver birch, common Rowan, Eurasian aspen, and other hardwood species (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). In central and southern Europe, it occurs with numerous additional species, including European Black pine, Mountain pine, Macedonian pine, and Swiss pine.

In the eastern part of its range, it also occurs with Siberian pine among other trees.

Scots pine is the National tree of Scotland, and it formed much of the Caledonian forest, which once covered much of the Scottish highlands. Overcutting for timber demand, fire, overgrazing by sheep and deer, and even deliberate clearance to deter wolves have all been factors in the decline of this once great pine and birch forest (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). Nowadays, only comparatively small areas of this ancient forest remain, the main surviving remnants being Glen Affric, Rothiemurchus, and the black wood of Rannoch. Plans are currently in progress to restore at least some areas, and work has started at key sites. The name is derived from Latin *pinus* via French *pin* (pine). The name is sometimes mis-spelled “scotch”, a form that gives offence in Scotland and should be avoided.

Scots pine is the most widely distributed conifer in the world, with a natural range that stretches from beyond the Arctic Circle in Scandinavia to southern Spain and from western Scotland to the Okhotsk Sea in eastern Siberia. Despite this wide distribution, Scots pine forests in Scotland are unique and distinct from that elsewhere because of the absence of any other native conifers (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). Scots pine (*P. sylvestris*), also called Scotch pine, is an introduced species in North America, brought here from Europe probably in colonial days. Although it is used for both pulpwood and sawlogs, its principal value in the United States appears to be as a Christmas tree, as an ornamental, and for erosion control. Scots pine has been widely planted in the United States, especially in the Northeast, Lake States, Central States, and Pacific Northwest. It is now considered naturalized in parts of New England and the Lake States (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). The species has also been planted across southern Canada. Scots pine is the most widely distributed pine in the world. It grows naturally from Scotland almost to the Pacific Ocean and from above the Arctic Circle in Scandinavia to the Mediterranean. Its altitudinal range is from sea level to about 2440 m.

Scots pine is adapted to a wide variety of climates as indicated by its extremely large natural range. Scots pine survives in the Verkhoyansk Mountains of eastern Siberia where winter

temperatures have been recorded as low as -64°C . The primary distribution of Scots pine indicates that it is a tree of the continental climates. In some areas, it grows where the subsoil is permanently frozen (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). Scots pine can also survive high temperatures, and it is found at middle altitudes in the Mediterranean region.

Like most trees, the Scots pine has special mycorrhizal associations with fungi, whereby the hyphae, or threadlike filaments, of the fungi wrap around the root tips of the tree, and through this an exchange of nutrients takes place (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). The fungi, which are unable to make direct use of the sun's energy themselves, receive carbohydrates and sugars which the pine has produced through photosynthesis, while the tree receives certain nutrients and minerals from the fungi, which it is unable to access directly in the soil. Through this mutualistic or symbiotic relationship, both the tree and the fungi benefit and are able to grow better than they would in the absence of the other (Krugman and Jenkinson, 1974; Lanner, 1998; Lindroth *et al.*, 1999). Scots pine is known to have mycorrhizal associations with over 200 species of fungi in Scotland, and these include the chanterelle (*Cantherellus lutescens*), a relative of the common chanterelle which only occurs in the pinewoods, and the extremely rare greenfoot tooth fungus (*Sarcodon glaucopus*). Glen Affric is one of only three locations where this species has been observed in the United Kingdom.

1.1.3 Monterey pine

P. radiata (Family Pinaceae) is known in English as Monterey pine in some parts of the world (mainly in the United States, Canada, and the British Isles), and Radiata pine in others (primarily Australia, New Zealand, and Chile) (Millar, 1983, 1993, 1998). It is a species of pine native to coastal California in three very limited areas in Santa Cruz, Monterey, and San Luis Obispo Counties, and (as the variety *P. radiata* var. *binata*) on Guadalupe Island and Cedros Island off the west coast of Baja California, Mexico. It is also extensively cultivated in many other warm temperate parts of the world.

Along the California coast it has escaped from cultivation, and from there into southern coastal Oregon it shows signs of naturalizing. It has been introduced as a timber tree in vast areas of New Zealand (where it is the most common tree), Australia, Chile, Southwest Europe and South Africa (Kafton, 1985; Kinloch and Dupper, 1987; Kuang *et al.*, 1998; Karhu *et al.*, 2006). *P. radiata* grows to between 15 and 30 m in height in the wild, but up to 60 m in cultivation in optimum conditions, with upward pointing branches and a rounded top. It is closely related to Bishop pine and Knobcone pine, hybridizing readily with both species; it is distinguished from the former by needles in 3s and from both by the cones not having a sharp spine on the scales (Bollmann and Sweet, 1976; Butcher *et al.*, 1984; Burdon *et al.*, 1992a, b).

1.2 Botanical Description

1.2.1 Loblolly pine

Loblolly pine is the most important and widely cultivated timber species in the southern United States. Because it grows rapidly on a wide range of sites, it is extensively planted for lumber and pulpwood. Loblolly pine comprises over half of the standing pine volume in the south. Sonderegger pine (*Pinus* × *sondereggeri* H.H. Chapm.) is a natural hybrid between Loblolly pine and Longleaf pine (*Pinus palustris* Mill.), and occurs throughout the southeast (Neale and Sederoff, 1989; O'Brien *et al.*, 1996; Newton *et al.*, 2001). Its needles are in bundles of three, sometimes twisted, and measure 12–22 cm long: an intermediate length for southern pines, shorter than those of the Longleaf pine or Slash pine, but longer than those of the Shortleaf pine and Spruce pine. The cones are green, ripening pale buff-brown, 7–13 cm in length, 2–3 cm broad when closed, opening to 4–6 cm wide, and each scale bearing a sharp 3–6 mm spine.

Loblolly is one of the fastest growing pines, and is often chosen to use for convenient landscape screening. In urban areas, stands of loblolly are used as wind and noise barriers. The tree grows rapidly during its juvenile phase and can reach 30 m (100 ft.) tall. It is particularly prized for its straight trunk, which contains no knots for up to 9 m (30 ft.). Early colonists used the tree's resin

to boil into pitch or tar (Neale and Sederoff, 1989; O'Brien *et al.*, 1996; Newton *et al.*, 2001). Loblolly's evergreen leaf is usually between 12.7 and 25.4 cm (5 and 10 in.) long, dark green or green-yellow, with three needles per fascicle. It is monoecious and therefore bears both male and female flowers on the same plant. Male flowers are red to yellow, cylindrical, produced in clusters at the tips of branches, and on the preceding year's growth. Female flowers are yellow-purple and form on the New Year's growth. This species' cones are ovoid, not precisely oval, and they are usually between 7.62 and 15.24 cm (3 and 6 in.) long, with sharp spines on the scales (Neale and Sederoff, 1989; O'Brien *et al.*, 1996; Newton *et al.*, 2001). The bark of the juvenile tree changes from a dark brown to a brownish-red color, and as the tree matures, the bark separates into deeply furrowed plates, sometimes 5.08 cm (2 in.) thick. Although the bark used to be discarded after the tree was harvested, now it is often sold as mulch. Loblolly's needles also are valued as mulch around plants that thrive in acidic soils, such as azaleas and camellias (Neale and Sederoff, 1989; O'Brien *et al.*, 1996; Newton *et al.*, 2001).

One of the most common trees on Assateague Island, loblolly can grow up to 30 m (100 ft.) tall and up to 0.9 m (3 ft.) in diameter; however, along the coast it seldom rises more than 15 m (50 ft.). Because of the shade from these trees, loblolly wood will have few smaller trees or shrubs (Pederick, 1970; Plessas and Strauss, 1986; Peters, 1990; Perry, 1991; Price *et al.*, 1998). Often during a storm you will find ponies and deer taking refuge in the wide spaces beneath them. Loblolly pine woods are also home to the Delmarva fox squirrel, and on the refuge, you will spot squirrel houses attached to these trees. Loblolly's yellow-green needles can become up to 22.86 cm (9 in.) long and usually grow in bunches of three. Cones up to 22.86 cm (9 in.) long can be found, each with scales tipped with prickles. Loblolly pine is a large evergreen tree that reaches heights of 90'–110'. It has a long, clear bole that is occasionally buttressed, ascending limbs, and a rounded, spreading crown (Pederick, 1970; Plessas and Strauss, 1986; Peters, 1990; Perry, 1991; Price *et al.*, 1998). Young trees retain lower branches much longer than Slash or Longleaf pines. The leaves are needlelike, 4'–9' long, and are borne in fascicles of three. They have a slight bluish-green

tinge, are stiff, and sometimes slightly twisted (Pederick, 1970; Plessas and Strauss, 1986; Peters, 1990; Perry, 1991; Price *et al.*, 1998).

The fruits of Loblolly pine are dark brown, oblong to cylindrical cones, about 3''–6'' long, sessile, and persistent on the trees for several seasons. Loblolly pine produces a large number of cones every year. The bark is grayish brown and furrowed with elongate, broad, irregular plates (Pederick, 1970; Plessas and Strauss, 1986; Peters, 1990; Perry, 1991; Price *et al.*, 1998). Young twigs are reddish brown and scaly. Buds at the ends of branches are much thinner than associated Slash and Longleaf pine. Loblolly pine prefers acid soils and full sun, but will adapt to a variety of sites, including fertile, upland fields, moist forests, or with mixed hardwoods. It is often found in association with Shortleaf pine (*Pinus echinata*).

1.2.2 Scots pine

Scots pine grows up to 25 m in height when mature, exceptionally to 35–40 m on a very productive site (in Estonia, are some trees ca. 46 m in height, at age 220 years in 2006). The bark is thick, scaly dark gray-brown on the lower trunk, and thin, flaky, and orange on the upper trunk and branches (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006). The habit of the mature tree is distinctive due to its long, bare, and straight trunk topped by a rounded or flat-topped mass of foliage. On mature trees the leaves ("needles") are a very attractive blue-green, 3–5 cm long and occur in pairs, but on young vigorous trees the leaves can be twice as long, and occasionally occur in 3s and 4s on the tips of strong shoots. The cones are pointed ovoid in shape and are 3–7 cm in length (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006). Over 100 varieties have been described in the botanical literature, but only three are now accepted, the typical var. *sylvestris* from Scotland and Spain to central Siberia, var. *hamata* in the Balkans, northern Turkey and the Caucasus, and var. *mongolica* in Mongolia and adjoining parts of southern Siberia and northwestern China. One other variety, var. *nevadensis* in southern Spain, may also be distinct.

After the end of the last Ice Age, approximately 10 000 years ago, Scots pine, like other trees, spread

northwards again from continental Europe into Britain. As the climate continued to warm, it spread into much of northern Scotland, reaching a maximum distribution about 6000 years ago, before declining about 4000 years ago for reasons that are not entirely understood (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006). Today, Scots pine has a natural range confined to the highlands in Scotland, with the native pinewoods covering approximately 17 000 hectares in a number of separate, isolated remnants—just over 1% of the estimated 1 500 000 hectare original area. In many of the remnant areas, Scots pine is growing on north-facing slopes, but the exact reason for this is not clear; the generally wetter conditions of such northerly aspects may have provided protection from fire, which was used to clear the forest in past centuries (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006).

Scots pine is unusual amongst conifers in having a number of different mature growth forms, ranging from tall and straight trunked with few side branches, to broad, spreading trees with multiple trunks. Eleven different growth forms, or habit types, have been identified for Scots pine in Scotland, and many of these can easily be seen in the pinewood remnants. Young stands of Scots pine display the characteristically conical shape of conifers, but as the trees mature, this gives way to the flat- or round-topped shapes that are typical of the pines in the ancient Caledonian Forest remnants (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999). The bark of the Scots pine is also quite variable, with the young bark on small branches being papery thin and often orange-red in color. The bark on the trunk of a mature Scots pine can vary from gray to reddish brown and forms layered plates or flakes up to 5 cm thick, with deep fissures in between. Several species of lichen commonly grow on the bark. The needles grow in pairs, are blue-green in color and are about 5 cm in length. They normally remain on the trees for 2–3 years, with the old needles turning yellow in September or October before they shed. Drops of sticky resin often cover the tree's buds, and also provide a natural preservative for the wood. If a Scots pine tree dies while it is still standing, the skeleton can persist for 50 or even 100 years before falling down; the high resin content in the sap makes the wood very slow to

decay (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006).

Male and female flowers occur on the same tree. They appear in May with the females on the tips of the higher and more exposed branches and the males clustered together, often *en masse*, on the branches just below. Pollination is by wind, and fertilized female flowers take 2 years to become a fully grown cone. The cones ripen in April, opening while they are still on the tree, and the tiny winged seeds, each weighing 0.005 g, are dispersed by the wind. Cone production is variable, with good seasons, in which a mature tree can produce 3000 cones, occurring every 3–5 years, while in between a tree will produce few cones or none at all (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006). The seeds are generally carried as far as 50–100 m from the parent tree, although in some situations, especially when there is snow on the ground and a frozen top layer forms, the seeds have been known to travel several kilometers over the smooth, icy surface.

The seeds require a high level of light to germinate and grow, so seedlings are found in open areas and clearings; as a shade-intolerant species, Scots pine does not regenerate under its own canopy (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999; Rogers *et al.*, 2006). In Europe, Scots pine grows on a wide variety of soil types. In Scotland, it is found on the most ancient rocks and also on the most recent glacial deposits. The cool, humid climate of Scotland, along with the nature of the parent material, which is usually siliceous and acidic, frequently results in a deep litter and raw humus layer. The soils exhibit various degrees of podzolization. Scots pine grows well on these soils but best growth is on freely drained sands and gravels, often on knolls and terraces. These soils have only a thin layer of raw humus and are weakly podzolized. Although Scots pine grows on peat land in certain areas, usually it is badly stunted (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999). Studies of the mineral nutrient content of the foliage of several Scots pine provenances at three sites in Michigan show that Scots pine has evolved an efficient mechanism to extract nutrients. Significant differences were found among seed sources in their ability to accumulate nitrogen, phosphorus, sodium, magnesium, and boron. Magnesium was one of the key minerals in Scots

pine nutrition at all three sites. The faster-growing seed sources accumulated higher levels of foliar magnesium (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999).

Scots pine is native to Europe and Asia. From the British Isles and Scandinavian peninsulas through central Europe south to the Mediterranean and east through eastern Siberia, Scots pine can be found at varying elevations. Scots pine was introduced to North America by European settlers and has long been cultivated, especially in the eastern United States and Canada. It is adaptable to a wide variety of sites and accordingly, has been widely planted for both Christmas tree and ornamental purposes (Righter and Duffield, 1951; Roy, 1966; Sigg, 1987; Sedjo, 1999). Although plantations have been established in the United States for the purpose of producing forest products, the species does not perform as well as in its native habitat.

1.2.3 Monterey pine

Monterey pine trees are 15–30 m tall, 30–90 cm in diameter, contorted to straight; crown broadly conic, becoming rounded to flattened. Their bark is gray to reddish brown, deeply V-furrowed, furrow bases red, ridges irregularly elongate-rectangular, and their flattened surfaces scaly (Sweet and Bollmann, 1976; Strauss and Conkle, 1986; Strauss and Doerksen, 1990). Their branches are level to down-curved or ascending, poorly self-pruning; twigs slender, red-brown, sometimes glaucous, aging gray, and rough. Monterey pine buds are ovoid to ovoid-cylindric, red-brown, ca. 1.5 cm, resinous. They have needles with 2 (var. *binata*) or 3 (type variety) per fascicle, spreading-ascending, persisting 3–4 years, (8)9–15(20) cm × 1.3–1.8(2) mm, straight, slightly twisted, deep yellow-green, all surfaces with fine stomatal lines, margins serrulate, apex conic-subulate; sheath 1.5–2 cm, base persistent (Strauss *et al.*, 1993; Smith and Devey, 1994; Spencer *et al.*, 1998). Seed cones mature in February, 2 years after pollination, mostly serotinous and persistent 6–20 (–40) years, numerous, solitary to whorled, spreading to recurved, curved, mostly asymmetric (usually symmetric in var. *binata* and occasionally so in var. *radiata*), ovoid before opening, broadly ovoid when open,

7–15 cm, yellow-brown, lustrous, scales rigid, stalks to 1 cm; apophyses toward outer cone base mostly increasingly mammillate (but not in var. *binata*), those on inward cone side and middle and apex of cone more level; umbo central, mostly depressed, with small central boss or occasionally with slender, deciduous prickle. Seeds are compressed–ellipsoid; body ca. 6 mm, dark brown; wing 20–30 mm $2n = 24$ (Fielding, 1957; Forde, 1966; Falkenhagen, 1991).

In the wild, Monterey pine in California is seriously threatened by an introduced fungal disease, pine pitch canker, caused by *Fusarium circinatum*, while var. *binata* on Guadalupe Island is critically endangered (less than 100 surviving trees) by uncontrolled grazing by goats released long ago on this nearly uninhabited island. The forests associated with Monterey pine are associated with other flora and fauna of note. In particular, the pine forest in Monterey, California was the discovery site for Hickman's potentilla, an endangered species (Storer *et al.*, 1999; Sutton, 1999; Siregar and Sweet, 2000). *Piperia yadonii*, a rare species of orchid is endemic to the same pine forest adjacent to Pebble Beach. Nearby in a remnant pine forest of Pacific Grove, is a prime wintering habitat of the Monarch butterfly (Hiebert and Hamrick, 1983; Griffin and Lindgren, 1985; Gorman *et al.*, 1992; Hillyard, 1997; Gordon *et al.*, 1998; Hodge and Dvorak, 2000). Monterey pine, *P. radiata*, is the most widely planted pine in the world. This species has become important for lumber and paper in Australia, New Zealand, Spain and large areas in Africa and South America. Rapid growth and attractive foliage quickly provide variety and contrast in the landscape. In their native habitat along the Pacific coast, Monterey pines are famous for their wind-swept, picturesque shape.

Monterey pine is an adaptable tree, but it does have specific cultural requirements. Temperatures below freezing for only a few hours are damaging to this species. Temperatures above 100 °F cause needle scorch and damage to new shoots. In the summer, Monterey pine is adapted to cool, foggy conditions. The tree's needles collect fog, dripping as much as 3.81 cm (1½ in.) per week to the root system. Monterey pine is susceptible to air pollution, particularly ozone injury. Monterey pine grows best in acidic, well-drained soils. A 10.16 cm (4 in.) layer of organic matter, such

as wood chips, is very beneficial. This species does not thrive on shallow soils and is prone to blow down on such sites. Monterey pine is considered susceptible to a wide range of pests, with bark beetles and root rots the most serious tree killers (Copes, 1981; Cromer *et al.*, 1982; Cotterill *et al.*, 1987; Cylinder, 1995; Cato and Richardson, 1996; Cato *et al.*, 2001). A total of 56 species of insects and 18 diseases are listed as attacking Monterey pine. Characteristics of Monterey pine include the following: (1) leaf: evergreen needles, 10.16–15.24 cm (4–6 in.) long, 3 per fascicle, slender; shiny green; persist 3 years, (2) flower: monoecious; male cones in yellow spikes; female cones dark purple, (3) fruit: egg shaped, 7.62–15.24 cm (3–6 in.) long, asymmetrical (especially at the base), shiny brown, often clustered in dense whorls, serotinous (remain closed on the tree for many years); cone scales thick and rounded, tipped with a small prickle, (4) twig: slender and dark orange, (5) bark: mature bark is dark reddish-brown; thick with deep ridges and furrows, (6) form: straight trunk with irregular, open crown, 15–30 m (50–100 ft.) tall and 0.3–0.9 m (1–3 ft.) in diameter; cone clusters are often conspicuous on branches and trunk.

1.3 Economic Importance

1.3.1 Loblolly pine

Loblolly pine is the most commercially important pine of the southeast where it is dominant on approximately 29 million acres and makes up over one-half the standing pine volume. This pine cannot survive the occasional severe winters of USDA zone 5 but has a solid hold on most of the southern forest. It is the most common plantation pine in the southern forest, but has a problem with fusiform rust disease (*Cronartium quercuum*).

Loblolly pine is used by human for furniture, pulpwood, plywood, composite boards, posts, poles, pilings, crates, boxes, pallets. Loblolly is also planted to stabilize eroded or damaged soils. It can be used for shade or ornamental trees, as well as bark mulch.

Loblolly pine is also used by different animals. White-tailed deer (*Odocoileus virginianus*), gray squirrel (*Sciurus carolinensis*), fox squirrel (*Sciurus niger*), bobwhite quail (*Colinus virginianus*), and

wild turkey (*Meleagris gallopavo*) all utilize both pure and mixed loblolly stands for shelter. Red-cockaded woodpeckers (*Picoides borealis*) also use these trees for foraging habitat and nesting, as do a variety of other bird species such as the pine warbler (*Dendroica pinus*), brown-headed nuthatch (*Sitta pusilla*), and Bachman's warbler (*Vermivora bachmanii*). Seeds are also eaten for food by songbirds and small mammals. Standing dead trees are frequently used for cavity nests by woodpeckers.

1.3.2 Scots pine

Scots pine is an important tree in the forestry industry. The wood is used for pulp and timber products. A seedling stand can be created by planting, sowing, or natural regeneration. Commercial plantation rotations vary between 50 and 120 years, with longer rotations in north-eastern areas where growth is slower. In Finland, Scots pine was used for making tar in the preindustrial age. There are still some active tar producers, but mostly the industry has ceased to exist. The wood is pale brown to red-brown, and used for general construction work. Scots pine has also been widely planted in New Zealand and much of the colder regions of North America; it is listed as an invasive species in some areas there, including Ontario and Wisconsin (Laitakarai, 1927; Heit, 1969; Lemmien and Botti, 1974).

A number of rare and special plants are particularly associated with the pinewoods of the Caledonian forest, and these include twin-flower (*Linnaea borealis*), one-flowered winter-green (*Moneses uniflora*), and orchids such as creeping ladies tresses (*Goodyera repens*) and lesser twayblade (*Listera cordata*). The shade provided by the canopy of mature Scots pines provides a good habitat for blueberries (*Vaccinium myrtillus*) and cowberries (*Vaccinium vitis-idaea*), and dense carpets of these cover the forest floor in many areas. They also play a successional role in the development of the hummocks that are commonly found in the pinewoods. These hummocks form over extended periods of time in the shade of the trees, when lichens and mosses colonize boulders or tree stumps. As these lower plants grow, humus or organic matter builds up, and this allows the blueberries and cowberries to become

established. Eventually a living mat of vegetation is formed, completely covering the underlying boulder or stump, and creating the gently rounded, hummocky forest floor, which is characteristic of many of the native pinewood remnants of the Caledonian forest (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974).

Like all trees, Scots pine attracts the attention of various insects. Some of these live in the fissures between the plates or flakes of the tree's bark, and these form a food source for birds such as the crested tit (*Parus cristatus*) and the treecreeper (*Certhia familiaris*), which specialize in winking them out of the cracks and crevices. Larvae of the pine weevil (*Hylobius abietis*) burrow into the wood of the tree, and other insects live on the pine's foliage—aphids suck the sap, and caterpillars of species such as the sawfly (*Neodiprion sertifer*) and pine looper moth (*Bupalus piniaria*) eat the needles (right). Wood ants (*Formica aquilonia*) feed on these caterpillars, thereby helping to protect the trees from defoliation. These ants live in large social colonies, and their mounds of fallen pine needles and forest detritus are a characteristic feature of the pinewoods. A variety of birds are associated with Scots pine in Scotland, ranging from common insect- or seed-eating species like the chaffinch (*Fringilla coelebs*) and siskin (*Carduelis spinus*) to large raptors such as the golden eagle (*Aquila chrysaetos*). Black grouse (*Tetrao tetrix*) and capercaillie (*Tetrao urogallus*) both live in the pinewoods and eat the buds and shoots. The capercaillie became extinct in Scotland in the 18th century, but was successfully reintroduced from Scandinavia in 1837 and is primarily associated with the native pinewoods today. The only bird that is endemic to the United Kingdom (i.e., found here and nowhere else in the world) is the Scottish crossbill (*Loxia scotica*), which is confined to the pinewoods (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974). It is sometimes called the "Scottish parrot" because of its crossed mandibles, which it uses to pry open the tightly fitting scales of the Scots pine's cones. The seeds inside form the mainstay of the diet for this rare bird.

Mammals associated with the pinewoods include the red squirrel (*Sciurus vulgaris*), which also extracts and eats the seed from pine cones while they are still on the trees; mice and voles,

which feed on pine seeds which have fallen to the ground, and the pine marten (*Martes martes*), which eats voles, red squirrels, and small birds, and relishes blueberries in late summer. Larger mammals found in the pinewoods include the wild cat (*Felis silvestris*), badger (*Meles meles*), fox (*Vulpes vulpes*), roe deer (*Capreolus capreolus*), and red deer (*Cervus elaphus*). Both roe and red deer browse on Scots pine seedlings, eating the needles and leader shoot of young trees, and the overgrazing pressure from their expanded numbers in the last 150 years has prevented the natural regeneration of the native pinewoods throughout the Highlands (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974). Red deer also damage or kill sapling Scots pines by debarking or thrashing them with their antlers, particularly in late spring when the new season's antlers are shedding their velvet. In a natural, healthy forest ecosystem, the deer numbers would be in balance with the regenerating trees in the forest, but the imbalance in our pinewoods has created a "generation gap" in Scots pine, with no trees younger than 150 years in most locations, until fencing or intensive deer-culling measures were initiated in the last 10–20 years.

In the past, pinewoods supported a wider range of large mammals, including the wild boar, European beaver (*Castor fiber*), lynx (*Felis lynx*), moose (*Alces alces*), brown bear (*Ursus arctos*), and the wolf (*Canis lupus*), but in Scotland these have all been extirpated—the wolf was the last to disappear, when the last individual was shot in 1743. Little known until relatively recently, the native pinewoods of the Highlands have become the subject of various restoration and regeneration programs, and the future prospects for this unique part of Scotland's natural heritage now look better than they have done for centuries. Many of the best remnants of the pinewoods have active restoration measures underway in them and research projects are elucidating more of the interconnections and relationships that make up this boreal forest ecosystem. In Europe and throughout several countries in Asia, Scots pine is an important species of high economic value. Forest stands containing Scots pine are managed to produce pulpwood, poles, and sawlogs from which dimension and finish lumber is produced. Logs from trees of large diameters are processed into veneer and used in manufacturing plywood.

The species is also valued as an ornamental and landscape plant and has been widely planted in parks and gardens (Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974).

As a Christmas tree Scots pine is probably the most commonly used species in the United States. Because of its ease of planting, generally high planting survival and favorable response to plantation culture it has been widely planted throughout much of the eastern United States and Canada. For several years it was the favorite species of large eastern wholesale growers because of its excellent harvesting and shipping qualities. It is also a preferred species for many choose and cut growers in much of the eastern and central United States. When established in plantations usually 6–8 years are required to produce a 2.1–2.4 m (7–8 ft.) tree. The tree requires annual shearing, usually beginning at the second or third year following planting and continuing on through the year of harvest. Scots pine is host to a number of insect and disease problems, and continued protection from foliage and stem damaging agents is necessary. The species is not demanding with respect to fertility or moisture and supplemental fertilization or irrigation is not considered necessary. As a Christmas tree, Scots pine is known for its excellent needle retention and good keepability. It resists drying and if permitted to become dry does not drop its needles. When displayed in a water-filled container it will remain fresh for the normal 3–4 week Christmas season (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974). Like all natural trees it is readily recyclable and has many different uses following the Christmas holidays.

1.3.3 Monterey pine

Monterey pine is a fast-growing tree, adaptable to a broad range of soil types and climates, though does not tolerate temperatures below about -15°C . Its fast growth makes it ideal for forestry; in a good situation, *P. radiata* can reach its full height in 40 years or so. It was first introduced into New Zealand in the 1850s; today, over 90% of the country's plantation forests are of this species. This includes the Kaingaroa forest on the central plateau of the North Island, which is the largest planted forest in the world. Australia also has

massive radiata pine plantations; so much so that many Australians are concerned by the resulting loss of native wildlife habitat. A few native animals, however, thrive on *P. radiata*, notably the yellow-tailed black cockatoo which, although deprived of much of its natural diet by massive habitat alteration, feeds on *P. radiata* seeds. *P. radiata* has also been introduced to the Valdivian temperate rain forests of southern Chile, where vast plantations have been planted for timber, again displacing the native forests. In areas such as New Zealand this tree has become naturalized, and is considered a weed in the native forest habitat where it has escaped from plantations.

1.4 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

1.4.1 Loblolly pine

Loblolly pine is found throughout much of the southeastern United States from New Jersey to central Florida and west into Tennessee, Kentucky, Texas, and Oklahoma. It grows primarily in coastal plains and some Piedmont regions. This is an easily seeded, fast-growing member of the yellow pine group and is an aggressive invader in fallow fields. It is widely grown in plantations for commercial timber production, but also has been planted to help stabilize soil and reduce erosion or as a noise and wind barrier. Loblolly pine has also been planted in mine reclamation areas; and due to its high litter and biomass productivity, it is being studied as a possible alternative source for energy. The wood, which is marketed as southern yellow pine, is primarily used for pulp and paper but also for lumber and plywood. It may be sold interchangeably with Shortleaf pine.

Loblolly pine stands are important for numerous wildlife species. The trees provide habitat for many animals, including white-tailed deer, wild turkey, gray squirrels, rabbit, quail, and doves. Many songbirds feed on the seeds and help propagate the trees through seed dispersal. Red crossbills depend on Loblolly pine seeds for up to 50% of their diet. Other birds that frequent the trees include pine warblers, Bachman's warblers, and brown-headed nuthatches. Osprey and bald eagles often nest in tall loblolly trees. Two

endangered species that also use these pines are fox squirrels, which eat the cones, and red-cockaded woodpeckers, which will sometimes nest in old growth trees.

1.4.2 Scots pine

Scots pine is the most widely planted pine introduced in North America. It is also the preferred large-volume Christmas tree in the United States—approximately 30% of the 35 million Christmas trees harvested annually are Scots pine. Because it survives on poor droughty sites, Scots pine has been used to control erosion in many areas. However, the poor vigor of many of these stands on dry, infertile sites has made them susceptible to serious insect attack and many of them have little potential to produce timber. Scots pine has also been used to a large extent in ornamental plantings. It grows better than red pine on compacted clay soils frequently found around home sites. Because Christmas tree plantations are a ready source of trees, many trees are removed from these plantations as ornamental stock. Scots pine has also been planted along roadsides throughout the Lake States. They are similar in fiber and wood characteristics to red pine and are usable for both pulpwood and saw logs (Rudolph and Lemmien, 1959; Schreiner *et al.*, 1962; Steinbeck, 1966; Skilling and Nicholls, 1974; Skilling, 1981).

In Europe, seed source studies on Scots pine go back almost 200 years, and the literature on genetic variation is large. In the United States, an international seed source trial was conducted in 1938. This trial included trees grown from seed collected in Scandinavia and north-central Europe. In 1961, seeds from 162 natural stands and 24 plantations in Europe and Asia were out-planted in 12 test plantations in Michigan. The results of these seed source studies show the extreme importance of beginning with the correct seed source. The fastest-growing varieties from central Europe grew 2.5 times as tall and produced 15 times as much wood as the slowest-growing variety. In Michigan, the variety *carpatica* from eastern Czechoslovakia was most suitable for timber production because of its fast growth and good stem form. The next best was variety *haguenensis* from Belgium, Vosges Mountains of

France, and adjacent West Germany. However, these varieties may perform poorly in other parts of the United States. Information on performance of many seed sources is now available for most of the Lake States and the Northeast (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a, b; Wright and Wilson, 1972; Skilling, 1981).

This difficulty appears to be partly due to grazing by deer and domestic animals. Successful regeneration has been achieved, however, with the uniform or shelter wood compartment system, which also appears to be successful in the Scandinavian countries. The best regeneration is found in stands with the following characteristics: large seed supply, open or light tree canopy, light understory ground cover, and exposed mineral soil or no continuous layer of raw humus. When scleroderris canker (*Gremmeniella abietina*) is present in the Scots pine over-story, the advance reproduction can be completely eliminated. Although Scots pine is primarily a monoecious species, some shoots, branches, and even entire trees are predominantly of one sex. Male flower primordia are formed in late summer at the base of the bud that will make the next year's growth. During the winter their presence can be noted as a slight swelling, and the preferred male catkins are easily visible if a bud is dissected. About 2 weeks after growth begins in the spring, the male catkins enlarge to 0.6–0.7 cm (0.2–0.3 in.) long and shed pollen. The male catkins are borne at the base of the twigs, replacing leaf clusters. They are most common in the lower part of the crown and on short lateral twigs. Because they replace leaves, an excess of pollen production can lead to sparse foliage. A Pennsylvania breeder who selected for early flower production for two generations obtained a variety that produced plentiful pollen but few needles, and it was worthless as a Christmas tree (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974).

Female flower primordia are also formed in late summer but are microscopic. They are borne at the tips of buds for the next year's growth. There may be one, two, or three on a single bud. They first become visible after the buds expand in the spring. The primordia enlarge into female flowers or strobili about 2 weeks after growth begins in the spring, at a time when the new

growth has completed 75% of its elongation for the season. Because of this, shearing of the outside branches such as is practiced by Christmas tree growers removes all female flowers. Indeed, trees sheared in June will not produce seed for the next 3.5 years. Flowering occurs in late May or early June. On any one tree nearly all pollen is shed and nearly all the female flowers are receptive during the same 2- or 3-day period. In any one stand most trees flower within a day or two of each other. Trees of different provenances may differ in blooming time by several days, however; trees of northern provenances bloom the earliest (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a, b; Skilling and Nicholls, 1974; Skilling, 1981).

Pollen production tends to be concentrated on short lateral twigs in the lower half of a tree crown. Female flowers are borne on the most vigorous shoots. They tend to be concentrated on upper branches but may occur in any part of the crown receiving full sunlight. Pollination occurs in early summer, at a time when the female strobili are from 0.6 to 0.7 cm (0.2–0.3 in.) long. Shortly after pollination, the scales of the female strobili thicken, and the pollen grains germinate and send out a short pollen tube. At this time the female strobili become reflexed instead of pointing forward (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974). For the next 12 months the germinated pollen remains dormant and the female strobili grow little. A little more than a year after pollination, the germinated pollen grains renew growth and fertilize the ovules. In June, soon after fertilization, the conelets rapidly elongate and reach full size by early summer. Seeds mature and cones ripen in early October. The cones require alternating periods of dry and wet weather to open and shed few seed until early winter. Indeed, many seeds are retained on the tree until early spring.

Seeds from any one tree can be sorted visually by color into those that are full and those that are empty—empty seeds are much lighter in color than full ones. On any one tree the full seeds are fairly uniform in color and size, but both traits vary considerably from tree to tree. Trees from the same stand may produce seeds ranging from tan to almost black and from all one color to speckle. Seed size varies in a geographic pattern—seeds from the extreme northern latitudes are half the

size of those from the southern part of the range. Individual trees in Michigan, under favorable growth conditions, begin to produce male and female flowers from 5 to 8 years, although the average is between 10 and 15 years.

Form is as much a matter of site as of variety. On some sites most trees grow crooked whereas on other sites trees of any variety are usually straight. The tendency for a variety to be straight or crooked depends on its susceptibility to a particular pest or other damaging agent, and on the presence of that pest or damaging agent in that locality. For example, when the Zimmerman moth (*Dioryctria zimmermani*) is present in high numbers, Greek trees, which are generally not attacked, are straight, while Belgian trees, which are very susceptible, are very crooked. Where pine grosbeaks are present in large numbers, the Belgian trees, which are resistant to this pest, are straight whereas trees of the Riga variety are likely to be crooked. Poor quality sites seem to have a larger number of pests and a larger number of poorly formed trees than good quality sites. To promote closer branching and denser crowns for Christmas tree production, the trees are sheared by removing the tips of all the new shoots. Following shearing, the leaf fascicles near the cut ends develop adventitious buds. These buds are not formed if shearing is done during late summer (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a,b; Wright and Wilson, 1972; Skilling and Nicholls, 1974; Skilling, 1981).

In nature, Scots pine does not reproduce vegetatively. It is not difficult, however, to graft scions from the larger trees onto potted understock of Scots pine. In a Swedish study, cuttings from young seedlings (50–100 days old) rooted readily, but cuttings from shoots of 3-year-old plants rooted poorly. Scots pine shows tremendous variation in yield, both by site and by geographic seed source. In seed source tests, some varieties grew 2.5 times as fast as others on the same site (Wright and Bull, 1963; Wright *et al.*, 1966a,b; Steinbeck, 1966; Skilling and Nicholls, 1974; Skilling, 1981). The average height of 150-year-old trees in Scotland is from 13.7 to 16.8 m (45–55 ft.). On well-drained sites, an occasional tree as tall as 22.9 m (75 ft.) is found. In a Michigan study in which dominant crop trees were released, the released trees averaged 13.7 m (45 ft.) in height

and 18 cm (6.9 in.) in d.b.h. at 21 years. The plantation was grown from seed from Magdeburg, Germany, and the soil is a fox sandy loam on a well-drained site. A 32-year-old, unthinned Scots pine plantation in the same area averaged 19 cm (7.3 in.) in d.b.h. and 18.6 m (61 ft.) in height. This seed source was probably central Europe. A Scots pine plantation in northern New York averaged 26.0 m (85.5 ft.) tall and 48 cm (19 in.) d.b.h. at age 74–77 years. At 42 years the unthinned portion of the stand averaged 23 cm (9.2 in.) in d.b.h. (diameter at breast height) and contained a volume of 263.8 m³ ha⁻¹ (3768 ft.³/acre). Basal area was 36.0 m² ha⁻¹ (157 ft.²/acre). The area receiving five light thinnings at 5-year intervals to a basal area of 19.5–21.8 m² ha⁻¹ (85–95 ft.²/acre) had an average d.b.h. of 30 cm (11.8 in.) but volume was only 155.2 m³ ha⁻¹ (2217 ft.³/acre) and basal area was 25.7 m² ha⁻¹ (112 ft.²/acre). The heaviest thinning with five thinnings at 5-year intervals to a basal area of 14.9–17.2 m² ha⁻¹ (65–75 ft.²/acre) produced an average d.b.h. of 34 cm (13.3 in.) with 117.5 m³ ha⁻¹ (1679 ft.³/acre) of volume and 20.7 m² ha⁻¹ (90 ft.²/acre) of basal area. The bulk of the root system consists of horizontal roots close to the surface. The majority of these horizontal roots are within 20 cm (7.8 in.) of the surface. The horizontal root system is smaller on good soils than on poor soils. The depth of the horizontal root system is also related to soil moisture—it is deeper on the drier soils. On vigorous trees, the length of the longest horizontal roots ranged from 4.5 m (14.8 ft.) for 14-year-old trees to 17.1 m (56.0 ft.) for 52-year-old trees. Root systems on rocky soils are usually shorter than on sandy soils. The size of the stem and the length of horizontal roots are closely interdependent (York and Littlefield, 1942; Rudolph and Lemmien, 1959; Schreiner *et al.*, 1962; Steinbeck, 1966; Skilling and Nicholls, 1974; Skilling, 1981).

The regeneration cut is made to coincide with a heavy seed year. This can be predicted 1 year in advance because the cones take 2 years to mature. At the time of regeneration, the number of overstory trees is reduced to approximately 50/hectares (20/acre) by one or two fellings to provide the required light conditions for young seedlings and to reduce root competition for water and nutrients. The seed trees normally are felled when the reproduction is well established—usually within 5–10 years. Scots pine is an introduced

species that has been widely planted for the purpose of producing Christmas trees. It is an extremely hardy species which is adaptable to a wide variety of soils and sites. As a Christmas tree, it is known for its dark green foliage and stiff branches that are well suited for decorating with both light and heavy ornaments (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a, b; Wright and Wilson, 1972; Skilling and Nicholls, 1974; Skilling, 1981). It has excellent needle retention characteristics and holds up well throughout harvest, shipping, and display. The needles of Scots pine are produced in bundles of two. They are variable in length, ranging from slightly over 2.54 cm (1 in.) for some varieties to nearly 7.62 cm (3 in.) for others. Color is likewise variable with bright green characteristic of a few varieties to dark green to bluish tones more prominent in others. The undersides of Scots pine needles are characterized by several prominent rows of white appearing stomatal openings. The bark of upper branches on larger, more mature trees displays a prominent reddish-orange color, which is very distinctive and attractive. Large amounts of cones are likewise produced that often persist on the tree from 1 year to the next. Like most pines two growing seasons are required to produce mature cones. On excellent sites within its native range mature trees may reach a trunk diameter of 76.2 cm (30 in.) or more and individual trees may exceed 37.5 m (125 ft.) in height (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a, b; Wright and Wilson, 1972).

Scots pine is reproduced from seed. More than 35 different seed sources or varieties are commercially recognized. Seed is obtained by international collectors and marketed through reputable seed dealers. A few seed orchards have been established in the United States from which seed is locally collected. For Christmas tree production purposes seed is usually sown in the spring and the resulting seedlings are allowed to grow for 2 years in the nursery bed before they are lifted and sold to Christmas tree producers. There has been some research by university personnel to identify and produce genetically improved planting stock, although these efforts have not been totally successful (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974).

1.4.3 Monterey pine

Monterey pine has a very restricted distribution. It is native to just five areas: the Año Nuevo-Swanton area (San Mateo and Santa Cruz counties), the Monterey Peninsula and Carmel (Monterey county), Cambria (San Luis Obispo county), and Guadalupe and Cedros Islands off Baja California in Mexico. The species was more widely distributed at various times in the past. Fossil cones or needles of the Monterey pine have been recorded from 20 localities in California, including the famous La Brea Tar Pits. Most of the localities are near the coast and range from Tomales Bay in the north to Chula Vista near San Diego and on the Channel Islands (Vogl, 1973; Tjosvold and McCain, 1988; Toro and Gessel, 1999; Vogl *et al.*, 2002). The one inland locality is in Riverside county. The fossils range in age from middle Miocene through Pleistocene. The cones of this species are distinctive and one of the easiest to recognize from fossils. They are asymmetrical with large, smooth, bulbous umbos (knoblike protuberances) on the scales. The scales do not have sharp prickles like some pine species. Most of the Pleistocene cones are carbonized (also called coalified). Basically, the wood has turned into coal. In this process, the molecules that make up the cellulose begin to break down after prolonged burial. Hydrogen and oxygen atoms are released in the form of water, carbon dioxide, and methane gas. This leaves behind carbon—hence the term carbonized (Wheeler and Guries, 1982; Wheeler *et al.*, 1983, 1995; White, 1999).

The evolutionary history of the Monterey pine (*P. radiata*) and its close relative, the Bishop pine (*Pinus muricata*), first piqued the curiosity of paleontologists in the early 1900s. In the 1930s, Herbert Mason suggested that the disjunct populations of these pines could be explained by Tertiary-age islands. He hypothesized that the populations had been isolated on offshore islands millions of years ago, and that after these islands became part of the mainland, the pine's insular distribution persisted. It was a reasonable theory at the time, but geologic studies in recent decades have found no evidence for such islands, at least in central and northern California. In addition, later discoveries show that Monterey pine was much more widely distributed only a few tens or hundreds of thousands of years ago, well after

the end of the Tertiary Period. In a series of studies during the last half of the 1900s, pale botanist Daniel Axelrod used geologic, fossil, and associated floristic evidence to better piece together the evolutionary and geographic history of Monterey pine. According to Axelrod, the closed-cone pines originated in Central America from an ancestor related to the modern *Pinus oocarpa*. The closed-cone pines spread northward into California about 15 million years ago. By this time, *P. radiata* had already evolved into a distinct species. Monterey pine's widespread fossil distribution along the California coast led Axelrod to believe that it flourished throughout the Pleistocene. He theorized that it was not until a warm, dry period 4000–8000 years ago that it was driven to near extinction, surviving in the form of five small populations. He called this warm period the Xerotherm. Today it is more commonly referred to as the Climatic Optimum or early Holocene warm period (Whitlock *et al.*, 1993; Wikler and Gordon, 2000; Wikler *et al.*, 2000).

A few years ago, Connie Millar, a scientist with the United States Forest Service, developed a revised theory of the pine's history based on new fossil evidence and a refined understanding of climate change during the Pleistocene. Marine sediments and ice cores from the Atlantic, Pacific, and Arctic oceans have now given us high-resolution climate records going back hundreds of thousands of years. Pale temperatures have been determined from the shells of fossil organisms and from gases trapped in the ice, sometimes datable to individual years (Wilcox and Miller, 1975; Wilcox, 1983; Wilson, 1990; Wu *et al.*, 1998a). These scientific breakthroughs have enabled scientists to paint a much more detailed picture of climate change than was ever before possible. These records show that there have been at least 11 ice ages over the past million years. Each lasted for about 90 000 years. Between the glacial periods were warm interglacial periods of about 10 000 years, like the one we live in now. The Climatic Optimum (Axelrod's "Xerotherm"), when temperatures were even warmer than at present, appears to have had analogs in other interglacial periods. This casts doubt on Axelrod's theory, which relied on the premise that the early Holocene warming was unique. The previous interglacial period (125 000–111 000 years ago), for example, apparently had peak temperatures at

least two degree centigrade warmer than the period 8000–4000 years ago. Another important line of evidence has come from fossil pollen. Pollen is extremely durable and can last for millions of years (Zagory and Libby, 1985; Yang and Yeh, 1993; Wu *et al.*, 1998b, 1999). The tiny grains have distinctive morphologies and in many cases are identifiable to species. Fossil pollen samples collected from cores of lake, bog, and marine deposits can show what plants were living nearby and how the flora in a particular area changed over time with changes in climate (Old *et al.*, 1986; Owen, 1998; Ostrowska *et al.*, 1998).

For her study of Monterey pine, Millar drew upon pollen evidence from sediment cores in the Santa Barbara Channel. According to Millar, Monterey pine "was least abundant during full interglacials (i.e., the Holocene and previous interglacials), when oaks dominated coastal habitats, and was also uncommon during the cold periods of the glacials, when junipers dominated. Monterey pine, as well as other coastal pines, increased dramatically in abundance and shifted in coastal location during climate periods intermediate between these extremes—i.e., at times such as the end of the ice ages (climate warming), during 'interstadial periods' (warmish intervals within the ice ages), and at the end of interglacials (climate cooling)." She also found that, "Times of abundance of Monterey pine correlated also with increases in charcoal abundances in the sediment cores, corroborating that fire plays an important role in dispersal and spread of Monterey Pines by opening cones and preparing seed beds (Pawsey, 1964; Pederick, 1967; Reyes and Casal, 2001; Rogers, 2004)."

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

1.5.1 Loblolly pine

Although Loblolly pine falls prey to many insect pests, including southern pine beetle, pine engraver beetle, pine tip moths, and seedling debarking weevils, it also provides rich habitat for animals and other plant species. Forests and stands of trees provide excellent habitat for wildlife, including white-tailed deer, gray squirrels, rabbits, and birds such as wild turkey and bobwhite quail. Warblers

and nuthatches thrive in Loblolly pine forests, and old-growth areas sustain other species such as the red-cockaded woodpecker. In the Bay region the trees provide important nesting habitat for bald eagles and osprey. In areas that are subject to severe erosion along the Bay and tidal tributaries, loblolly pines are frequently used for soil stabilization in areas that are subject to severe erosion (Echt *et al.*, 1998).

In several areas on the Assateague refuge, you will note that the loblolly pines have been cut down and burned. Unfortunately, a southern pine beetle infestation hit the refuge in 1994, and the dead trees have had to be destroyed. New pines are growing naturally, and in many of the devastated areas, oaks and other hardwoods have been planted.

1.5.2 Scots pine

Scots pine in North America is subject to a number of agents that can severely damage or kill the trees. Some of these agents are not present in Europe and Asia and, as a result, the species has not yet had an opportunity to develop genetic resistance. Fire and wind can damage the trees (Dickson and Winch, 1970; Genys, 1976; Dorworth, 1977). Young stands have thin bark and are heavily damaged by fire. Older trees with thicker bark are moderately resistant. Scots pine has more branches per whorl than red or white pine and this large number of branches make the tree weak at the nodes. During severe wind storms, trees may snap off at the nodes 3–6 m (10–20 ft.) above the ground. Wildlife and insects are also damaging. The pine grosbeak feeds on the terminal and lateral buds of Scots pine causing numerous small crooks. Trees of Scandinavian provenances are heavily attacked. In Christmas tree plantations, this feeding can cause major economic losses; a single year's feeding can reduce the tree harvest by 50%. This is a minor problem to timber growers, however (Dickson and Winch, 1970; Genys, 1976; Dorworth, 1977). On occasion, porcupine seriously damage Scots pine plantations by girdling young trees, causing dead tops.

The pine root collar weevil (*Hylobius radialis*) is a major cause of tree death in young plantations in the Lake States. The weevil girdles the tree at the base, killing it within 3 to 4 years. The damage is especially severe on dry sandy soils. The fast-

growing central European trees are particularly susceptible (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974). In Michigan, on low quality sites, mortality frequently reaches 70–80%. The pine root tip weevil (*Hylobius rhizophagus*) causes serious damage in Michigan on Scots pine Christmas trees grown from stump culture. These trees result from leaving the lower limbs on cut trees to grow into a second tree crop. The pine root tip weevil larvae feed on the roots and root tips, resulting in reduced height growth and flagged shoots, and eventual death. In some cases, the pine root tip weevil and the pine root collar weevil attack some Scots pine stands simultaneously, causing more mortality than expected from either insect alone (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974). The European pine sawfly (*N. sertifer*) causes moderate damage in Christmas trees and ornamental plantings. Heavy defoliation reduces growth from 10% to 20%. The fast-growing Scots pine variety *uralensis* shows some resistance to this insect while the slow-growing variety *iberica* is most susceptible (Laitakarai, 1927; Mosher and Wilson, 1977; Heit, 1969; Lemmien and Botti, 1974).

If Scots pine is pruned in midsummer, the Zimmerman pine moth may be attracted to the fresh pitch. The larvae feed in the cambial region, causing masses of coagulated pitch and frass to collect. Feeding by several larvae at the same whorl may kill the tree top or the entire tree. Partially girdled stems frequently break at the weakened area during storms (Paul, 1916; Read, 1971; Nicholls and Skilling, 1974; Ruby and Wright, 1976; Wright *et al.*, 1976). The white pine weevil (*Pissodes strobi*) burrows into terminal shoots and kills them. This insect is very damaging to trees on light soils but causes only minor damage on better sites. The eastern pine shoot borer (*Eucosma gloriola*) also burrows in the pith of new growth. In Michigan plantations, this insect is universal but causes only minor damage. The pine spittlebug (*Aphrophora parallela*) is a serious pest in many Scots pine Christmas tree plantations (Paul, 1916; Read, 1971; Nicholls and Skilling, 1974; Ruby and Wright, 1976; Wright *et al.*, 1976). Heavy infestations of spittlebugs may cause twig, branch, and tree mortality. In one 19-year-old Scots pine plantation in southern Michigan, the pine spittlebug has apparently acted

as the vector for the fungus disease *Sphaeropsis sapinea*; mortality is now 25% and is continuing. *Lophodermium* needlecast caused by the fungus *Lophodermium seditiosum* is the most serious disease of Scots pine Christmas tree plantations. The major loss is due to premature defoliation resulting in unsalable Christmas trees. In general, the longer needle provenances are resistant to this disease. The problem is minor in forest stands (Paul, 1916; Read, 1971; Nicholls and Skilling, 1974; Ruby and Wright, 1976; Wright *et al.*, 1976).

Scots pine is also a host for brown spot needle disease of southern pines (*Scirrhia acicola*). This disease, like *Lophodermium*, causes premature defoliation and is primarily limited to Christmas tree plantations. The long needle provenances are also more resistant to this disease (Rudolph and Lemmien, 1959; Schreiner *et al.*, 1962; Steinbeck, 1966; Skilling and Nicholls, 1974; Skilling, 1981). Western gall rust (*Endocronartium harknessii*) is common on Scots pine in the Lake States and the Northeast. Individual trees may have several hundred galls. In most cases damage is limited to branch mortality and growth loss. As described earlier, Scots pine is susceptible to scleroderris canker. This disease is present in many areas in Europe, and as a result, certain Scots pine provenances show some resistance. Scots pine is more resistant to scleroderris canker than red pine, and in some areas, red pines have been eliminated from the stand while Scots pines are still alive. Scleroderris canker can be spread on cut Scots pine Christmas trees. Therefore, state quarantines have been established to prevent the movement of this disease into noninfected areas (Rudolph and Lemmien, 1959; Schreiner *et al.*, 1962; Steinbeck, 1966; Skilling and Nicholls, 1974; Skilling, 1981). When southern seed sources of Scots pine are planted too far north of their normal range, severe foliage winter injury develops. This winter injury causes both branch and tree mortality. In the Lake States, a large number of Christmas tree plantations have been destroyed by this problem. Many of these problems in Scots pine plantations are the result of planting this species on very poor sites or planting the wrong seed source (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a, b; Wright and Wilson, 1972). Scots pine has the inherent ability to produce excellent, straight-boled stands under the proper conditions. Hundreds of Scots pine plantations throughout

the Lake States and the Northeast are equal to or better than the best red pine stands. When Scots pine is planted on very poor sites, however, or when improper seed sources are used, damage by insects is so severe as to make the final stand useless for timber production (York and Littlefield, 1942; Wright and Bull, 1963; Wright *et al.*, 1966a, b; Wright and Wilson, 1972).

1.5.3 Monterey pine

Monterey pine is the most common pine in the southern hemisphere, where no pines are native (except that *Pinus merkusii* barely crosses the line in Sumatra). The three remaining native stands of var. *radiata* are infected and under threat of extinction from pitch canker, a fungal disease native to the southeast United States and found (in 1986) to have been introduced to California. When trees begin to die of the disease, they attract bark beetles that provide a pathway for infection of other trees. In some stands, 80–90% of trees are infected. If the disease is introduced in agroforestry areas dependent upon *radiata* pine, such as New Zealand, it could have catastrophic effects in those countries as well (Furnier and Adams, 1986; Eldridge, 1996). Its cones are serotinous, i.e., they remain closed until opened by the heat of a forest fire; the abundant seeds are then discharged to regenerate the burned forest. The cones may also burst open in hot weather (Hood and Libby, 1980; Hong *et al.*, 1993; Gascon *et al.*, 2000).

There is no evidence that Monterey pine was ever continuously distributed along the California coast at any one time over the past two million years. Instead, its populations were always fragmented. The species expanded, shifted, or colonized new sites during periods of favorable climate. Its range contracted and some forest stands died out during periods of unfavorable climate. Many biologists are concerned about the survival of the five native stands of Monterey pine (Kinloch, 1992; Jorgensen and Hamrick, 1997; Jacobs *et al.*, 2000). On Guadalupe Island, for example, the population has long been threatened by goats. In 1964 only 320 trees remained. By 1992 the population had dwindled to 150. On the mainland, urbanization, fire suppression, genetic contamination, and pine pitch canker (a deadly fungal disease ravaging many stands) all threaten

the trees (Jones *et al.*, 1996; Johnson *et al.*, 1997; Jayawickrama and Carson, 2000). Domesticated Monterey pines are common in the United States as garden trees, and small ones are used for Christmas trees. Although the species is not likely to become extinct, it is important that the wild populations be preserved. In other parts of the world, domesticated Monterey pines are a major forest tree. In fact, it is the world's most planted conifer. Over 10 million acres have been planted. There are 2 million acres in Australia, 3 million in New Zealand, and millions more in Chile, Uruguay, Argentina, Spain, South Africa, and Kenya. Selective breeding has produced fast-growing trees with straight trunks—ideal for the forest industry (Nicholls and Eldridge, 1980; Ohmart and Voight, 1981; Nowak and McBride, 1991). Most of these countries have active breeding programs to develop still better strains for lumber and paper manufacturing. DNA studies have shown that the material from which these foreign plantings were developed was not very diverse genetically. On the contrary, the native forests are genetically diverse. According to Millar, the diverse germplasm in these native populations have “inestimable value”. It could, for example, help with the development of strains that are more resistant to certain insect pests and diseases (Lill, 1976; Krupkin *et al.*, 1996; Lavery and Mead, 1998).

Given the Pleistocene history of the species, Millar recommends expanding conservation efforts beyond the five native populations. For example, “neo-native” forests could be planted in areas where the pine lived only a few thousand years ago such as near Point Reyes, Point Sur, Santa Barbara, and San Diego. This would help ensure survival of genetically diverse forests (Lindsay, 1932; Matheson, 1980; McDonald and Laacke, 1990; Matziris, 1995). When Dr. Millar spoke to the Monterey Bay Paleontological Society a few years ago, she told the tragic story of a park along the northern California coast where all the Monterey pines were cut down. The trees were perfectly healthy, but were removed because they were a “non-native” species. Yet, a few thousand years ago the trees might have grown there naturally. One of the great values of fossils is that they enable us to see the present from the perspective of geologic time (Murphy, 1981; Moran and Bell, 1987; Moran *et al.*, 1988;

Mouradov *et al.*, 1999a). As Monterey pine clearly shows, this perspective can give us a greater appreciation for the present and help us plan better for the future. When one visits the Monterey Peninsula or drives by the trees along Highway 1 near Año Nuevo State Reserve, it is easy to see their remarkable cones, and consider their clever survival strategy. They are not relicts of the Ice Age on the verge of dying out, but instead are trees lying in wait for the next shift toward a cooler climate (Mouradov *et al.*, 1999b; Mouradov and Teasdale, 1999; Mathews and Campbell, 2000).

2. DEVELOPMENT OF TRANSGENIC PINES

Genetic modification of conifers through gene transfer technology is now an important field in forest biotechnology. Particle bombardment and *Agrobacterium*-mediated transformation have been used on gene transfer in conifers. The use of particle bombardment has produced stable transgenic plants in *Picea abies*, *Picea glauca*, *Picea mariana*, and *P. radiata*. Transgenic plants have been produced from *Larix decidua*, *P. abies*, *P. glauca*, *P. mariana*, *Pinus strobus*, *P. taeda*, and *P. radiata* via *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation has advantages over particle bombardment such as a simpler integration pattern and a limited rearrangement in the introduced DNA. At present, genetic transformation of conifers has been directed toward improving growth rate, wood properties and quality, pest resistance, stress tolerance, and herbicide resistance. All of these could drive forestry to enter a new era of productivity and quality.

2.1 Donor Genes: Source, Isolation, and Cloning, and Designing Transgenes

Since the first transgenic tree was obtained in 1987 (Fillatti *et al.*, 1987), stable transformation has been developed for many forest tree species and used to transfer economically important genes conferring traits such as virus, insect, and herbicide resistance (Birch, 1997; Herschbach and Kopriva, 2002). However, transgenic conifers were only reported 10 years ago (Huang *et al.*, 1991) and are restricted to a very few species (Ellis *et al.*,

1989, 1991; Charest *et al.*, 1991, 1993, 1996; Walter *et al.*, 1994, 1998, 1999, 2002, 2004; Levee *et al.*, 1997, 1999; Wenck *et al.*, 1999; Klimaszewska *et al.*, 1997, 2001; Le *et al.*, 2001; Tang, 2001; Tang *et al.*, 2001; Gould *et al.*, 2002). Genetic transformation using *Agrobacterium* is the most efficient procedure for introducing DNA into the genome of many plant species (Smith and Hood, 1995). Several infectious *Agrobacterium* strains that resulted in tumor development in numerous coniferous species have been identified since 1986 (Sederoff *et al.*, 1986; Morris *et al.*, 1989; Stomp *et al.*, 1990, 1991). However, there are only a few reports of the regeneration of stably transformed conifers using *Agrobacterium* (Huang *et al.*, 1991; Levee *et al.*, 1997, 1999; Wenck *et al.*, 1999; Newton *et al.*, 2001; Tang *et al.*, 2001; Gould *et al.*, 2002). Only a few stable transformants have been obtained biolistically (Charest *et al.*, 1996; Walter *et al.*, 1998, 1999; Clapham *et al.*, 2000). Recent progress made in somatic embryogenesis in conifers provides opportunities to produce transgenic plants in a number of species that will lead to their application in forest industry (Sutton, 2002; Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

With the various gene transfer methods currently available, simple placement or transfer of DNA into a plant cell is no longer a limiting factor (Birch, 1997). However, both the mechanism for DNA transfer to a plant cell and targeting of the DNA to a complex tissue or organ competent for regeneration is still a major limitation (Herschbach and Kopriva, 2002). Since the aim is usually to produce transgenic trees with better wood properties, which can only be evaluated in the adult tree, there exists a long lag time between the actual accomplishment of transformation and the evaluation of its success or failure (Merkle and Dean, 2000; Pena and Sequin, 2001; Herschbach and Kopriva, 2002).

There are many genes available for use in conifer transformation experiments. However, most of those that have been used are reporter genes establishing a model transformation system, and very few have been used for novel phenotypes or for tolerance to various stresses (Walter *et al.*, 2002). Transgenic plants in the field of forest biotechnology has been subjected to several reviews (Tzfira *et al.*, 1996, 1998; Merkle and Dean, 2000; Pena and Sequin, 2001; Van Raemdonck

et al., 2001; Herschbach and Kopriva, 2002; Walter *et al.*, 2002; Tzfira and Citovsky, 2002). In this chapter, we provide an overview of the current state of knowledge on genetic transformation in conifers, and their applications in future tree improvement and forest production.

2.2 Methods Employed

2.2.1 *Agrobacterium*-mediated transformation in conifers

The molecular mechanism of Ti-plasmid transfer, integration, and expression has been a key research area in plant genetic engineering (Koncz and Schell, 1986; Tzfira and Citovsky, 2002). Research related to these areas includes molecular and genetic analyses of the transfer DNA (T-DNA) transfer machinery in *Agrobacterium tumefaciens*, regulation of the *vir* genes of *A. tumefaciens* that direct this transfer, and a molecular analysis of the form of T-DNA that is transferred (Tzfira and Citovsky, 2002). The integration pattern for foreign genes introduced into plant cells via *Agrobacterium*-mediated transformation is, in general, strikingly different from the pattern resulting from particle bombardment (Hoekema *et al.*, 1983; Stachel *et al.*, 1985; Sheikholeslam and Weeks, 1987; Godwin *et al.*, 1991; Hood *et al.*, 1993). The process of foreign gene transfer from *Agrobacterium* into plant cells includes several essential steps: (1) bacterial colonization, (2) induction of the bacterial virulence system, (3) T-DNA processing, (4) bacterial attachment, (5) generation of the T-DNA transfer complex, (6) T-DNA transfer and nuclear targeting, and (7) integration of T-DNA into the plant genome (Sheng and Citovsky, 1996).

Crown gall is a neoplastic disease caused by the infection of dicotyledonous plants by virulent strains of the gram-negative soil bacterium, *A. tumefaciens* (Birch, 1997). During the infection process, part of a bacterial plasmid, called the tumor inducing (Ti) plasmid, is transferred from the bacterium to the plant, where it stably integrates into the nuclear DNA (Tzfira and Citovsky, 2002). This process is the only known natural occurrence of DNA transfer between kingdoms (Sheng and Citovsky, 1996; Birch, 1997). Bacterial DNA transferred to the plant not only

induces the formation of a tumor, but also contains information that directs the plant cell to produce and secrete compounds that the *Agrobacterium* can utilize as a source of carbon and nitrogen (Sederoff *et al.*, 1986; Diner and Karnosky, 1987; Sheng and Citovsky, 1996; Dillen *et al.*, 1997).

The process begins when a plant is wounded. A wounding event, which often occurs at the base of the stem of the plant, causes the plant to release compounds that attract *Agrobacterium* to the damaged cells (Citovsky *et al.*, 1989). When *Agrobacterium* reaches the plant, it attaches to a receptor on the surface of the plant cell and begins to synthesize cellulose, resulting in a strong connection between the two cells (Sheng and Citovsky, 1996). Subsequently, phenolic compounds secreted by the plant are recognized by a protein kinase on the surface of the bacteria (a kinase is an enzyme that phosphorylates specific target proteins and sometimes itself). In this case, the kinase (VirA, where “vir” is for virulence) autophosphorylates in response to the phenolics and then transfers the phosphate to another protein (VirG) within the bacterium (Tzfira and Citovsky, 2002). In its phosphorylated state, VirG is able to induce transcription of genes on the Ti plasmid (Citovsky *et al.*, 1989; Rossi *et al.*, 1996).

The Ti plasmid encodes for proteins that function in the DNA transfer process in addition to containing the actual DNA that is transferred to the plant (Tzfira and Citovsky, 2002). An endonuclease specifically cleaves the Ti plasmid, which results in the release of a piece of single-stranded T-DNA of approximately 20 kb (Sheng and Citovsky, 1996). The T-DNA remains single stranded and is coated with proteins that serve both to protect the DNA and target it to the plant cell nucleus by virtue of nuclear localization signals found within the proteins (Tzfira and Citovsky, 2002). Once inside the plant nucleus, the T-DNA may integrate into the plant chromosome. The integrated T-DNA gene products direct the plant to synthesize auxins and cytokinins that cause plant cells to grow in an unregulated manner resulting in tumor formation (Hansen *et al.*, 1994). Additionally, the T-DNA causes the plant to synthesize opines that the bacteria can use as a carbon and nitrogen source (Hansen *et al.*, 1994; Sheng and Citovsky, 1996).

Agrobacterium-mediated or direct transformation methods have been used for more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medical, and fruit trees, and pasture plants (Birch, 1997). Furthermore, efficient methodologies of *Agrobacterium*-mediated gene transfer have been established mainly for dicotyledonous plants. However, *Agrobacterium*-mediated gene transfer has been applied to about 25 coniferous species from the genera *Abies*, *Larix*, *Libocedrus*, *Picea*, *Pinus*, *Pseudotsuga*, and *Tsuga* (Table 2). Reporter genes and important trait genes have been tested in conifers by *Agrobacterium*-mediated transformation. The procedure of *Agrobacterium*-mediated genetic transformation (Figure 1) is widely used in conifers. These studies demonstrated that several factors are important for the application of *Agrobacterium*-mediated gene transfer in conifers and other plants (Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

2.2.2 Transformation via particle bombardment

The ability to deliver foreign DNA directly into regenerated cells, tissues, and organs appears to provide the best method for achieving truly genotype-independent transformation bypassing *Agrobacterium* host specificity and tissue culture-related regeneration difficulty (Birch, 1997). There is no biological limitation to the actual DNA delivery process, so genotypic specificity is not a limiting factor (Birch, 1997). Recent advances in the transformation of crop plants with particle bombardment have demonstrated that DNA can be inserted into virtually any tissue and cell that is impacted by the particle (Bommineni *et al.*, 1993). Indeed, foreign genes were expressed in all conifer tissues that have been tested thus far by exposure to particle bombardment, including embryos, seedlings, megagametophytes, xylem, pollen, needles, buds, cell suspension cultures, embryogenic callus, cell aggregate cultures, and roots (Table 3). While almost all of this expression was transient, it yielded valuable information on factors involved in the expression of introduced genes in the various tissues competent for regeneration (Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

Table 2 *Agrobacterium*-mediated transformation in pine

Species	<i>Agrobacterium</i> strains ^(a)	Plasmid vectors	Gene expression	References
<i>Pinus banksiana</i>	<i>A.r.</i> A4/R100	A4/pRiA4b	Stable expression	McAfee <i>et al.</i> , 1993
<i>P. contorta</i>	<i>A.r.</i> LBA9402	pHRGPnt3-GUS	Stable expression	Lindroth <i>et al.</i> , 1999
<i>P. eldarica</i>	<i>A.t.</i> U3	U3	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. elliotii</i>	<i>A.t.</i> A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. halepensis</i>	<i>A.r.</i> LBA9402	p35SGUSINT	Stable expression	Tzfira <i>et al.</i> , 1996
<i>P. jeffreyi</i>	<i>A.t.</i> C58	C58	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. lambertiana</i>	<i>A.t.</i> Bo542/A28/Bo542kanr	pTiBo542/pEND4	Stable expression	Loopstra <i>et al.</i> , 1990
<i>P. lambertiana</i>	<i>A.t.</i> A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. maximartinezii</i>	<i>A.r.</i> A4	pRiA4	Stable expression	Villalobos-Amador <i>et al.</i> , 2002
<i>P. monticola</i>	<i>A.r.</i> A4/R100	A4/pRiA4b	Stable expression	McAfee <i>et al.</i> , 1993
<i>P. palustris</i>	<i>A.r.</i> A4, <i>A.t.</i> 208	pRiA4, wild Ti	Stable expression	Diner, 1999
<i>P. pinceana</i>	<i>A.r.</i> A4	pRiA4	Stable expression	Villalobos-Amador <i>et al.</i> , 2002
<i>P. pinea</i>	<i>A.t.</i> EHA105, GV3850, <i>A.t.</i> LBA4404, C58	p35SGUSint	Transient expression	Humara <i>et al.</i> , 1999
<i>P. ponderosa</i>	<i>A.t.</i> A136(pTiEU6)/K27/B3.73/K41	A136(pTiEU6)/K27/B3.73/K41	Gall formation	Morris <i>et al.</i> , 1989
<i>P. radiata</i>	<i>A.t.</i> C2/74/542	C2/74/542	Gall formation	Bergmann and Stomp, 1992
<i>P. radiata</i>	<i>A.t.</i> AGL1	pSKY1	Stable expression	Charity <i>et al.</i> , 2002
<i>P. radiata</i>	<i>A.t.</i> LBA4404	pBI121	Stable expression	Cerda <i>et al.</i> , 2002
<i>P. radiata</i>	<i>A.t.</i> A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. taeda</i>	<i>A.t.</i> M2/73/U3	M2/73/U3	Gall formation	Sederoff <i>et al.</i> , 1986
<i>P. taeda</i>	<i>A.t.</i> A281/542/C58/M2/73/U3	A281/542/C58/M2/73/U3	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. taeda</i>	<i>A.t.</i> EHA105, <i>A.t.</i> LBA4404, GV3101	pWWS006	Transient expression	Wenck <i>et al.</i> , 1999
<i>P. taeda</i>	<i>A.t.</i> LBA4404	pBI121	Stable expression	Tang, 2001
<i>P. taeda</i>	<i>A.t.</i> GV3101	pCV6NFHygGUSINT	Stable expression	Tang <i>et al.</i> , 2001
<i>P. taeda</i>	<i>A.t.</i> EHA101, 105	pGUS3, pSSLa.3	Stable expression	Gould <i>et al.</i> , 2002
<i>P. virginiana</i>	<i>A.t.</i> 542/M2/73/U3	542/M2/73/U3	Gall formation	Stomp <i>et al.</i> , 1990
<i>P. sylvestris</i>	<i>A.t.</i> 542/M2/73/U3	542/xM2/73/U3	Gall formation	Stomp <i>et al.</i> , 1990

^(a) *A.t.*, *Agrobacterium tumefaciens*; *A.r.*, *Agrobacterium rhizogenes*

Parameters that were found to influence successful particle and DNA delivery into regenerable tissue of plants included the condition of the explant prior to bombardment, environmental factors (including temperature and humidity), and influences of the parameters affecting transient activity of the marker genes (Birch, 1997). Additional important parameters also included depth of particle penetration and degree of tissue damage as a function of accelerating force and timing of selection. In *Pinus*, transient expression of the green fluorescent protein (*GFP*) gene in embryogenic masses of *P. strobus* via

particle bombardment has been achieved (Tian *et al.*, 1997). Transient expression of *uidA* (β -glucuronidase, Jefferson *et al.*, 1987) in cotyledon cells of *P. taeda* by particle bombardment has been reported (Stomp *et al.*, 1991), and these results demonstrated that microprojectile bombardment had potential for the production of transgenic plants in pine (Table 3). Transient regenerated pine has been obtained by transforming embryogenic tissues of *P. radiata* using particle bombardment (Figure 2) (Walter *et al.*, 1994, 1998), and more than 150 transgenic *P. radiata* plants were produced with 20 independent transformation

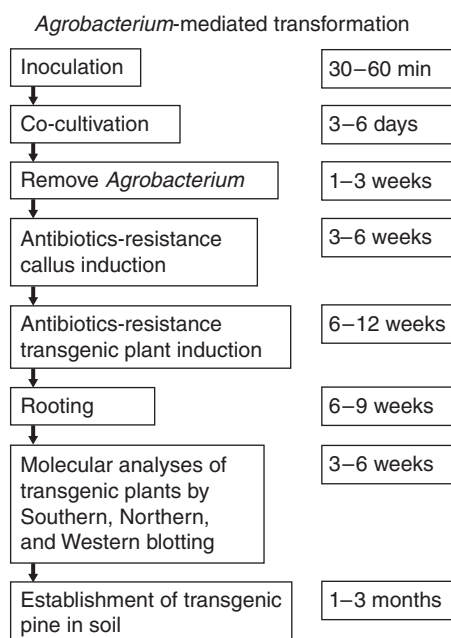


Figure 1 Procedure of *Agrobacterium*-mediated transformation

expression events with four different embryogenic clones (Walter *et al.*, 1998).

2.2.3 Transformation using electroporation

Transformation using electroporation as a method of direct gene transfer has potential for genetic improvement and for studies of gene structure and function in forest trees. This is especially attractive when used in conjunction with protoplasts that are capable of regeneration to somatic embryos or

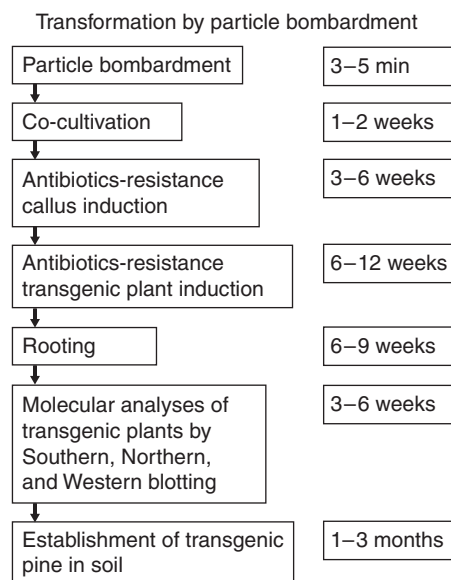


Figure 2 Procedure of transformation via particle bombardment

plantlets (Bekkaoui *et al.*, 1988, 1990; Tautorius *et al.*, 1989; Wilson *et al.*, 1989). Because electroporation avoids the host-range limitation of *Agrobacterium*-mediated transfer methods, it has the further advantage of being useful for the rapid evaluation of the functionality of plasmid construction, and for assessing transient gene expression, and stable transformation. Electroporation has been utilized to transfer genes into protoplasts isolated from embryogenic cell cultures of *P. glauca* (Bekkaoui *et al.*, 1988), *P. mariana* (Tautorius *et al.*, 1989), *P. taeda* (Gupta *et al.*, 1988), and *Larix × eurolepis* (Charest *et al.*, 1991), and from nonembryogenic

Table 3 Transformation of conifers by particle bombardment

Species	Bombarded tissues	Plasmid vectors	Gene expression	References
<i>Pinus griffithii</i>	Pollen cones	pBI221	Transient expression	Fernando <i>et al.</i> , 2000
<i>P. monticola</i>	Pollen cones	pBI221	Transient expression	Fernando <i>et al.</i> , 2000
<i>P. radiata</i>	Embryogenic cultures	pEmuGN, pCW103, p40CSD35SIGN, pCW5, pCW6, and pCW122	Transient expression	Walter <i>et al.</i> , 1994
<i>P. radiata</i>	Embryogenic tissue	pSeGer1, pAsGer1, pCW122	Stable expression	Bishop-Hurley <i>et al.</i> , 2001
<i>P. radiata</i>	Suspension cells	pBI221, pCAMVLN	Transient expression	Campbell <i>et al.</i> , 1992
<i>P. radiata</i>	Embryogenic tissues	pRC101, pCW122	Stable expression	Walter <i>et al.</i> , 1998
<i>P. strobus</i>	Embryonal masses	p35S-GFP, mGFP	Transient expression	Tian <i>et al.</i> , 1997
<i>P. taeda</i>	Cotyledons	pBI221	Transient expression	Stomp <i>et al.</i> , 1991

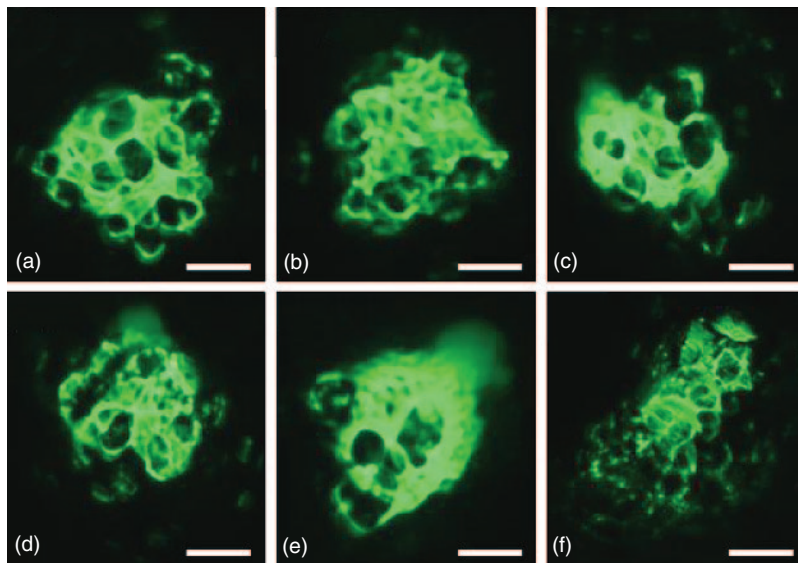


Figure 3 GFP transgenic pine calli derived from *Agrobacterium*-mediated transformation

cultures of *P. radiata* (Campbell *et al.*, 1992) and *Pinus banksiana* (Tautorus *et al.*, 1989). These experiments have demonstrated that 35S promoter (35S) of cauliflower mosaic virus (CaMV) and the nopaline synthase promoter function in conifer tissues.

The commonly used reporter genes, such as the firefly luciferase (*luc*) gene, GFP (Figure 3), and *uidA* (*GUS*) gene, can be used to assess gene activity in conifer protoplasts. Bekkaoui *et al.* (1990) reported that the level of foreign gene activity in electroporated *P. glauca*, *P. mariana*, and *P. banksiana* protoplasts is dependent on the promoter transferred, electroporation conditions, as well as on the target cell line under investigation. Gupta *et al.* (1988) reported that protoplast viability was reduced from 90% to 45–55% after electroporation. However, the transient expression of the *luc* gene was detected in protoplasts surviving 36 h after electroporation. Gene expression was improved by the addition of polyethylene glycol to the electroporation mixture. According to Tautorus *et al.* (1989), transient expression of the chloramphenicol acetyltransferase gene (*CAT*) in electroporated *P. mariana* and *P. banksiana* protoplasts was affected by the cell lines used, by voltage, temperature, and by the plasmid concentration and conformation. Increasing the plasmid DNA concentration resulted in higher

levels of transient *CAT* expression. A linearized plasmid gave 2.5 times higher levels of *CAT* enzyme activity than did circular plasmid in *P. banksiana*. A heat shock treatment of protoplasts for 5 min at 45 °C resulted in enhanced *CAT* gene expression for *P. mariana* and *P. banksiana*. Because of the difficulty in plant regeneration from conifer protoplasts, transformation using electroporation is mainly used to study transient expression of genes and factors influencing transgene expression (Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

2.3 Selection of Transformed Tissue

Three antibiotics carbenicillin, claforan, and timentin are usually used to eliminate *A. tumefaciens* during the genetic transformation of pine. *A. tumefaciens* strains, EHA105, GV3101, and LBA 4404, all harboring the plasmid pCambia1301, which carries the selectable marker gene, hygromycin phosphotransferase (*hpt*) controlled by the CaMV 35S promoter and terminator, and the *uidA* reporter gene (*GUS*) driven by the CaMV 35S promoter and the terminator of nopaline synthase gene, have been tested. Exposure to 350 mg l⁻¹ carbenicillin, claforan, and timentin, respectively for up to

6 weeks did not eliminate the *Agrobacterium*, while antibiotics at 500 mg l⁻¹ eradicated them from the co-cultivated zygotic embryos. All three antibiotics increased callus growth and shoot regeneration at 350 and 500 mg l⁻¹ each, but reduced callus growth and shoot regeneration at 650 mg l⁻¹ when compared with controls. Putative transgenic calli were selected for continued proliferation and differentiation on 4.5 mg l⁻¹ hygromycin-containing medium. Transformed calli and transgenic plants produced on a selection medium containing 4.5 mg l⁻¹ hygromycin were confirmed by GUS histochemical assays, by polymerase chain reaction (PCR), and by Southern blot analysis. These results are useful for future studies on optimizing genetic transformation procedures in pine (Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

Growth and differentiation of transgenic calli of pine were reduced by high concentrations of antibiotics (carbenicillin, claforan, and timentin, 650 mg l⁻¹ each), although it was observed that lower concentration (350 and 500 mg l⁻¹ carbenicillin, claforan, and timentin) caused an increase. These results indicate that the establishment of an efficient *A. tumefaciens*-mediated transformation protocol for stable integration of foreign genes into pine was also dependent on the selection of antibiotics. This could be useful for future studies on growth and differentiation of transformed cells as well as transferring reporter and/or functional genes to pine and other conifers (Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

2.4 Regeneration of Whole Plants

Since the first report of conifer transformation by Huang *et al.* (1991), significant advances have been made with *Agrobacterium*-mediated genetic transformation. In the last decade, successful transformation has been accomplished with *Larix*, *Picea*, and *Pinus*, although the transformation efficiency varies between species. Transgenic regenerated plantlets were obtained with *L. decidua*, *Pinus halepensis*, and *Pseudotsuga menziesii* (Dandekar *et al.*, 1987; Huang *et al.*, 1991; Tzfira *et al.*, 1996) infected by *Agrobacterium rhizogenes*, and embryogenic tissue of hybrid larch was transformed by *A. tumefaciens* (Levee *et al.*, 1997). According to Levee *et al.*

(1997), 1–2 transformation events per 100 co-cultivated embryogenic tissues were obtained with hybrid larch using *A. tumefaciens*. The addition of 100 µM coniferyl alcohol during the co-cultivation step increased the transformation frequency of kanamycin-resistant tissues where other substances known to be virulence inducers, such as acetosyringone and syringaldehyde, did not seem to have any effect (Levee *et al.*, 1997). Different bacterial strains (C58, pMB90, EHA101, and LBA4404) with various chromosomal backgrounds were tested, but none of them increased significantly the frequency of kanamycin-resistant tissues (Levee *et al.*, 1997). Tzfira *et al.* (1996) used *A. rhizogenes* strain LBA9402 to transform *P. halepensis* embryos, seedlings, and shoots. Mature embryos exhibited susceptibility to the *Agrobacterium* with more than 85% showing considerable transient β-glucuronidase (GUS) expression in the radicle. GUS expression was also observed in cotyledons, but at a lower rate in about 24% of the embryos (1–5 spots/embryo) (Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

Agrobacterium-mediated gene transfer is widely used in plant biotechnology laboratories; however, large-scale use of this organism in conifer transformation has been limited due to difficult propagation of explant material, selection inefficiencies and low transformation frequency (Wenck *et al.*, 1999). Wenck *et al.* (1999) have investigated co-cultivation conditions and different disarmed strains of *Agrobacterium* to improve transformation. They found that extra virulence genes including either a constitutively active *virG* or extra copies of *virG* and *virB*, both from pTiBo542, increased transformation efficiencies of Norway spruce 1000-fold from initial experiments where little or no transient expression was detected (Wenck *et al.*, 1999). In Loblolly pine, transient expression was increased 10-fold utilizing modified *Agrobacterium* strains (Wenck *et al.*, 1999). *Agrobacterium*-mediated gene transfer is a useful technique for large-scale generation of transgenic Norway spruce and may prove useful for other conifer species (Wenck *et al.*, 1999). During the same year, a genetic transformation procedure for white pine was developed after co-cultivation of embryogenic tissues with *A. tumefaciens* (Levee *et al.*, 1999). This efficient transformation procedure led to an average of four independently transformed lines

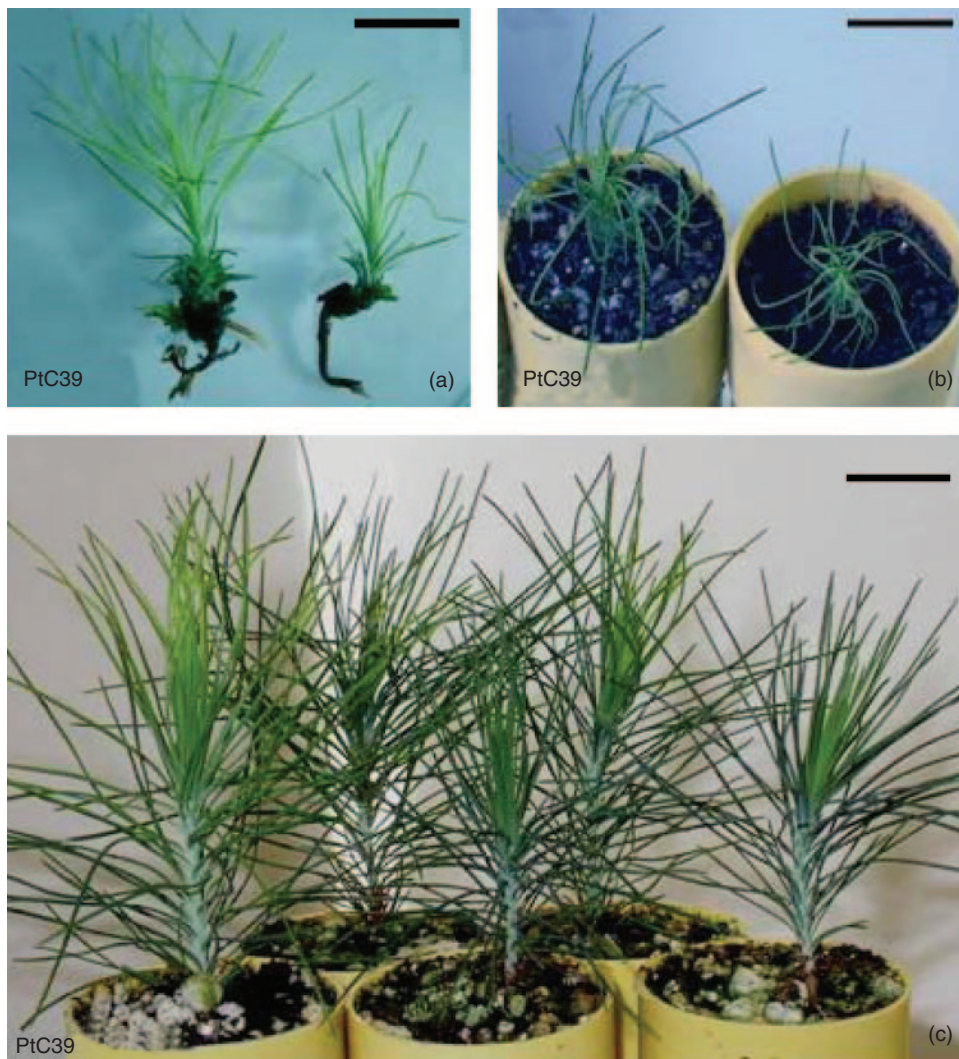


Figure 4 Transgenic pine established in soil

per gram of co-cultivated embryogenic tissue, and up to 50 transformed lines were obtained in a routine experiment (Levee *et al.*, 1999). Constructs bearing the *uidA* gene or the *GFP* gene were introduced and GUS or GFP activity was followed over time. The expression of the *uidA* gene was lowest with a 35S-*gus*-intron construct and was 20-fold higher with a 35S-35S-*AMVgus::nptII* construct. The addition of scaffold attachment region sequences surrounding the *gus::nptII* fusion did not significantly enhance the GUS activity (Le *et al.*, 2001). Transformed mature somatic embryos have been germinated

and plantlets have been acclimatized (Levee *et al.*, 1999).

Both family and species types are important for the application of *Agrobacterium*-mediated gene transfer in conifers (Figure 4). Embryos of 24 open-pollinated families of Loblolly pine (*P. taeda*) were used as explants to conduct *in vitro* regeneration (Tang *et al.*, 1998; Tang and Ouyang, 1999; Tang, 2000). *A. tumefaciens* strain, GV3101 harboring the plasmid pPCV6NFHygGUSINT, was used to transform mature zygotic embryos of seven families of Loblolly pine. The frequency of transformation varied among families. The highest

frequency (100%) of transient GUS-expressing embryos was obtained from family 11-1029 with over 300 blue spots per embryo. Ninety transgenic plants were regenerated from hygromycin-resistant calli derived from families WO3, 8-1082 and 11-1029, and 19 transgenic plantlets were established in soil. The presence of the *uidA* gene in the plant genome was confirmed by PCR, Southern blot, and plant DNA/T-DNA junction analysis (Park *et al.*, 2000; Tang, 2001; Tang *et al.*, 2001).

In *Picea*, transgenic plants of three species were produced after co-culture of embryogenic tissue with the disarmed strain of *A. tumefaciens* C58/pMP90/pBIV10 and selection on medium containing kanamycin (Klimaszewska *et al.*, 2001). Transformation frequencies were dependent on the species, genotype, and post-co-cultivation procedure. Of the three species tested, *P. mariana* was transformed at the highest frequency, followed by *P. glauca* and *P. abies*. The transgenic state of the embryogenic tissue was initially confirmed by histochemical GUS assay followed by southern hybridization. Transgenic plants were regenerated for all three species (Klimaszewska *et al.*, 2001). At the same time, Le *et al.* (2001) developed an efficient and reproducible procedure for the transformation of white spruce, *P. glauca* (Moench) Voss, embryogenic tissues by using *A. tumefaciens*-mediated gene transfer. They co-cultivated rapidly dividing white spruce embryogenic tissues with disarmed *A. tumefaciens* strains containing additional copies of the virulence regions from plasmid pToK47. The highest frequency of transformation was obtained with 5-day-old tissues grown in liquid medium and co-cultivated with *Agrobacterium* for 2 days in the medium containing 50 μ M acetosyringone. Transgenic plants were regenerated from transformed tissues within 4 months of co-culture (Le *et al.*, 2001).

Recently, a shoot-based and genotype-independent Loblolly pine (*P. taeda*) transformation method was established by *A. tumefaciens* mediated-gene transfer (Gould *et al.*, 2002). Shoots from seedlings aged 4–6 weeks and adventitious shoots from culture were inoculated with *A. tumefaciens* EHA101 (pGUS3), or EHA105 (pSSLa.3), subjected to selection and regenerated. The overall recovery of *P. taeda* shoots was 10–20%, while recovery of intact rooted plants was about 1% due to difficulty in rooting. The addition

of a shoot multiplication step and effective rooting protocols will improve the efficiency of this genotype-independent transformation method in *P. taeda* (Gould *et al.*, 2002). In *P. radiata*, Charity *et al.* (2002) demonstrated that an *A. tumefaciens*-mediated transformation protocol was developed for detached cotyledons of zygotic embryos resulting in up to 55% of the cotyledons transiently expressing the reporter gene *uidA*. Transient expression of *uidA* was improved when detached cotyledons were precultured on half-strength medium containing cytokinin for 7 days, wounded by vortexing and then vacuum infiltrated in a solution of *A. tumefaciens* (Charity *et al.*, 2002).

Particle bombardment-mediated transformation can be used to regenerate whole plants (Ellis *et al.*, 1991; Charest *et al.*, 1993; Walter *et al.*, 1994). The expression of foreign genes in conifers was observed on meristematic cells that have the ability to rapidly divide and have a high rate of metabolic activity. Cells with high metabolic activity are most likely to be active in endogenous gene expression with all the functions for gene transcription and translation actively expressed. Therefore, genes introduced into these cells would also have a higher probability of being expressed, since: (1) DNA replication may aid in the incorporation of foreign DNA, and (2) a certain phase (S phase) in the cell cycle may be a prerequisite for DNA integration into the genome. Transformation of conifers by particle bombardment included *Larix laricina* (Klimaszewska *et al.*, 1997), *L. decidua* (Duchesne *et al.*, 1993), *P. abies* (Robertson *et al.*, 1992), *P. glauca*, *P. mariana* (Duchesne and Charest, 1991; Charest *et al.*, 1996), *P. radiata* (Walter *et al.*, 1998), *P. strobus* (Tian *et al.*, 1997), *P. taeda* (Stomp *et al.*, 1991), and *P. menziesii* (Goldfarb *et al.*, 1991).

Stable transformation of *P. glauca* by particle bombardment and transgenic regenerated plantlets were obtained by transforming embryogenic cultures (Ellis *et al.*, 1991). Incorporation of the introduced genes into the genome was confirmed by PCR and Southern blot analysis of embryogenic callus and regenerated transformed plants, as well as spruce budworm feeding trials with transformed tissues (Ellis *et al.*, 1991). Stable transformation of *P. abies* tissue was obtained following bombardment of mature somatic embryos with pRt99Gus (Robertson *et al.*, 1992). Stable transformation of *P. mariana* by particle

bombardment has been accomplished by Charest *et al.* (1996). An efficient particle bombardment has been developed by stably transforming several *P. abies* embryogenic tissue lines. Transgenic *P. abies* plants from nine independent transformation events were recovered and were growing in a greenhouse for future investigations (Walter *et al.*, 1999). In addition, pollen grain transformation by particle bombardment has been successful. The grains were transformed and a cone from a selected female was pollinated. The production of transgenic individuals in this way avoids the need for in vitro regeneration. Normal cone development has been reported in *Pinus aristata*, *Pinus griffithii*, *Pinus monticola*, and *P. abies* (Table 3). However, transgenic plants have not yet been produced, and regeneration of transformed embryos is proving to be difficult (Fernando *et al.*, 2000).

In *P. radiata*, a particle bombardment system was established by genetically transforming embryogenic tissue (Walter *et al.*, 1998). The average number of stable, geneticin-resistant lines recovered was 0.5 per 200 mg fresh weight bombarded tissue. Expression of the *uidA* reporter gene was detected histochemically and fluorimetrically in transformed embryogenic tissue and in derived mature somatic embryos and regenerated plants. More than 150 transgenic *P. radiata* plants were produced from 20 independent transformation experiments with four different embryogenic clones (Walter *et al.*, 1998). Using the same method, an efficient biolistic transformation technology was developed for *P. abies* (L.) Karst (Walter *et al.*, 1999). Stable integration of a *uidA* reporter gene and a kanamycin-resistance gene (*nptII*) into the genome of *P. abies* was achieved, and more than 200 mature embryos were regenerated for every transformation event. Transgenic *P. abies* plants from nine independent transformation events were recovered and were awaiting field release (Walter *et al.*, 1999; Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

In addition, Clapham *et al.* (2000) established an efficient production method of transgenic plantlets of *P. abies* from embryogenic suspension cultures using a particle inflow gun (Finer *et al.*, 1992). Embryogenic colonies resistant to Basta appeared 2 months after bombardment. Of over 100 independent Basta-resistant sublines

tested, 65% expressed the co-transformed reporter gene, and over 80% of the sublines retained their embryogenic potential. Of 11 transformants analyzed, 4 contained transgenes in low copy number (1–3), the rest contained transgenes with up to 15–20 copies. Over 200 Basta-resistant sublines from four cell lines have been established, of which 138 are confirmed as transformed (Clapham *et al.*, 2000). Particle bombardment has also been used to produce transgenic plants in conifers for functional analysis of genes (Bishop-Hurley *et al.*, 2001) and promoters (Moyle *et al.*, 2002) and the development of early screening technologies for new introduced traits that promise to be of significant advantage to conifer biotechnology (Walter *et al.*, 2002).

2.5 Testing for Activity and Stability of Inheritance of the Gene, and Adverse Effects on Growth, Yield, and Quality

Applications of genetic engineering technology in tree physiology and biotechnology include: (1) alteration of tree form and performance, (2) insect resistance and herbicide resistance, (3) abiotic stress tolerance, and (4) modifying lignin content and composition. Insect resistance and herbicide resistance and modifying lignin content and composition have been widely studied in higher plants (Comai *et al.*, 1985; Bowler *et al.*, 1991; Baucher *et al.*, 1996; Pena and Sequin, 2001; Strauss *et al.*, 2001; Herschbach and Kopriva, 2002).

2.5.1 Gene expression

The activity of six different promoter-*GUS* (*uidA*) binary plasmid constructs has been analyzed in transgenic roots of *Pinus contorta* (Lindroth *et al.*, 1999). Transgenic roots were induced by infection with *A. rhizogenes* strain LBA9402, harboring a binary plasmid construct that contained one of the promoters: *Ubi-1* from *Zea mays*, 35S from CaMV, *cdc2a* and *sam-1* from *Arabidopsis thaliana*, *HRGPnt3* from *Nicotiana tabacum*, and *RSI-1* from *Lycopersicon esculentum* (Lindroth *et al.*, 1999). Promoters of broad tissue specificity (*cdc2a*, *Ubi-1*, and 35S) showed GUS staining in most cell types of all the species. The other three

promoters were expressed specifically in lateral root primordia. The studies of gene activity in primary transgenic roots allowed the screening of candidate promoters related to lateral and adventitious root formation within 3–6 weeks of inoculation in the angiosperm species and 2–3 months in *P. contorta* (Lindroth *et al.*, 1999). Recently, a putative promoter fragment of a *P. radiata* gene encoding a multifunctional *O*-methyltransferase (AEOMT) was isolated from genomic DNA (Moyle *et al.*, 2002). The isolated promoter was fused to the GUS reporter gene, and its expression profile was analyzed in transgenic tobacco and in transient transformation experiments with *P. radiata* embryogenic and xylogenic tissue. Histochemical analysis in transgenic tobacco plants revealed that the AEOMT promoter induced *uidA* expression in cell types associated with lignification, such as developing vessels, phloem and wood fibers, and xylem parenchyma as well as in nonlignifying phloem parenchyma (Moyle *et al.*, 2002).

Elfstrand *et al.* (2001) have studied the effects of an endogenous peroxidase-like gene from *P. abies*, *spi2*, on the development and growth of Norway spruce somatic embryo plants. Embryogenic cells transformed with *spi2* under control of the maize *ubi-1* promoter showed up to 40 times higher total peroxidase activity than the control cells; regenerated plants overexpressing *spi2* showed an increased total peroxidase activity (Elfstrand *et al.*, 2001). Overexpression of *spi2* resulted in increased sensitivity to stress, leading to a reduction in epicotyl formation and in height growth compared to control plants. Plants overexpressing *spi2* also showed a deeper phloroglucinol staining but similar levels of Klason lignin (Elfstrand *et al.*, 2001). In addition, adding extra copies of genes involved in *Agrobacterium* virulence and T-DNA transfer (*virG*, *virB*) to disarmed strains of *A. tumefaciens* increased transformation efficiencies for embryogenic *P. abies* (Wenck *et al.*, 1999).

2.5.2 Insect and herbicide resistance

Currently, insect resistance involves the introduction of a gene coding for *Bt* toxin from *Bacillus thuringiensis*. The *Bt* toxin inhibits the insect's digestive pathways. This gene has been successfully transformed into several species including larch

and conifers (Shin *et al.*, 1994) with varying degrees of success. Herbicide resistance has allowed the use of more efficient herbicides without concern for plant health in forestry, especially for higher intensity plantation systems. Herbicide resistance would be very useful especially in younger trees, where competition from weeds is the greatest (Shin *et al.*, 1994).

An example of herbicide resistance being introduced into a tree species is the work of Fillatti *et al.* (1987). A binary oncogenic strain of *A. tumefaciens* was introduced into a poplar clone. The strain carried a mutant gene (*aroA*), which codes for 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase that is less susceptible to the herbicide glyphosate (N-phosphonomethylglycine) than the wild-type protein. Using a method of Reiss *et al.* (1984), the neomycin phosphotransferase II (NPTII) activity of the plant tissue was measured to determine whether the tissue had been completely transformed. Analysis of the introduction of the *aroA* gene, coding for the EPSP synthase enzyme less susceptible to glyphosate, was carried out using Western blot analysis after Comai *et al.* (1985). Recently, Bishop-Hurley *et al.* (2001) have established a biolistic transformation procedure where embryogenic tissues of *P. radiata* and *P. abies* were co-transformed with two plasmid DNAs that contained the *bar* (bialaphos resistance) gene, specifying resistance to the herbicide glufosinate, and the *nptII* gene and the *uidA* reporter gene, respectively. Regenerated plantlets from *P. radiata* and *P. abies* transgenic lines were spray tested with commercial rates of Buster (glufosinate at 0.5, 1.0, 2.0, and 3.0 kg active ingredient per hectare). Transgenic plants survived and continued to grow with minor or no damage to their needles, whereas nontransgenic plants regenerated from the same cell lines died within 8 weeks of spraying (Bishop-Hurley *et al.*, 2001).

2.5.3 Wood modification

Modification of wood by altering its chemical structure is a way of improving its properties. Research and development in this field has become increasingly important because wood is a renewable energy source and industrial material (Chiang, 2002; Doblin *et al.*, 2002). The wooden

cell wall consists mainly of polymers including cellulose, lignin, and hemicellulose (Doblin *et al.*, 2002). The reactive hydroxyl groups on these polymers are responsible for many physical and chemical properties of wood. The basic chemistry of the cell wall polymers can be altered by wood modification, which can change important properties of the wood including decreasing lignin content, increasing cellulose content, and improving durability, dimensional stability, and hardness (Chiang, 2002; Doblin *et al.*, 2002; Li *et al.*, 2003). Modern molecular genetics technologies have created new opportunities for wood modification (Chiang, 2002; Doblin *et al.*, 2002; Li *et al.*, 2003). At present several processes for modifying wood have been demonstrated on a laboratory scale and continued advances in process development will see these materials becoming more commonplace in the near future (Chiang, 2002; Li *et al.*, 2003).

Lignin is the second most abundant organic compound on earth, and represents about 25% of the global wood biomass (Leple *et al.*, 1992). Although lignin is an important compound for wood development, it is an obstacle to efficient pulp and paper production because the lignin must be removed in order to extract the cellulose from the wood (Pilate *et al.*, 2002). This process is energy consuming and requires the use of polluting chemicals. It is of great interest to try and engineer trees to have a lower lignin component or a lignin type that is easily extracted without reducing tree growth rates or bole form (Baucher *et al.*, 1996; Pilate *et al.*, 2002). Lignin is a group of compounds formed from three precursors: 4-hydroxycinnamyl-alcohol (p-coumaryl alcohol), coniferyl alcohol, and sinapyl alcohol. Field and pulping performances of transgenic trees with altered lignification have been reported (Pilate *et al.*, 2002). Conifers could be engineered with lower lignin content, or more syringyl lignin instead of the guaiacyl lignin, although there is no report in this area right now (Herschbach and Kopriva, 2002).

Cellulose is the major component of plant cell walls with secondary cell walls having a much higher content of cellulose (Doblin *et al.*, 2002). The relationship between cellulose and lignin biosynthesis is complicated, but it is confirmed that inhibition of lignin biosynthesis in transgenic trees will increase cellulose biosynthesis

and plant growth (Chiang, 2002; Doblin *et al.*, 2002; Li *et al.*, 2003). Both lignin quantity and reactivity may be regulated by distinct monolignol biosynthesis genes, encoding 4-coumarate-CoA ligase (4CL) and coniferaldehyde 5-hydroxylase (CAlD5H) (Chiang, 2002; Li *et al.*, 2003). Li *et al.* (2003) used *Agrobacterium* to co-transfer antisense 4CL and sense CAlD5H genes into aspen (*Populus tremuloides*). They found that lignin reduction by as much as 40% with 14% cellulose augmentation was achieved in antisense 4CL plants; increases as great as threefold in the syringyl/guaiacyl ratio were observed without change of lignin quantity in sense CAlD5H plants (Li *et al.*, 2003). An increase in syringyl/guaiacyl ratio also accelerated cell maturation in stem secondary xylem in transgenic aspen (Li *et al.*, 2003). Their results suggest that this multigene co-transfer system should be broadly useful for plant genetic engineering and functional genomics (Chiang, 2002; Li *et al.*, 2003).

2.5.4 Bioremediation

Phytoremediation, the use of transgenic plants to remove contaminants from soil or water, promises to have a positive impact on environmental pollution and, in the long term, the preservation of natural forests (Herschbach and Kopriva, 2002). Overexpression of the bacterial mercuric reductase in yellow poplar resulted in transgenic plants that were resistant to toxic levels of mercuric ions and were able to release elemental mercury (Rugh *et al.*, 1998). Currently, scientists at the University of Washington are trying to transform Loblolly pine with *A. tumefaciens* expression vector harboring the rabbit cytochrome *P450 2E1* gene for detoxification of soils contaminated with ethylenedibromide (M.P. Gordon, personal communication).

2.5.5 Flowering control

Although the major traits introduced into tree species have focused on insect resistance, low lignin, and enhanced growth (Pena and Sequin, 2001; Strauss *et al.*, 2001), there are several possible risks from transgenes to the native forest ecosystems (Mathews and Campbell, 2000).

Increasing knowledge about the control of flower development in trees opens up strategies to reduce or prevent the danger of vertical gene transfer to the wild tree species via genetic engineering of sterility (Strauss *et al.*, 1995). Another problem specific to tree species, compared to conventional agricultural crops, is the necessity for long-term stability of the transgene over several vegetation periods (Fladung, 1999; Kumar and Fladung, 2001). In addition, risk assessment investigations aimed at root–soil interactions, transfer of transgenes to wild plants, and possibility of horizontal gene transfer must be part of field trial assessment with transgenic trees as discussed by McLean and Charest (2000) and Strauss *et al.* (2001).

2.5.6 Future applications in tree physiology and molecular improvement

Tree transformation techniques allow direct testing of some hypotheses in tree physiology that have been exceedingly difficult to resolve using other biochemical approaches. Using transgenic trees, we can directly test the roles of specific enzymes in metabolic processes (Elfstrand *et al.*, 2001), the functions of different promoters (Lindroth *et al.*, 1999), and the influences of matrix attachment region on plant transformation and developmental processes (Levee *et al.*, 1999). We can also directly identify specific genes from genomic sequences. Tree transformation technique provides a powerful new experimental tool for tree molecular improvement. With this technology, transgenic trees with useful phenotypes unachievable by conventional tree breeding can be produced. Tree transformation also allows the commercial value of improved tree lines to be captured by industrial investors, such as commercial plant lines expressing foreign genes conferring resistance to viruses (James *et al.*, 1993, 1998; Birch, 1997). The extent to which the other practical or commercial expectations of plant transformation can be met depends on the efficiency and predictability of production of lines with the desired phenotype, and without undesired side effects from the transformation process (Birch, 1997; Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

3. FUTURE ROAD MAP

3.1 Expected Products

Throughout human history, pines have provided a wealth of products beneficial to human society. The wood of many pine species has been used for structural lumber, production of paper and related products, fuel wood, posts, poles, and myriad other products. Pines have also provided a wide range of beneficial nonwood products including essential oils, resin, fragrant and attractive foliage, ornamental plants, decorative objects, edible seeds, flavorings, and medicinal products. In many cases, both wood and nonwood products from conifers have been overexploited to the point where extensive damage or loss of forest area has resulted. On the other hand, pines have been revered by many human cultures and have been used as both religious and political symbols. They are also the subject of a rich mythology and folklore and are well represented in arts. Genetic engineering of pine is expected to produce transgenic pines with novel traits such as (1) insect and herbicide resistance, (2) wood modification, (3) bioremediation, and (4) flowering control.

3.2 Addressing Risks and Concerns

Before the release of transgenic conifers to field, we have to achieve environmental and public acceptance. The activist groups see transgenic trees as setting a precedent toward broad and uncontrolled release of genetically modified plants in forests, and hence they are likely to fight vigorously against them (Strauss and Bradshaw, 2001; Fenning and Gershenzon, 2002). As long as ecological studies and development of improved options for genetic modification continue, we should find ourselves in a position to produce better kinds of transgenic trees, and have more answers to the questions being raised about their behavior in the environment. Although plant genomic research has been funded well in the United States to date, research support for plant genetic engineering risk assessment studies has been paltry by comparison (Strauss and Bradshaw, 2001).

The agencies primarily responsible for regulating biotechnology in the United States are

the US Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Food and Drug Administration (Strauss and Bradshaw, 2001). Products from transgenic organisms are regulated according to their intended use, with some products falling under the remit of more than one agency. In the case of transgenic trees the agency responsible is the USDA; the EPA is also involved when modified traits may have an environmental impact. The USDA regulates novel plant release through its Animal Plant Health Inspection Agency (APHIS). Applicants for transgenic plants releases must provide details of the organism, the genes transformed, their products, and the purpose of release (James *et al.*, 1998; Strauss and Bradshaw, 2001). In the case of field trials, the experimental design and precautions against accidental escape of the transgenic trees must also be included in the application. APHIS may also demand special precautions such as closed containers for transport to field site and field cages to minimize the risk of pollen escape. APHIS permits for release into the environment are usually issued or denied within 120 days and during that time state officials will inspect the facilities to determine security and operating conditions. Permits for field trials are renewed annually. Before commercialization, genetically engineered plants must conform to standards set by State and Federal marketing statutes. There are no national requirements for varietal registration of new plants (Strauss and Bradshaw, 2001).

The feasibility of managing gene flow and minimizing the risks of genetic pollution appear only to have been considered in the United States and Japan, and only under certain conditions (Strauss and Bradshaw, 2001). Some of the regulations are decidedly short term, allowing trials to be strictly managed yet failing to make provision for the same safeguards at general release (James *et al.*, 1998; Fenning and Gershenzon, 2002). Perhaps most worrying is the fact that while the regulations are rightly concerned with biosafety issues, they tend to overlook the impact that fast-growing, long-lived plants may have on-site productivity. Transgenic super trees possess all the characteristics of a good weed and risk becoming invasives, and very fast growing, nutrient-demanding plantations operated on short rotations could drive inappropriate plantation

development. Many in the forestry industry, as well as academic researchers, are interested in genetically engineering trees but are proceeding cautiously as they evaluate the technology's environmental consequences and gauge how consumers will react to products made from genetically modified trees (Rogers and Parkes, 1995; Mullin and Bertrand, 1998; Fenning and Gershenzon, 2002; Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

Forestry researchers are working on ways to create trees to resist pests, to make it easier to process pulp and paper products, and to assist in the restoration of endangered tree species (Strauss and Bradshaw, 2001; Fenning and Gershenzon, 2002). However, representatives from industry, academia, and environmental groups are expected to ask critical questions such as whether the benefits of developing genetically engineered trees outweigh any potential environmental risks, how broader historical and sociological factors play into the debate, whether current forestry practices will meet or exceed market demand for wood and paper products, and whether there should be changes in the regulatory process specific to the introduction of genetically engineered trees (Mullin and Bertrand, 1998; Strauss and Bradshaw, 2001), although questions about the potential ecological risks of introducing genetically engineered trees into the environment have been very complicated. In addition to examining the potential benefits and risks associated with genetically engineered trees, we also need to follow the current laws and regulatory practices that apply to the technology (James *et al.*, 1998; Fenning and Gershenzon, 2002). The potential environmental impacts of genetically engineered trees must be weighed against the costs of not pursuing the technology. Long-term field studies should be designed to examine not only novel gene stability and transgenic behavior but also tree crop-induced fluxes in soil nutrient status and soil water availability (Rogers and Parkes, 1995; James *et al.*, 1998; Mullin and Bertrand, 1998; Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

3.3 Expected Technologies

Although particle bombardment and *Agrobacterium*-mediated gene transfer methods are now

sufficiently developed to allow transformation of essentially any plant species in which regenerable cells can be identified (Birch, 1997), the key to transformation of conifers (a group of recalcitrant species) appears to be development of methods to expose many regenerable cells to nondestructive gene transfer treatments because the frequencies of transformation in currently established transformation protocols are low and the frequencies of undesired genetic change or unpredictable transgene expression are high in some conifers (Walter *et al.*, 1998; Wenck *et al.*, 1999; Tang *et al.*, 2001). These constraints may be addressed by improving tissue culture systems to enrich for regenerable cells accessible to gene transfer, by developing techniques to allow rapid detection and optimization of transformation event, and by developing highly efficient selection techniques. In the longer term, a more important goal than increased transformation efficiency is the development of transformation methods and constructs tailored for predictable transgene expression, without collateral genetic damage (Birch, 1997). To achieve a high proportion of useful transformants, we need to understand more clearly the factors contributing to undesired genetic change during the transformation process.

Establishment of model transformation systems is very important in conifers. Although *Arabidopsis* is a widely used model system in plant biology because of its small genome, small plant size, and rapid generation time, the features that make this model attractive for some genetic studies cannot be generally exploited in practical transformation systems for conifers (Birch, 1997). As our understanding of the genetic basis of agronomic traits increases, it is likely that this goal will be extended to the introduction of greater lengths of DNA encoding multiple genes. We will need to determine the capacity of available methods to introduce such lengths of DNA intact. Some of these questions may not be answered with model plants; we have to be prepared to select the models according to the questions, and test the answers for applicability to the practical targets (Birch, 1997). Success in transformation of *Pinus radiata*, *P. strobus*, *P. taeda*, *P. abies*, *P. glauca*, and *P. mariana* provides good opportunities to develop a conifer model system from some of them (Walter *et al.*, 1998; Levee *et al.*, 1999; Wenck *et al.*, 1999; Klimaszewska *et al.*, 2001; Tang *et al.*, 2001).

Plant transformation is already sufficiently developed to allow the testing and even commercialization of plants with novel phenotypes under simple genetic control (Birch, 1997). However, our understanding of the biological basis for efficient conifer transformation and improved technologies for predictable transgene expression without collateral genetic damage need to be extended and developed (Walter *et al.*, 1998; Wenck *et al.*, 1999; Tang *et al.*, 2001). Exciting scientific advances in exploring the mechanism of *Agrobacterium*-mediated transformation have been made, but the whole process of gene transfer from bacteria to the nuclei of plant cells is not fully understood yet (Sheng and Citovsky, 1996). As transformation projects are increasingly undertaken with the possibility of generating commercially useful products, scientists in turn must increasingly integrate social, legal, and economic issues as well as technical issues from the earliest stages of project design (Birch, 1997; Tang and Newton, 2003, 2004; Tang *et al.*, 2004, 2005, 2006).

Tissue culture is not a theoretical prerequisite for plant transformation, but it is employed in almost all current practical transformation systems to achieve a workable efficiency of gene transfer, selection, and regeneration of transformants (Birch, 1997). Transformation protocols using particle bombardment (Birch and Bower, 1994) or *Agrobacterium* (Van Wordragen and Don, 1992) have been published based on practical experiences in laboratories working on recalcitrant species. Unfortunately, there is no guarantee that a transformable plant cell type will prove regenerable, even in the hands of the most successful tissue culturist (Birch, 1997). For transformation of a specific coniferous species, we need to pay attention to strategies for selection, transgene expression and integrating components of transformation. Screening approaches are expensive unless the transformation efficiency is high (Kim and Minamikawa, 1996). The features useful for the regulated expression of transgenes in plants include: (1) appropriate transcriptional promoters and enhancers (Benfey *et al.*, 1990), (2) transcriptional terminators and 3-enhancers (Richardson, 1993), (3) polyadenylation signals, untranslated 5-leader, and 3-trailer sequences (Turner and Foster, 1995; Wu *et al.*, 1995), (4) transit sequences for appropriate subcellular

compartmentation and stability of the gene product (Hicks *et al.*, 1995), and (5) cryptic introns resulting in inappropriate RNA processing (Perlak *et al.*, 1991; Reichel *et al.*, 1996). It is commonly generalized that *Agrobacterium* produces simpler integration patterns than direct gene transfer, but both particle bombardment and *Agrobacterium*-mediated transformation approaches result in a similar range of integration events, including truncations, rearrangements, and various copy numbers and insertion sites. Furthermore, the frequency distributions of copy number and rearrangements vary with transformation parameters for both gene transfer methods (Grevelding *et al.*, 1993).

3.4 Limitations and Changes of Technologies in Relation to Public Perceptions, Industrial Perspectives, and Political and Economic Consequences

Genomic studies have provided exciting opportunities for crop molecular breeding and plant improvement because the genomic sequences for model species are available and genetic transformation methods based on *Agrobacterium* and particle bombardment have resulted in the production of large numbers of primary transformants with normal morphology and stable transgene expression in short-term trials (Meilan *et al.*, 2000; Lucier *et al.*, 2001). Based on the information from genomic sequences, we are able to clone, express, and analyze functions of any single or multigene that are relative to a specific metabolic process, signal transduction pathway, storage product accumulation, cell division, plant growth and flowering, and seed development. However, it is unknown to what extent transformation causes less obvious genetic damage, such that transgenic clones might need to be re-evaluated in long-term field trials to verify that their yield and adaptability characteristics remain intact (Meilan *et al.*, 2000). Because most industries wish to see tested, well-known clones transformed and used directly, this is a critical issue for transgenic trees (Dekker-Robertson and Libby, 1998; Meilan *et al.*, 2000).

The major technical challenge facing tree transformation biology is the development of methods

and constructs to produce a high proportion of plants showing predictable transgene expression without collateral genetic damage (Rogers and Parkes, 1995; Birch, 1997). The issue of stability of gene expression is important not only for the maintenance of delivered traits, but may also be essential for obtaining regulatory and public approval for genetically modified trees through use of genes that impart floral sterility for gene containment (Birch, 1997). If engineered sterility is essential for commercial use, a breakdown in gene expression could have economic, environmental, and legal consequences (Strauss and Bradshaw, 2001). Gene silencing has been an active area of research in recent years, and it is becoming clear that many of the same mechanisms that act in silencing transgenes are also important for defense against viruses and invasive DNA elements (Fire *et al.*, 1998; Fire, 1999). However, little is known about the mechanisms of gene silencing and the factors that correlate with expression stability (Fire *et al.*, 1998). The extent of somaclonal variation and transgene instability is expected to vary depending on the specific transformation system, tree genotype, and vector/gene constructs employed (Birch, 1997; Fenning and Gershenzon, 2002). Therefore, studies are likely to be best accomplished via public scientists working in partnership with industries that are considering commercial use.

The insect- and herbicide-resistance genes widely used in crop biotechnology could be of considerable value for tree plantations and have been shown to be highly effective in transgenic trees (Meilan *et al.*, 2000). However, the engagement of academic and government scientists with industries wishing to commercialize transgenic trees is likely to be essential to convince a wary public that scientific assessments and plantation monitoring programs have been conducted responsibly (James *et al.*, 1998; Mullin and Bertrand, 1998). Another significant hurdle for use of transgenic technology in forestry is the Intellectual Property Rights that dominate the biotechnology sector (Lucier *et al.*, 2001). Even the major companies, which own large patent estates, are often in court with their competitors, fighting over access to major technologies, and several of these companies have chosen to use their patents as weapons to restrict access to technologies and to extend their sphere of control. The tight control of

technology and information by a few companies is itself a major driver of the high perception of risk associated with biotechnology (Strauss and Bradshaw, 2001; Fenning and Gershenzon, 2002). Unless there are major changes in government and corporate policy about control of intellectual property, industries are likely to continue to face a hostile public and a skeptical scientific community (Strauss and Bradshaw, 2001).

Particle bombardment and *Agrobacterium*-mediated transformation have been successfully used for a wide range of conifers including *Abies nordmaniana*, *L. decidua*, *P. abies*, *P. glauca*, *P. mariana*, *P. radiata*, *P. taeda*, *P. elliotii*, and *P. menziesii*. Production of genetically engineered conifers with commercially useful traits such as herbicide, insect, and pathogen resistance has been accomplished. Further development of gene transfer technologies for conifers will allow for the assessment of xylem-specific genes, promoters, and specific fungal-resistance genes in transgenic conifers, and the production of herbicide-resistant forest tree germplasm. These will develop to form commercially applicable technologies. Genetic engineering programs are focused on developing technology platforms and providing research services that lead to a better understanding of tree development and gene function. Forest research has been and is still firmly committed to playing a positive scientific role in the public debate concerning the application of genetic engineering technology in forestry and environmental biology. The techniques previously used for genetic transformation of agricultural plants are now finding their way into the engineering of forest tree species. At present, traditional breeding programs proceed at a slow rate due to long maturation times and the slow growth rate of trees; however, biotechnological approaches have the potential to provide significant improvement in tree growth and quality. If these problems can be addressed, forestry will enter a new era of productivity and quality.

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Spruce

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1. INTRODUCTION

1.1 Distribution of the Genus *Picea*

The genus *Picea* A. Dietr. (spruce) consists of 34 species that are restricted to the northern hemisphere (Farjón, 1990). Most of the species occur in Asia, a couple of species in Europe, and up to 10 species in North America. Spruce has a transcontinental distribution in Asia, Europe, and North America. The transcontinental forest comprises the North American species, white spruce, *Picea glauca* (Moench) Voss; and black spruce, *Picea mariana* (Mill.) Britton *et al.*; and the Eurasian species, Norway spruce, *Picea abies* (L.) Karst.; and Siberian spruce, *Picea obovata* Ledeb. Other species are restricted to mountainous and subalpine regions (Wright, 1955; Rushforth, 1987; Farjón, 1990). Spruce species are adapted to a wide range of ecological habitats and climates. In the most northern latitudes of its distribution, where temperatures are low and the growing season is short, it is common to find pure stands of spruce. At warmer latitudes with longer growing seasons, spruce mixes with other deciduous angiosperm and gymnosperm species. During the cooler climate of the latest

ice age, an ice sheet spread across the northern hemisphere and had a great impact on species' distribution and population structure (Hewitt, 2004). In Europe, the mountain ranges of the Pyrenees and the Alps acted as barriers and thus caused a latitudinal dispersal, whereas in North America longitudinal mountain ranges and access to Central America made southward migration possible (Hewitt, 1996). The distribution of *Picea* today is largely influenced by the climatic oscillations during the Quaternary, and the effects on population structures and speciation have been studied (Lagercrantz and Ryman, 1990; Bucci and Vendramin, 2000; Vendramin *et al.*, 2000; Jaramillo-Correa *et al.*, 2004; Ledig *et al.*, 2004; Ran *et al.*, 2006).

1.2 Taxonomy

Spruce belongs to the family Pinaceae and the genus includes up to 34 species (Farjón, 2001; Ledig *et al.*, 2004). The intrageneric classification within *Picea* is considered difficult, and various authors differ as to species assignment to different subgroups. The relatively narrow range in morphology and ecological preference between species

and the high intraspecific morphological variation may compromise the reliability of the classification (Wright, 1955). Furthermore, many of the *Picea* species will hybridize in nature where species distributions overlap (Wright, 1955; Rushforth, 1987; Farjón, 1990). However, monophyly of *Picea* has never been debated (Wright, 1955; Rushforth, 1987; Sigurgeirsson and Szmidt, 1993).

Different subdivisions of the genus have been made on the basis of morphology and crossability studies. Many of the morphological characters overlap between species and there has been a lack of agreement on which trait to rely on. Traditionally, the genus is divided into three sections: *Picea*, *Casicta*, and *Omorika* (Schmidt-Vogt, 1977). Farjón (1990) divided *Picea* into sections: sect. *Picea* and sect. *Casicta*, each with two subsections (Table 1). Molecular approaches to resolve the intergeneric relationships of *Picea* do not support any of the previous subdivisions (Sigurgeirsson and Szmidt, 1993; Ran *et al.*, 2006). None of these authors suggest a new subdivision, but conclude that further studies are necessary.

1.3 Origin and History

Based on fossil records (LePage, 2001) and molecular clock estimation of the *matK* (maturase K) gene (Wang *et al.*, 2000), the origin of *Picea* could date back to the early Tertiary or late Cretaceous. The fossil record of *Picea* includes wood, leaves, cones, and seeds. The oldest record found is from Axel Heiberg Island in the Canadian Arctic and dates back to middle Eocene (ca. 45 million years BP) (LePage, 2001). The finding of three new fossil species at the Axel Heiberg Island also indicates that the genus had already diversified at that time (LePage, 2001). Most fossil findings from Asia are from the Oligocene, Miocene, and Pliocene, and the fossils found in Europe are even more recent, mostly from the Pliocene with one exception (LePage, 2001). Ledig *et al.* (2004) propose a North American origin for *Picea*, with migration to Asia across the Bering land bridge. They also suggest that the genus then spread from Asia further west to Europe, as the tropical to subtropical climate probably presented a barrier for dispersal through the DeGeer and Thulian Routes (Tiffney, 1985). Paleorecords contribute to evaluate species migration in response to climate

change; nevertheless, their accuracy is limited because of glacial refugia that are undetectable in fossil records (cryptic refugia). One example is the controversial unglaciated area extending from northeastern Siberia to the Yukon Territory. Recent genetic studies based on chloroplast DNA (cpDNA) support the existence of a glacial refuge in Alaska of white spruce (Anderson *et al.*, 2006). There are two main hypotheses regarding the origin of the genus (Wright, 1955; Sigurgeirsson and Szmidt, 1993; Ran *et al.*, 2006). Wright (1955) argues that the most likely center of origin is in Asia, with two independent migrations to the American continent. He bases his argument on the fact that Asia contains the largest species diversity, and that it harbors *P. koyamae*, which he considered to be the most basal species. However, these ideas conflict with the findings of Sigurgeirsson and Szmidt (1993) and Ran *et al.* (2006). The first molecular phylogeny of spruce at the genus level, using chloroplast DNA-restriction fragment length polymorphisms (cpDNA-RFLP), was constructed by Sigurgeirsson and Szmidt (1993) and their results indicate a North American origin. Based on a joint cp- and mtDNA (mitochondrial DNA) analysis, Ran *et al.* (2006) found, in agreement with Sigurgeirsson and Szmidt (1993) that the North American species Sitka (*P. sitchensis*) and Brewer spruce (*P. breweriana*) were basal to the other spruces in the phylogeny, and thus support a North American origin.

1.4 North American Species

Six of the 10 North American species occur in southwest United States and Mexico, and four are considered relicts (Ledig *et al.*, 2000, 2004). The economically important species, white and black spruce, are both found across northern North America and have a sympatric distribution. However, they have different habitat requirements, and natural hybridization has not been reported (Wright, 1955). White spruce has a more northern limit distribution. Attempts to introduce white spruce in Great Britain and Europe for reforestation have been unsuccessful in most cases. Red spruce (*P. rubens*) and Sitka spruce are also of significant economic importance and have a more restricted distribution. Red spruce's range covers Maine and surrounding territories and

Table 1 Subdivision and recognized species according to Farjón (1990)

Taxa	Common name	Distribution
Section <i>Picea</i>		
Subsection <i>Picea</i>		
<i>Picea abies</i> (L.) H. Karst.	Norway spruce	Europe
<i>P. alcoquiana</i> (Veitch ex Lindl.) Carrière		Japan
<i>P. asperata</i> Mast.		Asia
<i>P. aurantiaca</i> Mast.	Chihuahua spruce	Asia
<i>P. chihuahuana</i> Martínez		North America
<i>P. crassifolia</i> Kom.		Asia
<i>P. glauca</i> (Moench) Voss	White spruce	North America
<i>P. glehnii</i> (F. Schmidt) Mast.		Japan
<i>P. koraiensis</i> Nakai		Asia
<i>P. koyamae</i> Shiras.	Black spruce	Japan, Asia
<i>P. mariana</i> (Mill.) Britton <i>et al.</i>		North America
<i>P. maximowiczii</i> Regel ex Mast.		Japan
<i>P. meyeri</i> Rehder and E.H. Wilson	Siberian spruce	Asia
<i>P. morrisonicola</i> Hayata		Asia
<i>P. neoveitchii</i> Mast.		Asia
<i>P. obovata</i> Ledeb.	Oriental spruce	Europe, Asia
<i>P. orientalis</i> (L.) Peterm.		Asia
<i>P. retroflexa</i> Mast.		Asia
<i>P. rubens</i> Sarg.	Red spruce	North America
<i>P. schrenkiana</i> Fisch. and C.A. Mey.		Asia
<i>P. smithiana</i> (Wall.) Boiss.		Asia
<i>P. torano</i> (Siebold ex K. Koch) Koehne	Himalayan spruce	Japan
<i>P. wilsonii</i> Mast.		Asia
Subsection <i>Omorikae</i>		
<i>P. brachytyla</i> (Franch.) E. Pritz	Brewer spruce	Asia
<i>P. breweriana</i> S. Watson		North America
<i>P. farreri</i> C. N. Page and Rushforth		Asia
<i>P. omorika</i> (Pancic) Purk.	Serbian spruce	Europe
<i>P. spinulosa</i> (Griff.) A. Henry		Asia
Section <i>Casicta</i>		
Subsection <i>Sitchensis</i>		
<i>P. jezoensis</i> (Siebold and Zucc.) Carrière	Sitka spruce	Japan
<i>P. likiangensis</i> (Franch.) E. Pritz.		Asia
<i>P. purpurea</i> Mast.		Asia
<i>P. sitchensis</i> (Bong.) Carrière		North America
Subsection <i>Pungentes</i>		
<i>P. engelmannii</i> Parry ex Engelm.	Engelmann spruce	North America
<i>P. pungens</i> Engelm.	Blue spruce	North America

extends along the Appalachians. Hybridization between red and black spruce has been described based on random amplified polymorphic DNA (Perron and Bousquet, 1997). Sitka spruce takes its name from Sitka, the former capital of Alaska when it was a Russian territory. It is found along the west coast from central Alaska to northern California (Rushforth, 1987; Farjón, 1990). A genetic analysis based on sequence-tagged polymorphism markers indicates that there is a zone of introgression between Sitka and white spruce in British Columbia (Bennuah *et al.*,

2004). Sitka spruce has become the most widely used conifer for reforestation purposes in Ireland, Wales, Scotland, and the Highlands, because the climate in its natural range is humid and mild, similar to that of western Great Britain. *P. pungens* grows in the Rocky Mountains, withstanding cold and hazardous winters, and hot and dry summers.

1.5 European Species

The most economically important conifer tree species in Europe is Norway spruce. Its natural

distribution ranges across the Pyrenees, Alps, and Balkans, northward to southern Germany and Scandinavia, and eastward through the Carpathian Mountains and Poland to western Russia (Rushforth, 1987; Farjón, 1990). Here, Norway spruce meets Siberian spruce in a broad hybridization zone. Siberian spruce then extends across northern Asia. The taxonomic relationship between Norway and Siberian spruce is still unresolved. A third species, Serbian spruce (*P. omorika*) is found in Serbia and Bosnia and is endemic in a restricted area along the Drina River. The two species, Serbian and Norway spruce, are usually mixed within the range of Serbian spruce (Farjón, 1990). However, they never hybridize in nature.

1.6 Asian and Japanese Species

Asia harbors most of the species diversity in the genus *Picea*. Farjón (1990) points out, together with other authors (see Farjón, 1990) that if more was known about many of the Asian spruce, some would be considered subspecies and varieties rather than species. Ran *et al.* (2006) include 19 species from Asia, and their results suggest a repeated dispersal to this area because they are polyphyletic. There are six species in Japan, four of which are endemic. In two previous molecular phylogenetic studies (Sigurgeirsson and Szmidt, 1993; Ran *et al.*, 2006), all the Japanese species were sampled. In both studies, those species were placed into different clades. This indicates that the Japanese species do not have a single origin (Ran *et al.*, 2006).

1.7 Cytology

There seems to be little karyotype differentiation between the species in the Pinaceae family (Murray, 1998). The diploid chromosome number in *Picea* is $2n = 24$ (Murray, 1998; Siljak-Yakovlev *et al.*, 2002; Vischi *et al.*, 2003). The genome size in Norway spruce and Serbian spruce has been estimated at $2C = 37.2$ pg (picogram) and 33.8 pg, respectively; and the GC (guanine-cytosine) content in both species is about 41% (Siljak-Yakovlev *et al.*, 2002). Depending on the method used and the sample, the DNA content

in white spruce varies from 17 to 14 pg and from 22 to 34 pg in black spruce (Murray, 1998). The genome size in Norway spruce corresponds to $2C = 30 \times 10^9$ bp (base pair) (Murray, 1998; Vischi *et al.*, 2003). The chromosome location of ribosomal DNA (rDNA) has been determined using fluorescent *in situ* hybridization and the variation in number of rDNA sites between species. In Norway spruce, six rDNA sites have been found (Siljak-Yakovlev *et al.*, 2002; Vischi *et al.*, 2003), whereas eight, seven, and five sites have been found for Serbian, white, and Sitka spruce, respectively.

1.8 Economic Importance and Industrial Uses

In Canada, forest cover spans 245 million hectares (Mha), well over two-fifths of its land mass; the most common tree species are white and black spruce. About 197 Mm^3 of industrial round wood were harvested in 2002 and the value of forest products exported in 2002 was US\$22.5 billion, whereas forest imports were valued at US\$4 billion. Major forest products include pulp and paper, lumber, veneer, plywood and particle board, and shingles and shakes. In the European Union (EU), about 127 of Union's 323 Mha—39% of its 323 Mha—is forest cover, varying from 10% in the United Kingdom to 50–60% in Austria, Finland, and Sweden. The EU forests are predominantly coniferous; 85% of them categorized as being seminatural, 10% intensively managed plantations, and only 5% natural stands. Total harvested wood for 2000 is estimated at 287 Mm^3 for the EU.

However, in the context of climate change, an increasing number of pests may target forest tree species, including spruce. The ecological ranges of existing pests could expand, and introduced exotic pests could also proliferate in these new environments. Therefore, proper strategies and mitigation processes for maintaining forest health must be elaborated. In this regard, tree biotechnology would have a significant impact (Fladung and Dietrich, 2006). Although not clearly quantifiable in economic terms, the impact of carbon sequestration from the forest would not be negligible. Finally, both natural and planted forests clearly play an

important role in maintaining water and wildlife resources.

1.9 Traditional Breeding: Objectives, Tools, and Strategies

Tree breeding aims to develop improved trees for economically important traits. Strictly speaking, conventional breeding is done by selecting and crossing trees at the individual level (Eriksson and Ekberg, 2001). In a wider sense, breeding also includes selection at the species and provenance levels. Conventional tree breeding is a cyclical process that relies on the extent of genetic variation. Genetic gains are obtained by selecting the best trees in the breeding population. Genetic gain is an estimate of the genetic improvement after selection, which is reflected in a phenotypic improvement. If the proportion of additive component of genetic variance is high, the genetic gain after selection of the best individuals is expected to be high. Selection in the breeding population (backward selection) is monitored by the results of the genetic evaluation of the progeny. Selection can also be performed on the best open-pollinated offspring (forward selection). The first step of a breeding program is to identify the objectives, which will differ depending on the final use of the improved material. Conventionally, forest tree breeding is based on the evaluation of such traits as growth and straightness. Other economically important traits related to wood quality and energy content are not usually included in breeding programs because they are too complex and expensive to achieve. Once the breeding objective has been identified, the next step is to estimate the additive genetic variance and expected genetic gain. Selection decreases genetic diversity in the improved population. Such an important issue has to be considered when designing a forest tree breeding program. For example, in Scots pine (*Pinus sylvestris* L.), which has large undomesticated populations, breeding programs contain a large amount of genetic diversity, and it has been estimated that the genetic gain can increase by 10% in a second-cycle breeding population without decreasing the genetic diversity (Ruotsalainen, 2002).

1.10 Limitations of Conventional Breeding and Rationale for Genetic Engineering

Conventional tree breeding has been proven to be very efficient for traits with high heritability, but is inefficient in certain cases such as when heritability is low, the evaluation of the trait is difficult or very time consuming, and juvenile period is too long. In general, a single breeding cycle may require some 25 years. The use of molecular markers to assist in tree breeding (marker-assisted selection, MAS) was proposed as a tool to shorten the period required and, consequently, improve the efficiency of conventional plant breeding programs (reviewed by Dekkers and Hospital, 2002). This technique consists of indirect selection for the desired trait in early stages using molecular markers. The most important question for efficient application of MAS is to find a tight linkage between markers and putative genomic regions controlling the trait (called as quantitative trait loci or QTL). Detection of a tight linkage requires a large number of progeny sets tested under different environments and developmental stages (Asíns, 2002). So far, the progenies used to develop markers to assist selection of desired QTLs have been based on too small progenies (around 100 individuals) and partially informative molecular markers (dominant markers, e.g., amplified fragment length polymorphisms, AFLPs). Thus, the QTLs identified and their linkage with flanking markers might not necessarily be consistent. The rapid advances in molecular genetics will help resolve the main limitations concerning the practical application of MAS. New molecular technologies are getting cheaper and therefore, molecular markers can be applied to a greater amount of material with a considerable improvement in statistical power to find consistent markers to be applied in MAS. Future possibilities and potential impact of MAS are considerable from the perspective of the newly developed tools and technologies. Despite this potential, there are limitations to the application of MAS because the linkage between marker and QTLs can be lost during the breeding cycle due to recombination. As an alternative, selection can be done directly on desired alleles of known-function gene markers (the candidate genes, CG). The hypothesis is that known-function genes linked to QTLs could correspond to the loci controlling those traits

(Pflieger *et al.*, 2001). This is a straightforward approach that avoids loss of the desired QTL during recombination and will allow breeders to select directly for the desired trait. This technique for selecting CGs linked to important traits is now available for pine species (*Pinus* spp.), and its use is likely to increase in the next few years in different forest tree species (Moran *et al.*, 2002; Brown *et al.*, 2003; Pot *et al.*, 2005; Yu *et al.*, 2006). The CG approach could bypass some of the limitations of the application of molecular markers in a tree breeding program. Nevertheless, despite the advantage of selecting on the CG, introgressing the right allele through a breeding cycle will still be a long process. Genetic engineering as a tool to introduce the desired alleles in the genome of spruce from identified breeding populations is a promising alternative that should shorten the time to produce new improved trees for desired traits. At this stage, however, much remains to be accomplished in the context of CG identification and social acceptance of genetically engineered trees (see below).

2. GENETIC TRANSFORMATION AND TRANSGENICS OF SPRUCE SPECIES

There are several prerequisites for the production of transgenic plants. First, the foreign DNA must be delivered to plant cells and it must integrate in the genome. These transgenic cells must then be selected and multiplied, and finally regenerate into a plant. Therefore, development of efficient gene delivery techniques based on efficient *in vitro* plant regeneration protocols is a prerequisite for production of transgenics in any species.

2.1 Target Tissue for Transformation of Spruce Species

The first successes in genetic transformation of spruces were made after the discovery of somatic embryogenesis (SE), a tissue culture process that results in production of somatic embryos that readily convert to plants (publications on SE: Chalupa, 1985; Hakman *et al.*, 1985; Hakman and Fowke, 1987; Webster *et al.*, 1990; and on transformation: Ellis *et al.*, 1993; Charest *et al.*,

1996; Walter *et al.*, 1999; Wenck *et al.*, 1999; Clapham *et al.*, 2000; Klimaszewska *et al.*, 2001, 2004; Le *et al.*, 2001; Lachance *et al.*, 2007). In spruces, SE has also become an indispensable tool for tree improvement programs because the embryogenic tissue (ET) can be readily stored in liquid nitrogen during lengthy clonal field tests (Cyr, 1999). Genetic engineering in spruces relies on SE as the target material for gene delivery and transgenic regeneration, because it offers many advantages. First, embryogenic tissue can be rapidly proliferated on either semisolid or liquid media, providing a source of actively dividing cells that are the most competent for genetic transformation. Moreover, these cells can be easily handled in suspension culture and on various supports at very low cell densities that facilitate effective selection of transformed cells. Second, occurrence of chimeras is rare because a somatic embryo is usually derived from a single cell (Klimaszewska *et al.*, 2003). Third, it takes a relatively short time to recover a transgenic plant through SE. Finally, another significant advantage of SE is that a transformed embryogenic tissue line can be easily cryopreserved without any detectable effect on regrowth and transgene expression. This feature is critical because embryogenic cultures can progressively lose their somatic embryo maturation ability during the prolonged tissue culture period, or may get contaminated. Furthermore, high transformation efficiency is necessary for cost-effective, large-scale production of transgenic material needed for the high-throughput gene testing or extensive screening and final selection of lines that might eventually be accepted for commercial deployment. For all these reasons, in this review we focus on the protocols developed specifically for transformation of spruce embryogenic cells.

2.1.1 Somatic embryogenesis

Somatic embryogenesis in spruces is routinely initiated from immature or mature zygotic embryos; the formers are usually cultured enclosed in the megagametophytes. On average, the initiation frequency is always higher from immature embryos compared with mature embryos excised from either freshly collected or stored seed. This

response also varies depending on the species, exact developmental stage of an embryo, and the seed family, and can reach 20–80% (Cheliak and Klimaszewska, 1991; Park *et al.*, 1993; Högberg *et al.*, 1998). Explants from spruce plants have also been used to induce SE, with the oldest tree being a 3-year-old Norway spruce (Harvengt *et al.*, 2001). However, only the seed explants provide practical SE initiation efficiencies and ease of handling.

The most frequently used nutrient media for spruce SE include the one described by von Arnold and Eriksson (1981) or by Litvay *et al.* (1985). Media are supplemented with organic nitrogen (L-glutamine and casein hydrolyzate). For SE initiation and proliferation, the media typically contain 2,4-dichlorophenoxyacetic acid at 9.0–10 μM and benzyl adenine at 4.5–5.0 μM , and sucrose at 1% or 2%, and they are solidified with agar (0.8%) or gellan gum (0.4%). The somatic embryos remain at the early developmental stage (Figure 1a) unless subcultured onto a medium with abscisic acid (ABA) at 20–60 μM on which they mature after 5–7 weeks of culture. Spruce species respond to several maturation protocols: one of the more efficient was tested with three species and involved culture of the embryo masses in a layer spread over a filter paper disk (Whatman No. 2) placed on MLV, modified Litvay medium (Litvay *et al.*, 1985), with 60 μM ABA, 6% sucrose, and 0.6% gellan gum (PhytigelTM) (Klimaszewska *et al.*, 2001). After 7 weeks, the embryos (Figure 1b) were harvested and germinated at a high frequency on a medium without plant growth regulators and with 2% sucrose and 0.6% gellan gum. Somatic plants were developed after 3–4 months, and at this stage were moved *ex vitro*.

2.1.2 Cryopreservation

Long-term storage of spruce ET at ultra-low temperatures (–140 to –196 °C) is a routine and integral element of any SE program. Since the first publication on cryopreservation of white spruce ET by Kartha *et al.* (1988), the protocol has been modified for use with other spruce species. One of the current protocols entails incubating 2 g fresh mass embryogenic tissue in 7 ml of liquid culture medium (of the same composition as for

maintaining the growth of ET), supplemented with 0.4 M sorbitol for a period of 18–24 h. All subsequent steps are carried out on ice. Just before freezing, cold DMSO (dimethyl sulfoxide) solution (in a culture medium) is added to the cell suspensions to a final concentration of 5–10% (v/v). The cell suspension is then left on ice for 1 or 2 h, and then dispensed into cryovials, which are placed in alcohol-insulated containers that are pre-cooled for 2 h at –80 °C. The containers with vials are then placed at –80 °C for 1–2 h, during which a slow cooling of the cell suspension (approximately –1 °C min^{–1}) takes place. The vials are subsequently plunged into liquid nitrogen and stored in a cryofreezer. For regeneration, the contents of the vials are rapidly thawed in a water bath at 37 °C for 1–2 min and the cell suspension is poured over a filter paper disk placed on a thick pad of sterile blotting paper. The storage solution is allowed to drain for several minutes and the filter paper with cells is transferred onto semisolid medium. Growth of cultures typically occurs within 1–2 weeks after thawing.

2.1.3 Genetic transformation

The media for transformation and selection processes of transformed cells in spruces are usually the same as those used for ET proliferation, with appropriate modifications or additives (Klimaszewska *et al.*, 2004). Once transgenic clonal lines (transclones) are selected and stabilized, the cryopreservation, maturation, and germination of somatic embryos, as well as conversion to transformed plants (Figure 1c) follow the same protocols as developed for SE of each spruce species. The most frequently used gene delivery methods for stable transformation of spruces are DNA-coated particle bombardment (PB) and co-culture of cells or explants with disarmed strains of *Agrobacterium tumefaciens* (AT, *Agrobacterium*-mediated transformation). PB is a direct transformation method that involves bombarding the plant cells with small metallic particles coated with the gene(s) of interest. After the particles enter a cell, providing the cell remains viable, the foreign DNA randomly integrates into the genome. In the AT method, the gene(s)

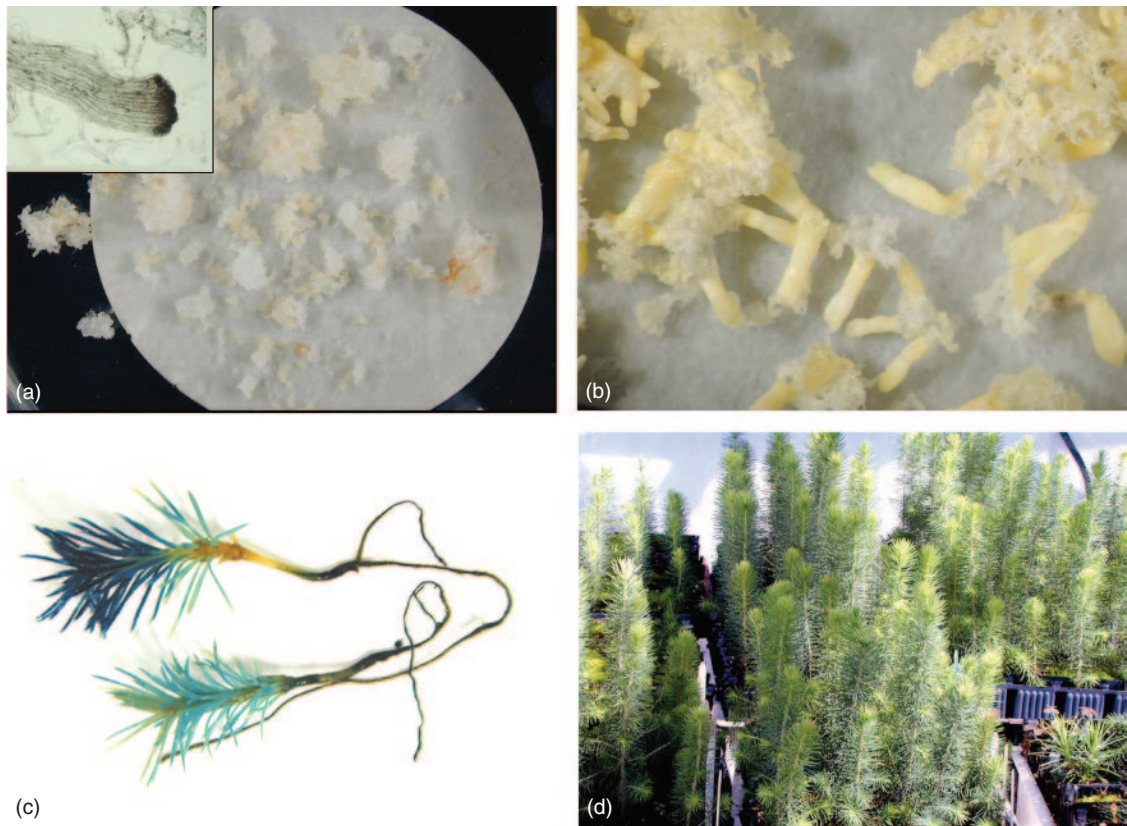


Figure 1 Genetic transformation of *Picea glauca* through co-cultivation with *Agrobacterium tumefaciens*: (a) selection of transformed cell colonies on a medium with kanamycin with an inset showing higher magnification (20 \times), (b) mature transgenic somatic embryos (magnification 8 \times), (c) expression of *uidA* by transgenic somatic seedlings visible following X-gluc histochemical assay (magnification 2.5 \times), (d) transgenic somatic seedlings in the greenhouse

of interest is inserted into the transfer-DNA (T-DNA) region of tumor-inducing plasmid (pTi) from a disarmed *A. tumefaciens* strain. After contact and attachment of bacteria to plant cells, the T-DNA is transferred to the nucleus and then stably integrated into plant genome (Tzfira *et al.*, 2004).

Transformation efficiency of spruces is affected by a number of factors related to the plant material, e.g., species, genotype, explant type, and source, as well as the developmental and physiological state of target tissue. A number of methodological issues are also critical for successful transformation and regeneration of any transformed plant, namely, the gene delivery method (AT or PB), the selection procedure of transformed cells, and the stress recovery following either physical damage caused by

particle bombardment or *Agrobacterium* infection. As well, physiological damage may occur during the long process of transformation and selection.

2.2 Transformation Using Particle Bombardment

White spruce was the first transgenic spruce species produced after PB transformation of mature somatic embryos that regenerated plants through secondary SE (Ellis *et al.*, 1993). Since this key report, protocols have been developed to produce transgenic plants in two other spruce species: black spruce (Charest *et al.*, 1996; Tian *et al.*, 2000) and Norway spruce (Walter *et al.*, 1999; Clapham *et al.*, 2000). As a physical method of gene transfer,

PB optimization mainly relies on modification of bombardment parameters such as microcarrier particle size and speed, distance to target, use of filter paper as a support to anchor the cells, and preculture of cells on preparation media to preserve cell integrity (Altpeter *et al.*, 2005). This direct, practical, and rapid physical method to deliver foreign DNA into the cells is considered to be independent of genotype, especially in transient gene expression experiments. Moreover, a gene construct could be delivered into virtually any organ, tissue, cell, or even cell compartment. Thus, this method appears best suited for large-scale studies of transient gene expression, such as promoter testing.

2.3 Transformation Using *A. tumefaciens*

The first success in AT transformation of a conifer and regeneration of transgenic plants was achieved by Levée *et al.* (1997) in hybrid larch (*Larix* spp.). Since then, AT has become the preferred method for spruce transformation rather than PB (Wenck *et al.*, 1999; Klimaszewska *et al.*, 2001; Le *et al.*, 2001). For example, it is presently being used exclusively for a functional genomics study in white spruce under the Arborea phase I project (<http://www.arborea.ca>). Over 20 different gene constructs were stably integrated and several translines for each construct were obtained. Combined with SE technology, close to 2000 transgenic trees were produced. One of the reasons for using the AT method of transformation is that it usually results in more predictable transgene integration patterns, with a higher frequency of single-copy and single-locus insertion events, and with less transgene fragmentation and rearrangement. The single insertion events tend to result in stable gene expression, whereas integration of multiple copies of a transgene may lead to both transcriptional (TGS) and post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999; Meyer, 2000).

Improvement of AT methodology requires that conditions for interaction of bacteria with plant cells, strains of *A. tumefaciens*, access to plant cells (wounding, vacuum infiltration), and co-cultivation parameters (plant cell and bacteria preparation, density ratios, duration, temperature, bacterial virulence inducers) be

optimized. Furthermore, after transformation is completed, the agrobacteria must be eliminated from the cultures before the subsequent *in vitro* steps of selection and regeneration of transformed plant material. It is also an important objective for field release of transgenic trees (see Section 3). The main concern is that the modified, laboratory strain of *A. tumefaciens* could be released into the soil from the roots of the transgenic host plant and subsequently infect other plants or transfer the genes to other microorganisms by horizontal gene transfer (Droege *et al.*, 1999; Stewart *et al.*, 2000). The persistence of *A. tumefaciens* in genetically engineered trees is of significant interest, because they are likely to remain in the environment for decades. Unfortunately, there are disproportionately fewer publications investigating the survival of residual *A. tumefaciens* in plant tissues after transformation, relative to those on transformation itself. However, a few studies have reported that *A. tumefaciens* persisted in the tissues of other transgenic plant species (herbaceous and trees) for up to 3 months after transformation (Mogilner *et al.*, 1993; Cubero and López, 2005). Recent studies on residual agrobacteria after transformation of ET of several conifer species including white spruce showed it to persist for up to 12 months despite the lack of visible bacterial growth on the culture medium (Charity and Klimaszewska, 2005). However, no agrobacteria were detected in mature somatic embryos or in needles, branches, stems, or roots of transformed plants grown in a greenhouse (Figure 1d) for up to 4 years following transformation. One of the critical issues that became apparent in that study was the limitations of the polymerase chain reaction (PCR) in detecting bacterial DNA in a background of pine needle DNA, which was approximately 10^9 – 10^{10} cells per gram fresh mass of tissue. Thus, rather than using the PCR method as the sole detection technique when determining if *A. tumefaciens* persists in plant tissues, it is recommended that an enrichment culture of bacterial cells released from macerated plant tissues on bacterial growth medium, followed by PCR of bacterial colonies, be used; Cubero and López (2005) also reached at this conclusion. Various strains have been tested in spruce transformation, such as succinamopine strains with C58 chromosomal background (EHA105, AGL1), octopine TiAch5 (LBA4404) or C58

(GV2260) strains, and various nopaline C58 strains such as EHA101, GV3101, C58pMP90 (= GV3101pMP90), and GV3850 (Hellens *et al.*, 2000).

2.4 Selection and Reporter Genes

As in most other plants, the success of transformation in spruce species depends on the efficient delivery and expression of selectable marker genes that subsequently allow selection of the transformed cells. Only negative selection with antibiotic- or herbicide-resistant genes has been reported in spruce. Three selection genes have been employed in stable transformation studies: (1) the commonly used *nptII* (neomycin phosphotransferase II) gene from *Escherichia coli* encoding neomycin phosphotransferase, which confers resistance to the antibiotics kanamycin or geneticin, (2) the *hpt* (hygromycin phosphotransferase) (or *aphIV*, aminoglycoside 3'-phosphotransferase IV) gene from *E. coli* encoding hygromycin phosphotransferase, an enzyme capable of detoxifying hygromycin B, a strong inhibitor of protein synthesis, and (3) the *bar* (bialaphos resistance) gene isolated from *Streptomyces* spp., encoding the phosphinothricin acetyl transferase responsible for resistance to phosphinothricin and bialaphos or the commercial herbicide formulations BastaTM or BusterTM. In each case, it is necessary to determine the optimal concentration of the chemical for effective selection of transformed cells. These concentrations are usually specific for each spruce species and type of target explant in order to allow selection of transformed cells while preserving their regeneration potential. Inefficient application (type, concentration, and timing of selection after transformation) of the selective agent may result in conditions that are toxic to both transformed and nontransformed cells, or that are insufficiently stringent, resulting in high frequency of nontransformed cells escaping the selection process (escapes).

Reporter genes facilitate visualization and quantification of gene expression by means of bioassays. The most frequently used genes are *uidA* (encoding β -glucuronidase) and *gfp* (encoding green fluorescent protein). Nontoxic, visual marker genes such as *gfp* may be useful in developing effective selection strategies for

transformed cells at the early stages without the need for antibiotic or other selective agent application.

2.5 Promoters, Gene Expression, and Silencing

Several constitutive promoters, such as CaMV 35S (cauliflower mosaic virus 35S), *nos* (nopaline synthase), *ubi* (maize *ubiquitin*), artificial *Emu*, and rice *actin*, have been used to control gene expression. In fact, these promoters have been tested in several coniferous species including pines and spruces (Tang and Newton, 2003). Overall, it was possible to achieve good levels of transgene expression, and in one case, long-term expression over 5 years was achieved in *P. glauca* using the *ubi* as well as the CaMV 35S promoters (Lachance *et al.*, 2007). In one specific study by Tian *et al.* (2003), constitutive expression of a tobacco cryptic promoter, *tCUP*, was demonstrated in white spruce. These data, together with other promoter evaluation in spruces, suggest that conifers (gymnosperms), despite their evolutionary history, can recognize 5' regulatory sequences derived from angiosperm.

Nevertheless, inducible promoters were proposed to offer more flexibility, as they allow the regulation of gene expression (reviewed by Gatz, 1997). Functional studies would benefit from the availability of a system to induce gene expression at defined developmental stages or under particular conditions. These promoters are quiescent in the absence of inducers, and therefore, ectopic transgene expression will not interfere with physiological activities and plant regeneration while the promoter is inactive. This approach is particularly important to obtain overexpression of a gene with regulatory function.

2.6 Targets for Genetic Engineering of Spruce

Recent reviews thoroughly described the potential applications of forest tree biotechnology, including genetic engineering (Boerjan, 2005; Nehra *et al.*, 2005; Fladung and Dietrich, 2006). Despite their wide ecological range and economical importance, relatively few studies have dealt with genetic

modification for adaptative or economical traits of spruces. Far more work has been done in coniferous species, such as pine, or hardwoods, such as poplar (*Populus* spp.). Nevertheless, successful approaches for increasing insect and microbial pathogen resistance were achieved. An endochitinase gene from the biocontrol fungus *Trichoderma harzianum* was stably expressed in black spruce and translines showed an increased resistance to the spruce root pathogen *Cylindrocladium floridanum* (Noël *et al.*, 2005). More recently, insect resistance using a *Bacillus thuringiensis* (*Bt*) synthetic gene was achieved in white spruce (Lachance *et al.*, 2007). Several translines engineered with the *Bt Cry1Ab* endotoxin gene showed total resistance in several insect feeding assays with the spruce budworm (*Choristoneura fumiferana*). A unique feature of this work pertained to a 5-year field trial that showed long-term stability of transgene expression. Interestingly, strong *Cry1Ab* gene expressing lines identified early in the selection process (young somatic seedlings) also showed strong *Bt* transgene expression after several growing seasons in the field.

2.7 From Single Gene Transformation to Functional Genomics

Biological processes of tree growth, development, reproduction, and defense are orchestrated by genetically programmed mechanisms, fine tuned to respond and adapt to environmental conditions. Several studies, mainly in model plant systems, have uncovered a gene whose putative biochemical function and expression patterns may be linked to specific processes of interest in plants. Gene functional analyses by overexpression have been used in a limited number of publications dealing with forest tree species, mainly in poplar (Campbell *et al.*, 2003). To overcome the complexity of the peroxidase isoenzyme family, a Norway spruce peroxidaselike gene, *spi 2*, was studied by ectopic expression using the ubiquitin promoter (Elfstrand *et al.*, 2001). Transgenic *spi 2* expressing Norway spruce lines showed up to 40 times higher total peroxidase activity, confirming the function of *spi 2*. Despite some negative physiological effects of *spi 2* overexpression, such as embryogenic development defects, transgenic trees were regenerated. These were significantly shorter and showed more intense

phloroglucinol staining in the xylem, but no measurable differences in lignin content compared with wild-type trees.

The fact that gene function is investigated individually provides only a limited view of the molecular landscape of the cell as well as the complexity of protein action and interactions. An important advantage derived from functional genomics approaches is the depth of analysis that may be accomplished through large-scale investigations of gene and protein functions. In recent years, continuous new technological platforms have appeared and created diverse opportunities for genome-scale gene discovery and expression studies, focusing largely on RNA transcripts, also referred to as the transcriptome.

To date, most gene studies based on loss or gain of function experiments in trees have been investigated in transgenic poplars. Poplar has traditionally been the tree of choice because of its ease with transformation procedures, but significant progress, as described above, has been made in the development of reliable protocols for commercially valuable conifers such as pine and spruce. The opportunity now exists to develop a genome-wide vision of gene function in trees with genomics resources that are being developed. During the last 5 years, two important forest genomics projects, Treenomix (<http://www.treenomix.ca>) and Arborea (<http://www.arborea.ca>), have developed important resources by establishing databases containing a large number of expressed sequence tags for spruces. These projects not only provided a valuable starting point for genomics studies on conifer species, but allow investigation in the field of metabolomics and functional genomics.

3. DEPLOYMENT OF TRANSGENIC TREES IN THE CONTEXT OF MULTIVARIETAL FORESTRY AND BIOSAFETY ISSUES

Owing to the recent achievements in SE of spruce species, its commercial potential has been explored (Adams *et al.*, 1994), and the deployment of trees produced by SE has begun. Although SE is the primary enabling technology for genetic transformation, its most important application is in implementing multivarietal forestry (MVF), which is defined as the deployment of tested tree

varieties in plantation forestry. It is also called clonal forestry, but with advances in conifer SE, MVF is considered to be a more descriptive term when applied to commercial plantation forestry (Park, 2004). In general, a clone refers to a genotype with its genetic copies or ramets, whereas a variety refers to a clone that has been selectively bred for certain attributes as in an agricultural variety. The implementation of MVF offers many advantages, and some important ones are as follows: (1) much greater genetic gain than from conventional tree breeding based on seed orchards, (2) flexibility to rapidly deploy suitable varieties with changing breeding goals and environments, and (3) ability to design and balance genetic gain and diversity in plantations.

3.1 Commercial and Ecological Applications

In order to practice MVF, it is essential that high-value tree varieties be developed. Development of high-value SE varieties is usually closely coordinated with multigeneration tree breeding programs, which generally adapt a form of recurrent selection and manage breeding populations for the next generation. In many well-established programs, the nucleus breeding strategy is used, involving a hierarchical structuring of the breeding population, where the top tier contains the elite (nucleus) parents. This strategy is adapted to obtain fast delivery of genetic gain from the elite crosses (Lstibůrek *et al.*, 2004). Therefore, it is best to start varietal development with the nucleus population using a system of controlled crosses. In typical tree breeding programs, controlled crosses among the selected elite parents will be performed to produce offspring. The resulting seeds are then subjected to SE initiation to develop SE lines. Once embryogenic lines are developed and proliferated, they are stored in liquid nitrogen, while field tests are established using the embryogenic lines retrieved from cryogenic storage. Field testing is the most important part of MVF and is essentially the process for identifying tree varieties for deployment. This provides an opportunity to produce genetically tested identical genotypes consistently over time, which is analogous to the production of agricultural varieties. In eastern Canada, for example, over 3000 embryogenic

varieties of spruces, including white, black, red, and Norway spruce, are tested in the field. The current annual deployment in MVF is at about 750 000 trees on selected sites, but the number is steadily increasing (G. Adams, JD Irving, Ltd, personal communication).

Potential benefits and recent advances in genetic transformation technology have led to intense interest in using the technology in commercial forestry, such as MVF. It is envisaged that genetic transformation is likely to be applied to already improved tree varieties as a value-added component of MVF. Since the introduction of value-added transgenic varieties is mainly to be within the context of MVF, it is important to develop ecologically sound MVF strategies before deploying transgenics trees. Therefore, all aspects of the general requirements for implementing MVF are equally applicable for deploying transgenic trees. The current MVF strategy using SE and cryopreservation is based on careful exploitation of indigenous genetic variability that exists in nature. However, the deployment of transgenic trees requires additional attention because when new foreign genes are introduced, gene function has been substantially altered. Therefore, it is important to critically evaluate and manage potential risks before transgenics are deployed.

3.2 Managing Biodiversity

In MVF, the management of diversity is a concern because of the perception that a narrow genetic base may result in MVF plantations being more vulnerable to diseases and insects than seedling plantations, and ultimately in plantation failure. For known diseases and insects, MVF has an advantage because resistant varieties may be developed in combination with improved economic traits. But, for unknown or introduced diseases and insects, protection is limited despite the high degree of genetic variability existing among forest trees. It is difficult, if not impossible, to design a protection scheme against unknown pests. However, it is generally assumed that the more varieties deployed in a plantation, the lower is the risk. The use of an increased number of varieties in a plantation will reduce genetic gain, so it will be necessary to balance

genetic gain and diversity, leading to the question of what is an appropriate number of varieties in an MVF plantation (Libby, 1982). This is a difficult question; however, using various approaches, scientists generally agree that 10–30 varieties mixed in a plantation should be sufficient for protection and still yield the benefits of MVF (Libby, 1982; Hühn, 1987; Zobel, 1993; Roberds and Bisher, 1997). Once an appropriate number of varieties is decided, the MVF strategy must consider the configuration of the deployed varieties, either as a random mixture or in varietal blocks (Libby, 1982). In general, a random mixture is appropriate when varieties are not well known or future pest situations are uncertain (Lindgren, 1993).

3.3 Regulatory Consideration

The purposes of using genetic transformation technology in forest trees include the incorporation of new genetic traits that are not accessible by the natural mode of breeding and the regulation of gene expression, such as gene silencing and overexpression. Potential benefits of transgenic trees in the context of MVF include the following: (1) increased ecological competence by engineering transgenes conferring resistance to diseases, insect attack, and abiotic factors such as drought and cold stress and herbicides (Tang and Newton, 2003); (2) improved product quality and productivity by modifying gene expression (Nehra *et al.*, 2005); and (3) derivation of new products (Mullin and Bertrand, 1998).

It is likely that the conferring of disease and insect resistance through transgenic technology will enhance MVF; however, additional biosafety issues have been identified for transgenic deployment. The major concern has been the possible spread of transgenes from transformed varieties to natural populations or related organisms. The obvious mode of gene flow is sexual reproduction through windborne pollen, which is difficult to control. Isolation barriers have been suggested, but these are likely to be only partially effective. Genetic engineering of flowering sterility (Strauss *et al.*, 1995) seems to be an attractive option to incorporate employing transformation, but it is extremely difficult to ensure the stability of the sterility, especially in view of forest trees' long

rotations (Mullin and Bertrand, 1998). Another concern about the escaped genes is the change in invasiveness due to the selective advantage conferred by the transgene (Dale, 1992; Raybould and Gray, 1994). Even if the transgene does not escape, it still may have an adverse effect on other organisms because of possible toxicological effects when consumed by other organisms or through nutrient recycling (Mullin and Bertrand, 1998). The introduction of novel traits by genetic transformation may lead to changes in forest management that could have an impact on ecosystem processes. The extensive use of value-added transgenic trees may reduce diversity, even further leading to the deployment of a single or only a few genotypes. Therefore, a scientifically sound management plan for plantation diversity in the context of MVF should be applied.

Even when a great deal is known about a transgenic line, an absolute guarantee of safety is not possible. Risk assessment in relation to the deployment of transgenic trees is needed to evaluate the risk management strategies intended to minimize risks (Mullin and Bertrand, 1998). Such risks trigger the regulation of environmental release in many jurisdictions. Transgenic trees require rigorous testing before they are released into the environment. Assessment at initial stages is usually performed in the laboratory, growth chamber, or greenhouse. Once transgenic plants show promise, the next step is to conduct a field test under confined conditions to restrict unintended consequences. The confined field trial phase is an important step for collecting relevant data on the characteristics of transgenic plants and biosafety evaluations. In many jurisdictions, e.g., Canada and the United States, a permit is required to conduct a confined field test and researchers must abide by its terms and conditions. Typically, a confined field test is reproductively isolated and requires monitoring of trial sites for current and postharvesting periods. After several years of a confined field trial, an unconfined environmental release may be approved. Approval is usually based on a thorough environmental safety assessment completed according to rigorous, internationally accepted procedure using the most current scientific knowledge (Finstad *et al.*, 2007).

In Canada, environmental release of plants with novel traits (PNT, includes transgenic trees) is regulated by the Plant Biosafety Office of the

Canadian Food Inspection Agency. In Europe, the European Community (EC) imposes strict rules on genetically modified (GM) organisms, and GM trees may only be introduced into the environment in conformity with Directive, 2001/18/EC. This directive and supplemental decisions provide detailed guidance on the objective, elements, general principles, and methodology of full environmental risk assessment. Provisions for risk assessment are founded on comprehensive scientific evaluation by each member state within the EC. In the United States, transgenic trees are regulated by the Animal and Plant Health Inspection Service (APHIS). Developers of transgenic trees must obtain prior authorization from APHIS for import, interstate transport, and field testing of these plants. Field testing is a precondition of deregulation, which is necessary for the transgenic trees to be commercialized (Sedjo, 2004).

Transgenic trees are the product of a powerful technology that promises many benefits. It is important to capture those benefits while ensuring public safety and maintaining environmental integrity. The deployment of transgenic trees is also a social issue and, despite scientific endeavors, there are knowledge gaps in developing an effective assessment and management of biosafety. This is an appropriate time to open dialog among scientists, the public, and regulators for careful integration of this powerful technology in forestry.

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Chestnut

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1. INTRODUCTION

1.1 History, Origin, and Distribution of *Castanea*

The *Castanea* genus is a member of the family Fagaceae. The fossil record indicates that Fagaceae was already well established by the Eocene (35–55 million years before present (BP)) and may have arisen in the Paleocene (55–65 million years BP) (Crepet and Nixon, 1989a, b; Kvacek and Walther, 1989). Fagaceae is divided into four subfamilies, seven genera, and approximately 1000 species. The first three subfamilies, Fagoideae, Trigonobalanoiceae, and Quercioideae, each contain a single genus, *Fagus*, *Trigonobalanus*, and *Quercus*, respectively (Jones, 1986). The fourth subfamily, Castaneoideae, is made up of four genera, *Chrysolepis* (2 species), *Castanopsis* (approximately 30 to 100 species), *Lithocarpus* (approximately 300 species), and *Castanea* (approximately 7 species) (Johnson, 1988).

One of the smallest genera in the Fagaceae family, *Castanea* is discontinuously distributed throughout the temperate regions of the northern hemisphere. Fossil evidences from Greenland, Alaska, and western North America indicate that this genus was once more widespread than at present (Jones, 1986; Lang *et al.*, 2007). The

Castanea genus is divided into three sections, *Eucastanon* (chestnuts) with five species: *Castanea mollissima* Blume, *Castanea sativa* P. Mill., *Castanea crenata* Sieb and Zucc., *Castanea dentata* (Marsh.) Borkh., and *Castanea seguinii*; *Balanocastanon* (chinkapin) with one species: *Castanea pumila* (L.) P. Mill.; and *Hypocastanon* (Henry chestnut or willow-leaf chestnut) with one species: *Castanea henryi* (Rutter *et al.*, 1990). The two main species discussed in this review, European chestnut (*C. sativa*) and American chestnut (*C. dentata*), both grow to large sizes and have a maximum lifespan of several centuries.

1.2 *C. sativa* Mill. (Sweet Chestnut, European Chestnut)

European chestnut is a tree species with a wide distribution and an important economic role in Europe. It is present in 25 European countries, covering an area of over 2 million hectares (Conedera *et al.*, 2004). *C. sativa* is considered the only native chestnut species in Europe. The trees are large (20–25 m in height) and spreading with a compact crown, and the nuts are normally larger than those produced by other *Castanea* species.

European chestnut probably originated in the Caucasus Mountains, between the Black and Caspian seas, and it is thought that the

Ponto-Caucasian region is the main genetic source of the species (Villani *et al.*, 1999). Palynological data (Huntley and Birks, 1983) indicate that *C. sativa* was widely distributed during the Tertiary period, being restricted to the southern refugia after successive glaciations. The pollen record also indicates a second, rapid expansion linked to human activity through the expansion of the Roman Empire, starting more than 2000 years ago (Robin, 2002). The Romans not only used chestnuts as a year-round food, but also as an important tool associated with viticulture development, providing fences, vineyard stakes, poles, baskets, etc. (Bounous, 2005).

At present, the European chestnut stands, mainly located in the southern countries, derive from the Turkish Mediterranean gene pool (Villani *et al.*, 1994, 1999). Cultivated for centuries, the European chestnut became an important part of the cultural heritage in many rural areas of the continent. Historically, chestnuts were used as a staple food, providing a major food source for many European mountain populations (Bellini, 2005). Chestnut wood has also been used in house framing, furniture, tannin production, and as a source of renewable energy. Since the end of the 19th century, many chestnut stands have been degraded due to diseases, the depopulation of rural areas as consequence of industrial development, and the abandonment of cultural practices. However, over the last 20 years, and in line with the sustainable agriculture policies followed in many countries, the chestnut has been managed for both timber and nut production, to improve its conservation. A return to the massive consumption of the nut may be inconceivable, but European chestnut stands may play an important role in rural tourism, recreational areas, protection from erosion, landscape, etc., in addition to its original productive role.

1.2.1 Economic importance

1.2.1.1 Nut characteristics, production, and uses

The nut quality of European chestnuts is highly variable, depending on the individual varieties, which are the result of the natural selection that has been carried out for centuries through cultivation.

On average, the nuts have a content of 40–44% water, 49% carbohydrates, 5% proteins, and 1% fat, providing an excellent nutritional product. In each country, hundreds of varieties have been identified and most are used for local consumption; in France and Italy, the most important industrial nuts (some of which are European \times Asian hybrids) are derived from cultivars called “marrons”. Currently, one objective is the selection of the best cultivars from the germplasm available, which would be helpful in countries such as the United States, Chile, Australia, and New Zealand, where efforts are being aimed at selecting the superior types from European varieties that are best adapted to local conditions. The majority of European nut production comes from old groves, which, after important interventions, are being transformed into new plantations through rational grafting techniques (Bellini, 2005). In some cases, appropriate rootstocks (tolerance to diseases, good compatibility, adaptation to different pedoclimatic conditions) have been selected for young plantations (Bounous, 2002).

According to the data recorded in 2000 (Bounous, 2002), world nut production was around 473 000 metric tons (t) per year, of which 201 200 t (42%) were collected in Europe. Italy was the leading country, producing 78 400 t (88% of nuts and 12% of marron varieties) followed by Turkey (70 000 t), Spain and Portugal (20 000 t each), and France (12 600 t). Part of this production is for export (mainly to the United States), part is consumed unprocessed in traditional European uses, such as roasting or boiling, and only 20% of the production is used in the food industry. Produce is consumed seasonally because of the limited shelf life due to the high metabolic activity of the nuts, which, under storage, undergo water loss and fungal infections, affecting their quality (Sacchetti *et al.*, 2005). In the food industry, the nuts reach the market all year round as different commodities such as dried chestnuts, flour, marron glacés, creams, puré, pastes, peeled and frozen products, sweets, desserts, and even beer and liquors (Bounous, 2005).

1.2.1.2 Timber

European chestnut is a fast-growing tree species that displays a technologically and aesthetically

appreciated timber with great natural durability that can be used for high-value products. Most of the timber is obtained through traditional management systems (simple coppice) that do not always respond adequately to new market requirements that demand larger sized quality stems. Currently there is not as much demand for smaller logs. In some countries under the support of the European Union, new plantations producing medium- to large-sized logs can attain interesting profitability levels, around 125 Euros/m³ of wood, referring to trees with a diameter at breast height greater than 30 cm (Nunes *et al.*, 2005). Proper management of coppice stands is under study, taking into account factors such as rotation period, thinning, and final number of shoots per hectare (Patricio *et al.*, 2005).

1.2.1.3 Other products

The tannin content of the European chestnut varies from 1.8 to 12% (dry weight). Tannin extraction and purification was an important industrial activity between the 1850s and the 1950s, but even today countries such as Italy and France still maintain at least one company dedicated to this purpose (Robin, 2002; Zanuttini and Cielo, 2002). Chestnut culture provides additional associated products, among which honey, mushrooms, carbon, and cosmetics are the most important.

1.3 *C. dentata* (Marsh.) Borkh (American Chestnut)

The American chestnut is the largest chestnut species native to North America. Its original range covered most of the eastern United States and part of southern Canada (Little, 1977). Within its native range, and especially in the Appalachian Mountains, the American chestnut was often the dominant overstory species, often making up 25% to 50% of the stand (Jaynes, 1974). The preblight population size is at best a plausible guess, but numbers as high as 3.5 billion mature trees have been published (Anagnostakis, 1982; Roane *et al.*, 1986). Like the European chestnut, the American chestnut was used for timber, tannin, and nut production. In some regions, the nuts were so plentiful that domestic livestock was released into

the chestnut stands in the fall to forage (Rutter *et al.*, 1990).

The dominating force affecting the American chestnut throughout the 20th and the beginning of the 21st century was and is the chestnut blight. The fungus, *Cryphonectria parasitica* (Murr.) Barr., brought near-total destruction to the American chestnut as both a nut and timber crop (Hepting, 1974). Today, essentially no American chestnuts are harvested commercially and only a few cubic meters of lumber are sold annually. Although gone from the overstory, *C. dentata* is not extinct. The species still forms a significant component of the understory on many sites throughout its original range but it is extremely rare to see a large mature tree. In New York State, which is toward the northern limit of the original range, there are an estimated 4.2 million American chestnut trees 2.5–12.5 cm in diameter. But too few trees reach the 38 cm class to be recorded in the USDA (United States Department of Agriculture) Forest Service inventory (Alerich and Drake, 1995). The blight has reduced this once-dominant overstory species to an early succession stage shrub (Rutter *et al.*, 1990).

1.4 Traditional Breeding: Objectives, Tools and Strategies, and Achievements

European and American chestnuts have been the focus of attention by professional and amateur plant breeders, nurserymen, and pathologists for more than a century. They have been selected for various traits, moved from numerous points of origin, hybridized, and redistributed across the landscape. This comes after centuries of domestication by native people of both continents. The following is a brief exploration of the subject.

1.4.1 European chestnut breeding

The root rot or ink disease (caused by *Phytophthora* spp.) and chestnut blight (caused by *C. parasitica*) are the two most important diseases that affect European chestnut. Ink disease was first observed in Spain during the middle of the 19th century (Fernández de Ana, 2002), and the first breeding program to develop tolerant/resistant Euro-Japanese hybrids was initiated in Spain as

early as 1921 (Vieitez *et al.*, 1996). In France, a similar program was later developed, and a great number of first-generation hybrids have been selected—the rootstock for disease resistance and the cultivar for fruit quality. In the last years of the 20th century, an upsurge of ink disease occurred in various areas of Europe (Turchetti and Parrini, 1993; Abreu, 1996; Anselmi *et al.*, 1996), probably due to the winters being drier and warmer than usual, putting the trees under water stress during growth resumption the following spring (Turchetti and Maresi, 2005). Preliminary genetic studies suggest that at least two genes (which are incompletely dominant) seem to be responsible for resistance (Saleses *et al.*, 1993; Anagnostakis, 2001).

In Europe, chestnut blight was first detected close to Genoa (Italy) in 1938 and today it is still spreading in most European countries, except for Great Britain and some Northern countries (Vannini *et al.*, 2002). The mortality caused in both orchards and coppices is not as severe as in American chestnut stands, probably due to the higher natural tolerance of the European species, proper stand management, biological control of the disease, etc. The pioneering findings of Grente (1965, 1975, 1981) in Italy on the hypovirulence phenomenon allowed for the application of a biological control program against the disease (Heiniger and Rigling, 1994; Robin *et al.*, 2000; Anagnostakis, 2001). Hypovirulence is a virus-mediated attenuation of the pathogen virulence caused by *Cryphonectria hypovirus*, which accounts for the low severity of the disease. Although in some countries there is no proper disease management, there is a natural regulation of chestnut blight by spontaneous hypovirulence (Guérin and Robin, 2003). Consequently, the trees have coexisted with the disease for decades without there having been disastrous effects for the groves, although in countries such as Spain and Portugal, there has been continuous damage from the disease (Turchetti and Maresi, 2005). At present, conventional breeding programs aimed at identifying resistant individuals to ink and blight diseases are only underway in France (Ramos Guedes-Lafargue *et al.*, 2005) and Spain (Rodríguez *et al.*, 2005).

Large backcross breeding programs have not been, and probably will not be, carried out in Europe to obtain disease-resistant but nearly pure

European chestnut trees. By means of conventional breeding programs, a number of first-generation hybrids have been planted in different European countries. This in turn may have led to the loss of specific characteristics of *C. sativa*.

The chestnut population in Europe is highly complex due to its different expansion areas, selection of varieties, depletion caused by diseases, cultural abandonment, use of pure Asian species, and the spread of first-generation Euro–Asian hybrids resistant or tolerant to diseases. Many researchers in Europe are currently focusing their work on verifying the genetic variability of *C. sativa* in both natural populations and cultivated varieties, which will be of great relevance for the study and the conservation of biodiversity and adaptive potential. For these purposes, molecular markers are being used (Villani *et al.*, 1999; Casasoli *et al.*, 2001; Marioni *et al.*, 2003; Aravanopoulos *et al.*, 2005), which will help in the understanding of both short- and long-term evolution, in the studies of migratory routes, and in adaptive traits such as phenology, juvenile growth, and carbon isotope discrimination. The identification of quantitative trait loci will be an important tool for developing early selection methods.

1.4.2 American chestnut breeding

American chestnut breeding began in the 1800s. Originally, breeding objectives were to increase nut size in American chestnut by hybridizing it with European or Asian species, all of which produce larger nuts (Merkel, 1905; Van-Fleet, 1914; Jaynes, 1974; Anagnostakis, 1992). With the discovery of the chestnut blight in 1904 (Merkel, 1905) and the gradual realization of what a devastating problem it was becoming (Collins, 1912; Stoddard and Moss, 1913; Gravatt, 1924; Gravatt and Marshall, 1926; Zimmerman, 1926; Gravatt and Gill, 1930), focus shifted almost entirely to finding sources of blight resistance. Many strategies were considered. Blight-infested stands were searched for canker-free trees in hopes of finding naturally resistant American chestnut trees. Other chestnut species were imported and evaluated in field trials in hopes of finding a replacement species (Illick, 1921). Thousands of crosses were made between *C. dentata* and other chestnut species, with particular emphasis on *C. mollissima* and *C. crenata* in

hopes of transferring resistance into the American chestnut population (Graves, 1938; Graves, 1950; Berry, 1960; Diller *et al.*, 1964; Jaynes, 1964). By the 1960s, the various breeding programs had been curtailed or abandoned. Although differences in susceptibility were identified, no completely resistant pure *C. dentata* individuals had ever been located (Diller and Clapper, 1965; Jaynes, 1968). Hybrids between Chinese and American chestnut were intermediate in resistance, had lost considerable frost hardiness, and had acquired the spreading form typical of the Chinese chestnut parents used in the crosses (Burnham, 1988).

In the early 1980s, Charles R. Burnham, an emeritus corn cytogeneticist, and Philip A. Rutter, a previous Ph.D. student of evolutionary ecology, examined the literature on chestnut blight and chestnut breeding. After more than a year of collaboration, they decided that the American chestnut was a near-perfect candidate for the backcross breeding method (Burnham, 1981, 1990; Rutter and Burnham, 1982). It flowered early so that generations could be turned over relatively quickly for a tree species and the limited backcrossing that had been done in earlier breeding programs indicated that only a few major genes controlled resistance (Burnham, 1988). Burnham's reputation as a scholar was such that he was able to secure the serious attention of other plant scientists, and with their backing they formed a nonprofit organization, The American Chestnut Foundation (TACF), to carry out their backcross breeding program (Rutter and Burnham, 1982). Rutter began making controlled pollinations of chestnut in 1982. Over the next few years TACF acquired a property near Meadowview, Virginia, hired a staff with expertise in both the pathology of *C. parasitica* and chestnut breeding, acquired plant materials from the earlier breeding programs (Anagnostakis, 1992), and began backcrossing on a large scale.

Classical backcross breeding uses a single variety as a recurrent parent, usually a well-known variety with strong market acceptance and another, often a noncommercial variety or a related weedy species as the nonrecurrent parent. The two varieties are crossed and then rounds of backcrossing to the commercial variety and selection among the progeny for the trait of interest are carried out. The theoretical end result after six or more rounds of backcrossing and selecting followed by several generations of selfing, is that

99% of the genes are derived from the recurrent variety and the gene controlling the trait of interest has been transferred (Allard, 1960).

The modified backcross breeding strategy developed by TACF used Chinese chestnut as the nonrecurrent parent, but instead of using a single recurrent parent, they have been backcrossing onto a different set of American chestnut parents for each generation. The last backcross will be carried out by each of the state chapters of the Foundation onto locally selected parent trees. Rather than selfing to achieve homozygosity of the blight resistance genes, the backcross populations will be intercrossed. The end result is intended to be a blight-resistant but highly genetically diverse set of regionally adapted chestnut populations deriving approximately 93% of its genes from *C. dentata* (Hebard, 1992). Diskin *et al.* (2006) reported that a sample of the third backcross generation strongly resembled the American chestnut seedlings in a composite score of leaf and twig characters, indicating that the American chestnut phenotype will be recovered.

C. dentata flowers early for a tree species; therefore, only 3 to 5 years are needed per generation. As of 2007, TACF had carried out three generations of backcrossing, followed by one of intercrossing. The resulting progeny, designated BC₃F₂ are being evaluated for blight resistance (Hebard, 2006).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

The modified backcross breeding program being carried out by TACF is a skillful adaptation of a thoroughly proven plant breeding technique. Using molecular markers and linkage mapping Kubisiak *et al.* (1997) found that two or possibly three major loci control blight resistance, well within the capabilities of the backcrossing method. There are, however, some fundamental limitations to backcross breeding or other traditional tree breeding approaches.

1.5.1 Precision of gene transfer

Both backcross breeding and genetic engineering are methods of transferring a small number of

genes from one species or variety to another. They differ in the degree of precision of the transfer process. Backcrossing begins with the hybridization of the two parents. This transfers one copy of each gene from both parents into the hybrid progeny. Elimination of genes from the nonrecurrent parent depends on the processes of dilution and selection. After three backcross generations, independent assortment predicts that a population will contain, on average, approximately 7% of the genome of the nonrecurrent parent. Assuming that the *Castanea* genome contains 25 000 genes, roughly the same number as in *Arabidopsis thaliana* (The *Arabidopsis* Genome Initiative, 2000), 7% would be approximately 1700 genes. *C. dentata* and *C. mollissima* diverged from a common ancestor roughly 42 million years ago, so they share many identical alleles. According to Lang *et al.* (2006), the sequence divergence among *Castanea* species is roughly 1% (range among species was 0–2.4%). The backcrossing method will, therefore, leave behind the equivalent of 17 Chinese chestnut genes at random locations and with unknown functions. In practice, genes are arranged in linkage groups that segregate together until separated by crossover events. Often these crossover events occur at some distance from the gene(s) of interest. When this happens, selection for the trait of interest transfers several to many linked genes. This phenomenon is called linkage drag (Young and Tanksley, 1989). Backcross breeding, therefore, introduces approximately 17 random genes and an unknown number of linked genes of unknown function. These traces of the nonrecurrent *C. mollissima* parent genome are probably harmless; however, their existence should be recognized as a fundamental characteristic of backcrossing.

Agrobacterium-mediated transformation decreases in efficiency as the size of the transfer DNA (T-DNA) region increases; therefore, molecular biologists package the genes they want transferred as tightly as possible. The construct used to transform *C. dentata* contained approximately 6 kb of DNA and three well-characterized genes (described elsewhere). One of the ironies in the debate over genetic engineering is that critics often raise the argument about “unknown genetic effects”, while it is actually conventional breeding that introduces a greater degree of genetic uncertainty.

1.5.2 Relative speed with which new genes can be transferred

Development of transformation systems for both *C. dentata* and *C. sativa* has taken approximately two decades (Maynard *et al.*, 1998). This is roughly the same amount of time it has taken TACF to reach the BC₃F₂ generation. However, now that transformation systems are in place, the time required to transfer a new set of genes into chestnut somatic cell lines and regenerate acclimatized somatic seedlings ready for blight screening could be as little as 2 years. With backcrossing, there are two fundamental limitations: how quickly trees can be induced to flower and how quickly the resulting progeny can be scored for blight resistance each generation. Both of these steps have been reduced, but they are unlikely to be reduced much further. Therefore, if a new blight resistance allele were discovered in another chestnut species, it would require an additional 15–20 years to make the new cross and again reach the BC₃F₂ generation.

2. DEVELOPMENT OF TRANSGENIC CHESTNUTS

2.1 European Chestnut Transformation and Regeneration

2.1.1 Genetic transformation through organogenesis

The first attempts to transform European chestnut made use of both hypocotyl segments from *in vitro* germinated seedlings and stem segments of *in vitro* grown shoots, which were co-cultured with *Agrobacterium tumefaciens* (Seabra and Pais, 1998, 1999). In the two experiments, the transformation efficiency ranged from 1% to 2%, whereas the percentage of escapes (shoots developed in media with high concentration of kanamycin but with no *nptII* (neomycin phosphotransferase II) integration) reached 97%. Integration of the genes was transient as after 1 year of *in vitro* culture maintenance, the transgenic character of the cultured shoots was lost (R. Seabra, personal communication).

Plant regeneration systems based on adventitious bud induction have not proved reliable in

European chestnut (San-José *et al.*, 1984; Seabra and Pais, 1999). However, the use of meristematic tissue from apical or axillary buds as a target tissue for genetic transformation is of increasing interest for species identified as recalcitrant for somatic embryogenesis and/or adventitious shoot regeneration (Paz *et al.*, 2004; Petri and Burgos, 2005). The pre-existing meristems should be stimulated to proliferate by complete or incomplete tissue disorganization or dedifferentiation followed by regeneration of multiple shoots. We have investigated the shoot production response of “preconditioned” cotyledonary node explants cultured for 4 weeks on media supplemented with thidiazuron (TDZ). These explants showed an altered meristematic axillary region, with cell proliferation giving rise to expanded meristems, which, upon TDZ shoot-induction treatment, efficiently produced multiple shoot buds with no intervening callus phase (San-José *et al.*, 2001). The ability of this “target meristematic tissue” to form multiple shoots suggests that chestnut cotyledonary nodes are still promising explants for the production of transgenic plants.

Genetic transformation experiments were carried out on European chestnut by co-culturing cotyledonary node explants with *A. tumefaciens* harboring reporter genes (Corredoira *et al.*, 2005). The influence of preconditioned medium, the strain/plasmid combination, and the co-culture period were assayed. Following 12 weeks of successive subcultures in TDZ- and benzyladenine-containing media (4 and 8 weeks, respectively), 2.3% of the explants developed kanamycin-resistant shoots. Although this transformation system needs to be optimized and transgenic chestnut plants have yet to be obtained, the use of the “stimulated” organogenic pathway would be an interesting alternative to the embryogenic regeneration system.

2.1.2 Genetic transformation through somatic embryogenesis

The use of somatic embryos and embryogenic tissue as the target material for genetic transformation has resulted in important breakthroughs in the genetic engineering of trees, as somatic embryos contain a large proportion of dividing cells and have the potential to regenerate complete plants.

In European chestnut, somatic embryogenic cultures have been induced from immature zygotic embryos (Vieitez *et al.*, 1990; Vieitez, 1995; Sauer and Wilhelm, 2005; Corredoira *et al.*, 2006) and from leaf sections of *in vitro* cultured shoots (Corredoira *et al.*, 2003). Embryo proliferation by secondary embryogenesis and moderate rates of germination and plant recovery were also reported (Corredoira *et al.*, 2006). Making use of these embryogenic cultures, an efficient genetic transformation protocol for *C. sativa* has been described for the first time (Corredoira *et al.*, 2004) by means of the co-culture of somatic embryos with different strains of *A. tumefaciens* carrying marker genes. Small clumps (4–7 mg) of two to three somatic embryos in the globular or heart stages that were isolated from embryogenic cultures and maintained by secondary embryogenesis were used. The *Agrobacterium* strains C58C1pMP90 and EHA105 were used for transformation experiments. Strain C58C1 carries a pBI121 plasmid containing an *nptII* marker gene driven by the *nos* (nopaline synthase) promoter for kanamycin selection and the β -glucuronidase (*gus*) reporter *uidA* (β -glucuronidase) gene, driven by the cauliflower mosaic virus (CaMV) 35S promoter. Strain EHA105, carrying either a pUbiGUSINT or a p35SGUSINT plasmid, was also used; these binary vectors contain the *nptII* gene driven by the *nos* promoter and the *uidA* reporter gene (*gus*) driven by either the maize (*Zea mays* spp.) ubiquitin (*Ubi-1*) or the CaMV 35S promoter. Somatic embryo clumps were immersed in the bacterial suspension, blot-dried on sterile filter paper, and transferred to embryo-proliferation medium. Following different periods of co-culture (3 or 4 days), the embryos were washed with sterilized distilled water containing 500 mg l⁻¹ cefotaxime, and were transferred to fresh proliferation medium supplemented with 300 mg l⁻¹ carbenicillin, 200 mg l⁻¹ cefotaxime, and 150 mg l⁻¹ kanamycin (selection medium). After 12 weeks of culture, with subcultures to fresh selection medium at 15-day intervals, surviving explants that developed somatic embryos were evaluated on the basis of GUS activity to determine the transformation efficiency, defined as the percentage of initial explants that developed GUS-positive embryogenic cultures (Figure 1). Cotyledonary-stage regenerated embryos were isolated from GUS-positive lines and subcultured

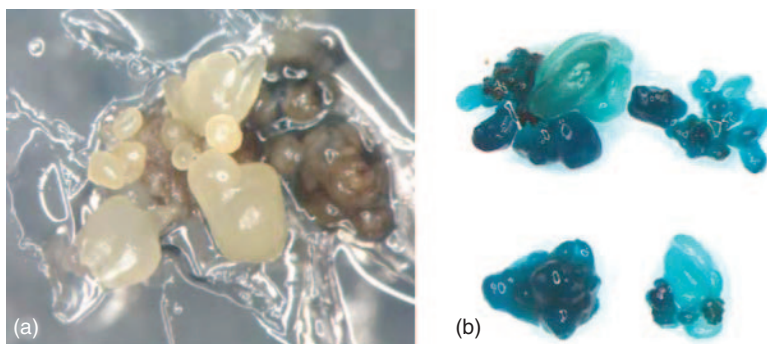


Figure 1 Genetic transformation of European chestnut: (a) kanamycin-resistant somatic embryos after 12 weeks on selection medium; (b) histochemical GUS analysis of somatic embryos transformed with *Agrobacterium tumefaciens* strain/plasmid combination EHA105/p35SGUSINT

on selection medium to proliferate and to establish embryogenic transgenic lines. In order to confirm the presence of the *nptII* and the *uidA* genes in GUS-positive embryogenic lines, polymerase chain reaction (PCR) and Southern blot techniques were used (Corredoira *et al.*, 2004).

2.1.3 Regeneration of whole plants

In general, it has been observed in European chestnut that the conversion rate of transformed embryos is lower than that obtained in the corresponding untransformed line. Transformed embryos were germinated according to Corredoira *et al.* (2003) with the germination medium supple-

mented with 75 mg l^{-1} kanamycin. Although low plantlet conversion rates (6.3–11%) were recorded, shoots from germinating embryos with shoot development only were successfully multiplied by axillary shoot proliferation, which allows the production of unlimited number of transgenic shoots to be rooted. Rooting frequencies higher than 50% were obtained for shoots derived from different transgenic embryogenic lines. This constitutes a valuable alternative for plant regeneration from transformed germinating embryos in which plantlet conversion is not achieved. Transgenic chestnut plants were acclimatized in phytotron and grown in the greenhouse. The presence of transferred genes in leaves from these plants was also verified by the GUS assay and PCR analysis (Figure 2).

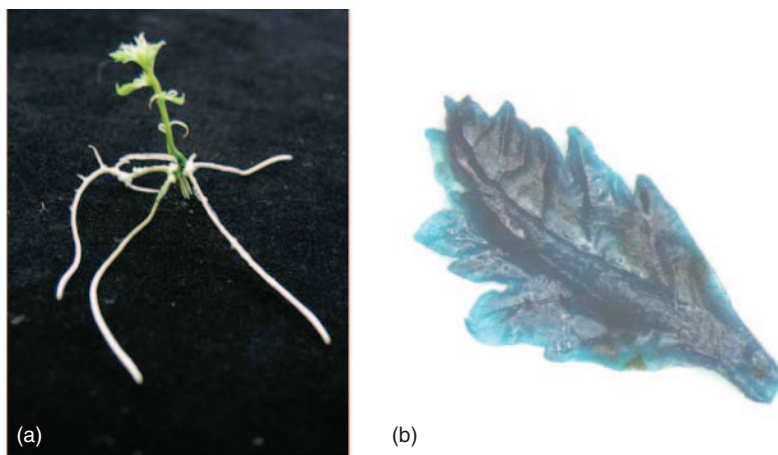


Figure 2 (a) Transgenic plantlet originated by the rooting of a shoot developed from a transformed embryo; (b) histochemical GUS analysis of a leaf excised from a transformed plant

2.1.4 Optimization experiments

In order to improve the protocol for genetic transformation of European chestnut a number of parameters were evaluated.

In the first experiment, the effect of bacterial strain/plasmid combination (EHA105/pUBIGUSINT, EHA105/p35SGUSINT, and C58C1/pBI121) and the length of the co-cultivation period (3 or 4 days) were investigated. The transformation efficiency was clearly related to the strain/plasmid combination used. The highest transformation frequency (25%) was achieved when the explants were co-cultured for 4 days with the strain/plasmid combination of EHA105/pUBIGUSINT. Four days of co-culture was a more effective period than 3 days although significant differences were only observed with the strain EHA105/pUBIGUSINT. The selection efficiency (defined as the percentage of kanamycin-resistant explants that developed GUS-positive embryogenic cultures) was relatively high (71.4%). This indicates that the kanamycin concentration (150 mg l^{-1}) in the selection medium and the subculture regime during 12 weeks appears to be an efficient selection system. Amplification of specific primers to the *nptII* and *gus* genes was detected in all of the GUS-positive transgenic lines analyzed (Corredoira *et al.*, 2004).

To investigate the effect of paramomycin as another potential selection agent, various concentrations ($25\text{--}200 \text{ mg l}^{-1}$) of this antibiotic were assayed and 150 mg l^{-1} of paramomycin was found to inhibit the growth of somatic embryos. Subsequently, somatic embryos of two embryogenic lines, one with high transformation capacity and other with low transformation capacity, were transformed with the strain EHA105/pUBIGUSINT and were cultured on selection medium with 150 mg l^{-1} of either kanamycin or paramomycin. The transformation efficiency was higher in both lines: 30% and 8% for kanamycin versus 13.3% and 1.7% for paramomycin. These results suggest that kanamycin is more efficient than paramomycin for selection of transgenic somatic embryos in European chestnut.

Acetosyringone, when added to various stages during the transformation process, has been shown to enhance transformation efficiency in some species (Wenck *et al.*, 1999; Lopez *et al.*, 2004).

However, when acetosyringone was applied to the bacterial suspension culture overnight and during the co-culture period, it was significantly detrimental in the two concentrations (100 and $200 \mu\text{M}$) evaluated (Corredoira *et al.*, 2004). The temperature during the co-culture period and the effect of wounding of the somatic embryos have also been analyzed. The temperature regime did not significantly affect the transformation efficiency, which ranged from 23.3% (at 28°C) to 28% (at 25°C). Neither was there a significant increase in the transformation capacity when somatic embryos were wounded by pricking them with a hypodermic needle, although higher values were scored using unwounded material.

To determine the effect of bacterial density on the transformation frequency, chestnut somatic embryos were inoculated with *Agrobacterium* suspensions of different optical density (OD) values (0.3, 0.6, and 0.9 at 600 nm). Transformation efficiency was highest when somatic embryos were infected with the bacterial culture in the exponential phase of growth ($\text{OD}_{600} = 0.6$) (Corredoira *et al.*, 2005).

Different developmental stages of chestnut somatic embryos, including isolated globular embryos, small embryo clumps (2–3 embryos at globular or heart stages), and isolated cotyledonary embryos, were used to assess the effect of the explant type used as target material on transformation. The transformation efficiency obtained with isolated globular embryos and embryo clumps (30.0% in both explant types) was significantly higher than that obtained with cotyledonary embryos (6.7%). The low transformation frequency of this type of explant might be related to its lower proliferation capacity through secondary embryogenesis, in comparison to the other explants. In addition, the genotype of the embryogenic line also had a remarkable influence on the transformation capacity of European chestnut. Of the six embryogenic lines evaluated, lines C12-H1 and C13-F1 produced higher transformation frequencies (21% and 33.3%, respectively) than lines CI-1, CI-3, CI-6, and C785L-1, with values of 3.3, 5.0, 1.7, and 10.0%, respectively.

To facilitate the management of the transformed embryogenic lines, a protocol for the long-term storage of this germplasm should be defined. Three European chestnut transgenic cell lines

were successfully cryopreserved using a single vitrification procedure, with embryo recovery frequencies ranging from 52% to 65%. The stable integration of the *uidA* gene into the transgenic plants that were regenerated subsequent to cryopreservation was confirmed (Corredoira *et al.*, 2007).

In conclusion, a reliable protocol for genetic transformation of European chestnut with marker genes has been defined. It should also be noted that the expression of the inserted transgenes has remained stable for 3 years in both somatic cell lines and regenerated plants.

Attempts to transform European chestnut with genes of interest are in progress. In this respect, García-Casado *et al.* (2000) have purified a thaumatinlike protein (CsTL1) from mature cotyledons of European chestnut; they have also demonstrated that the isolating protein has antifungal activity *in vitro*. The overexpression of this gene in chestnut would increase the tolerance or resistance to the diseases. The 1 kb CsTL1 complementary DNA (cDNA) (provided by Dr. Allona, ETSI Montes, UP Madrid, Spain) was used to perform a PCR reaction with specific CsTL1 primers (5'-GAGCTCGGGTAACC-3' and 5'-GTGGATCCCCCGGG-3'). Each primer carried a restriction endonuclease site (*Bam*HI and *Sac*I, respectively) to allow directional cloning of the PCR product. In order to obtain the gene sequence of the CsTL1, the PCR product was digested with *Bam*HI and *Sac*I and the resulting fragment was purified on an agarose gel. This fragment was subcloned into the *Bam*HI and *Sac*I cloning sites of the binary vector pBI121

(Clontech, Palo Alto, California, USA), replacing the preexisting *uidA* gene. The resulting vector was designated p35SCsTL1 and transferred into the *A. tumefaciens* disarmed strain C58C1 using the freeze and thaw method (Holsters *et al.*, 1978). The recombinant bacteria were selected on Luria broth (LB) medium containing 50 mg l⁻¹ kanamycin, 25 mg l⁻¹ gentamicin, and 20 mg l⁻¹ rifampicin.

2.2 American Chestnut Transformation and Regeneration

2.2.1 *Agrobacterium* strain and transgenes

The American chestnut somatic embryo cell lines described by Polin *et al.* (2006) were transformed using *A. tumefaciens* strain EHA105 containing the plasmid construct pΔVspB-OxO, which included a germinlike *oxalate oxidase* gene (*OxO*) (Dratewka-Kos *et al.*, 1989) to enhance blight resistance, a phosphinothricin acetyltransferase gene (*bar*) as a selectable marker, and a green fluorescent protein gene (*mgfp5-ER*) (Haseloff and Siemering, 1998) as a visual marker (Figure 3). The putative blight-resistance-enhancing gene, *OxO*, is from wheat (*Triticum aestivum*). It is driven by the promoter region of a soybean (*Glycine max* (L.) Merr.) vegetative storage protein (VspB4) (Mason *et al.*, 1993) and has an *Act2* (rice actin-2) terminator derived from the *actin-2* gene of *A. thaliana*. The VspB promoter was found to have a 726-bp deletion that reduced *OxO* expression and thus the gene construct originally named pVspB-OxO was renamed pΔVspB-OxO. (For a

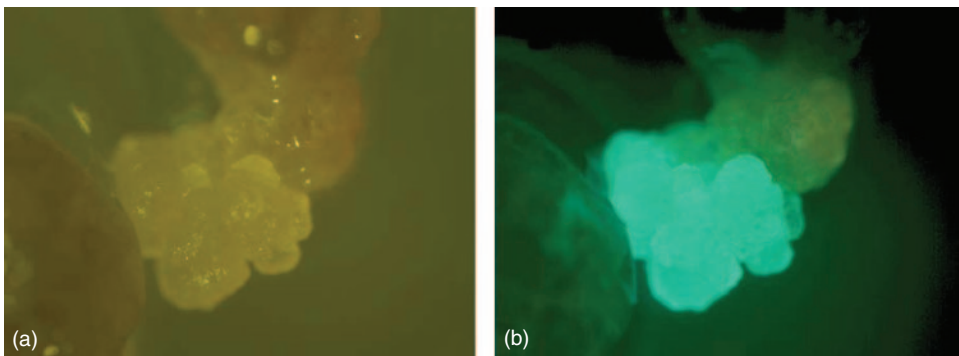


Figure 3 Genetic transformation of American chestnut: (a) PPT-resistant somatic embryos viewed under white light; (b) same somatic embryo cluster viewed under UV light showing embryos expressing GFP

full description of the *OxO* gene and the VspB promoter, see Section 3.5.2) The selectable marker gene, *bar*, is from *Streptomyces hygroscopicus* and has a Ubi-3 promoter and terminator from the ubiquitin-ribosomal protein gene (*ubi3*) in potato (*Solanum tuberosum* L.) (Garbino and Belknap, 1994). The *bar* gene confers tolerance to herbicides containing phosphinothricin (PPT), such as Finale[®], and was not only included in the vector as a selectable marker but is also intended to be used as a gene of interest in nursery management of the transgenic seedlings in subsequent breeding program. The screenable marker, *mgfp5-ER*, is from *Aequorea victoria* and has a CaMV 35S promoter from the cauliflower mosaic virus and a nos 3' terminator from the T-DNA region of *A. tumefaciens*. The *mgfp5-ER* gene has been extensively modified to improve expression in plants. It has had a cryptic intron removed, is targeted to the endoplasmic reticulum, has improved thermotolerance, and has modified spectral properties so that it can be excited at either 400 nm or 470 nm (Siemering *et al.*, 1996; Mankin and Thompson, 2001).

Andrade *et al.* (2005, 2006) used the *A. tumefaciens* strain AGL1, harboring the plasmid pCambia 2301 (carrying the *nptII* selectable marker and *uidA* reporter gene) to transform American chestnut embryogenic cultures, from which transgenic somatic seedlings were later regenerated.

2.2.2 Genetic transformation through organogenesis

Research to develop a transformation system for American chestnut followed a remarkably similar path to that described for European chestnut (Sections 2.1.1 through 2.1.4). Initial research focused on transforming leaf discs. Although a few transgenic calli were produced, no transgenic shoots were ever recovered (Maynard, 1991a). Attempts to transform and regenerate shoots from the meristematic regions of shoot tips were similarly unsuccessful (Maynard, 1991b). Small pilot studies with cotyledons and embryos excised from mature nuts were also carried out with little success. Occasionally, a callus cell line expressing the marker genes was produced; however, no

cell lines were ever identified that were both organogenic and transgenic (Maynard, 1991a).

2.2.3 Genetic transformation through somatic embryogenesis

The first reports of transgenic American chestnut tissues (as opposed to undifferentiated callus) were by Carraway *et al.* (1994) who used micro-projectile bombardment (biolistics) to transform proembryogenic masses derived from immature zygotic embryos. Once attention was focused on embryogenic cultures as the target tissue of choice for transformation, a number of variations were tested and progress was relatively rapid (Merkle, 1994; Carraway and Merkle, 1997; Merkle and Dean, 2000; Polin *et al.*, 2006).

Both biolistic and *Agrobacterium*-mediated transformation were evaluated. In a series of gene gun (Helios Gene Gun, Bio-Rad Laboratories, Hercules, California, USA) experiments, optimizing gas pressure, particle size, and a 4-h prebombardment treatment in a high osmotic potential medium, improved the transient expression manyfold, however, no stable transformation events were recovered (Polin, 2004).

As of March 2007, the US Department of Agriculture electronic database, AGRICOLA, contained more than 3200 citations for "*Agrobacterium* transformation". Researchers are frequently reporting modifications to the basic technique as they attempt to insert genes into new plant species. One such modification, described as a desiccation step, was first reported for wheat by Cheng *et al.* (2003). Desiccation was key to transforming American chestnut somatic embryos and keeping them embryogenic (Polin *et al.*, 2006). In the desiccation procedure, after 1 h of inoculation with *Agrobacterium*, the somatic embryos were transferred to a Petri dish containing a slightly moistened sterile filter paper, but lacking the traditional semisolid tissue culture medium. The embryo clumps were placed in small stacks (approximately 5 mm in size) on the filter paper. The Petri dishes were wrapped with cling film and co-cultivated in the dark at room temperature for 2–3 days. The postdesiccation steps followed standard *Agrobacterium* transformation protocols. Embryos were transferred to an *Agrobacterium*-lethal medium containing carbenicillin and

cefotaxime for a week and then transferred to a selection medium containing the same antibiotics plus PPT to eliminate any nontransformed plant cells. Putative transformants were transferred to fresh selection medium every 2 weeks. Over a period of 2–6 months, vigorously growing embryo clusters that showed uniform green fluorescent protein (GFP) fluorescence under a Nikon Stereoscopic Zoom Microscope SMZ1500 with an En GFPLP 83458M filter (Nikon Instruments, Inc., Melville, New York, USA) were selected as putative transformation events. Confirmation of transformation was done with Southern hybridization analysis (Polin *et al.*, 2006).

To increase the rate of transformation, several modifications to the basic desiccation procedure were tried. Acetosyringone had a positive effect when added to the *Agrobacterium* inoculum in a virulence-induction step, but as was observed for European chestnut, it had no effect or a slightly negative effect when added to the co-cultivation medium (Rothrock, 2006). Rothrock *et al.* (2007) found that wounding the embryos by any of several methods (vortexing with fine sand, vacuum infiltration, or sonication) had no effect or had a negative effect on transformation frequency. Wounding also induced a higher proportion of callus rather than additional embryos. The two clones tested gave slightly different results. For one clone that multiplied as tiny loosely aggregated embryos, the best treatment among those examined was to simply add the *Agrobacterium* inoculum to the Petri dish containing intact and undisturbed somatic embryos. The method was named “plate flooding” (Rothrock *et al.*, 2007). For the second clone that multiplied as relatively large well-aggregated embryos, the desiccation method and plate flooding gave similar results and both were superior to the wounding treatments.

2.2.4 Regeneration of whole plants: three alternatives

After stable transgenic somatic embryo cell lines have been identified, it is necessary to regenerate them into whole plants. At least three alternatives exist. Somatic embryos can be induced to germinate and grow into somatic seedlings (Robichaud *et al.*, 2004; Andrade and Merkle, 2005), somatic embryos can be induced

to produce shoots and those shoots multiplied, rooted, and acclimatized (Xing *et al.*, 1997, 1999; Polin *et al.*, 2006), or those clones that are recalcitrant to rooting treatments can be inserted into germinating nuts, a process called nutgrafting (McGuigan, 2007).

2.2.4.1 Somatic embryo/somatic seedling

Somatic embryo/seedling (SE/SS) technology has been promoted as the approach holding the most promise for operational *in vitro* mass propagation of elite genotypes (including transgenics) of a number of forest tree species (e.g., Gupta *et al.*, 1993; Merkle and Trigiano, 1994). Commercially important conifers, in particular, have been targeted for propagation via somatic embryogenesis and this approach is now at the point of commercialization for some top commercial pine species (Sutton, 2002; Nehra *et al.*, 2005). Somatic embryogenesis may ultimately provide the most efficient route for mass propagation of blight-resistant American chestnut as well, whether the blight resistance is conferred by transgenes or by conventional breeding. Following the first report of somatic embryogenesis in American chestnut (Merkle *et al.*, 1991), progress on germination and conversion of the somatic embryos was slow. Carraway and Merkle (1997) produced a few somatic seedlings, but these failed to survive transfer to *ex vitro* conditions. Through a combination of embryogenesis and micropropagation, Xing *et al.* (1999) established American chestnut plantlets in the field. Robichaud *et al.* (2004) produced the first field-plantable American chestnut somatic seedlings by supplementing the medium during embryo development and maturation with asparagine and/or glutamine. However, germination rates were still low, averaging 9%, and conversion frequencies were even lower, averaging 0.4%.

From approximately 2004 through 2006, American chestnut somatic seedling production efficiency was increased by over two orders of magnitude following testing of additional treatments that positively affected both germination and conversion. One major advance was the use of embryogenic suspension cultures, which could be size fractionated on stainless steel screens and plated on nylon mesh overlaid on semisolid

basal medium. This allowed for the production of relatively synchronous, singularized embryos, rather than clusters of fused embryos (Andrade and Merkle, 2005). By applying 12 weeks of cold stratification at 4 °C to these embryos, conversion frequency for one American chestnut line rose to 47%, and by combining cold treatment and activated charcoal in the germination medium, germination and conversion of embryos of another line were boosted to 77% and 59%, respectively (Andrade and Merkle, 2005). Preliminary data indicate that further increases in somatic seedling production efficiency may be possible for some genotypes by applying even longer cold stratification treatments and light quality treatments during germination (Johnson *et al.*, 2007). Hundreds of American chestnut somatic seedlings derived from these cultures have now been acclimatized and grown in the greenhouse. A subsample of these somatic seedlings grown in a Georgia nursery following a year in the greenhouse reached heights of up to 1.5 m following their first season of growth (Johnson *et al.*, 2007). While additional improvements are needed to make SE/SS technology useful for mass propagation of blight-resistant American chestnuts, the results are promising. As is proving to be the case with pine somatic seedlings, a robust SE/SS system for American chestnut should provide a number of advantages over micropropagation, including higher multiplication rates and the potential for scale-up and delivery via bioreactor and synthetic seed technologies, reducing labor costs (Merkle and Dean, 2000).

2.2.4.2 Germination/micropropagation

Not all American chestnut somatic embryo cell lines are able to germinate into seedlings. Some need to produce shoots first, and then those shoots multiplied, rooted, and acclimatized. Polin *et al.* (2006) observed significant differences among six cell lines tested in transformation efficiency, ease of regeneration of shoots, ease of rooting, and in production of whole plants. In pilot studies, more than a dozen cell lines derived from different trees were evaluated to identify two or three that could be reliably transformed, regenerated into shoots, rooted and finally, acclimatized (Polin *et al.*, 2006). For some somatic cell lines that were difficult or impossible to germinate, it was possible to regenerate whole plants if even a single embryo could be induced to regenerate a shoot (Polin *et al.*, 2006).

Once rooted (Figure 4), the plantlets were potted in a 2:1:1 peat moss:vermiculite:perlite mix. Clear-plastic Magenta GA-7 vessels were inverted over each plant to provide 95% or higher relative humidity, similar to what the plants had been exposed to *in vitro*. The plants were placed in a growth chamber for acclimatization. As the plantlets grew and began to develop new leaves, the lids were removed in stages, first lifting them 1 cm for an hour at a time, then to 1 cm for a day, followed by removing them completely for an hour and finally removing them completely for a full day. If at any point during the acclimatization period the plant began to wilt, the lid was replaced until the plant either recovered (at which point the

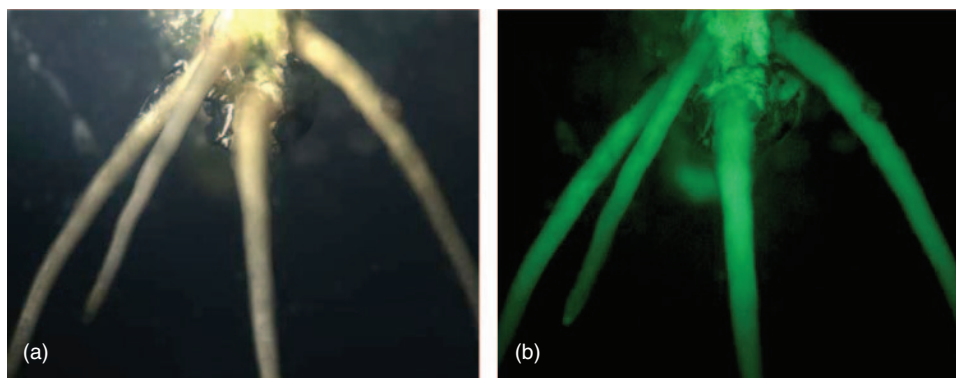


Figure 4 Rooting of transgenic American chestnut: (a) roots formed on transgenic chestnut shoots viewed under white light; (b) same roots viewed under UV light showing GFP expression

process was repeated) or the plant died. After the plants became accustomed to the growth chamber conditions, they were moved into a greenhouse (first for 1 h a day than for 2 h a day and so on until they were adjusted to the drier, high-light conditions) (LaPierre, 2003; Maynard *et al.*, 2006).

2.2.4.3 Nutgrafting

As described by Polin *et al.* (2006) for American chestnut, some cell lines will readily multiply as shoot cultures but those shoots fail to root. Nutgrafting (or nurse-seed grafting) is an alternative to *in vitro* rooting. Moore (1963) was the first to describe propagation of American chestnut by nutgrafting. He used one- or two-bud hardwood scions inserted into a slit made between the two cotyledons of germinated chestnuts. We are attempting to adapt the technique for use with scions grown *in vitro*. In a modified procedure taken from Greenwell (2002) and Schibig (2003), the roots of germinating chestnuts were severed from the cotyledons and a wedge-shaped cut was made at the base of each tissue cultured shoot. The shoot was then inserted into a slit cut in the cotyledons. The graft was placed in a Magenta GA-7 vessel filled with moistened sphagnum moss. Two nutgrafts were successful out of approximately 40 attempts using transgenic American chestnut shoots (McGuigan, 2007).

2.3 Testing Transgenic Chestnuts for Blight Resistance

2.3.1 Greenhouse screening for blight resistance

Traditionally, blight resistance is measured on trees that are 3 years old or older using a cork-borer inoculation method (Griffin *et al.*, 1983). Powell *et al.* (2007) developed a technique that can differentiate resistant Chinese chestnut from susceptible American chestnut using seedling stems that are only 3–4 mm in diameter (Figure 5). This technique will be used as an early indication of enhanced blight resistance. All transgenic events will be inoculated with two virulent strains of *C. parasitica*, EP155 and EP42, to ensure the results are not due to a particular strain of the pathogen. Chinese and American chestnut seedlings will be used as positive and negative controls, respectively. In pilot studies, susceptible plants were girdled by a canker in approximately 4 weeks, while resistant plants formed callus in the wounds and survived (Powell *et al.*, 2007).

2.3.2 Field testing of transgenic American chestnut trees

In the spring of 2006, two transgenic American chestnuts were planted in the field in Syracuse, New York (Figure 6). Two more were planted in the fall of 2006. The four plants were all from

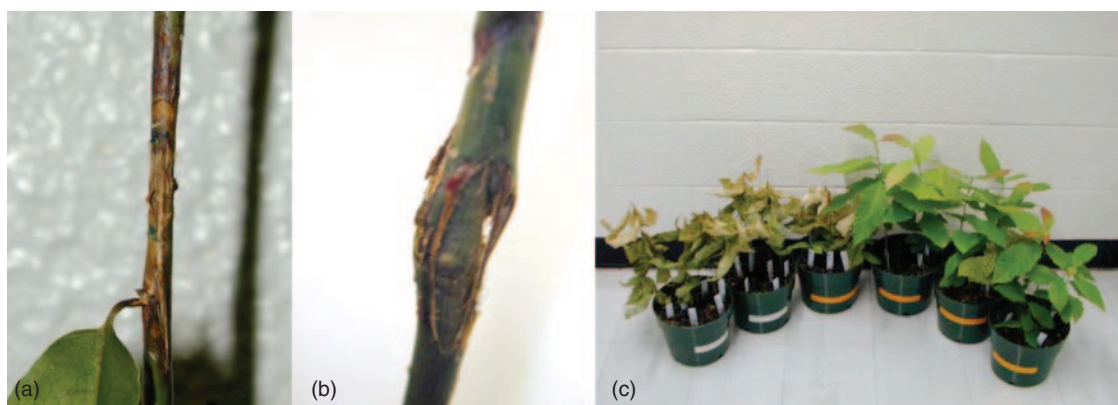


Figure 5 Example of a chestnut seedling resistance assay: (a) killing canker on susceptible American chestnut; (b) healing canker on resistant Chinese chestnut; (c) inoculated American chestnut seedlings on left showing wilting tops and inoculated Chinese chestnut seedlings on right surviving



Figure 6 The first two transgenic American chestnuts were planted in the field June 7, 2006 in Syracuse, NY, USA derived from somatic cell line WB275-27, transformation event LP 28 and given the variety name “Wirsig”

the same parent clone (WB275-27 obtained from H. Dayton Wilde at MeadWestvaco Corporation, Summerville, South Carolina, USA) and the same transformation event (LP 28). This transformation event was given the variety name “Wirsig” in honor of Arlene and Stan Wirsig, two of the original members of the New York State Chapter of the American Chestnut Foundation. One tree from the spring planting died soon after planting, but the second tree grew a new shoot approximately 45 cm high. By late October, it had set apparently normal resting buds and dropped its leaves. One tree from the fall planting was accidentally destroyed. The second set bud soon after planting. It appeared to enter dormancy normally in the fall, but did not survive the winter, possibly due to its small size at planting. A third planting of approximately 15 trees is planned for the spring of 2007.

2.4 Specific Regulatory Measures Adopted

All recombinant-DNA research described in this review has been carried out in laboratories certified by their respective Institutional Biosafety Committees. The 2006 field trial of American chestnut was initiated after submitting a Release Notification and receiving acknowledgement (No. 06-090-21n) from the USDA, Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (APHIS, BRS).

3. FUTURE ROAD MAP

3.1 Expected Products

For American chestnut, the primary improvement objective is simple—blight resistance. This could

be broadened to enhancing resistance to other important chestnut pathogens. At least initially, no attempt will be made to enhance nut size, reduce flowering age, or to improve any of the other traits that might be of interest to the horticulture industry. The European chestnut and the Chinese chestnut have almost entirely supplanted the American chestnut for human consumption in North America. It is unlikely that this market will be recaptured in the near future.

One approach to breeding for durable disease resistance is to incorporate multiple pathogen resistance-enhancing genes. This approach, called “gene stacking” or “gene pyramiding”, holds great promise for long-lived tree species like the American chestnut. One desirable feature for stacking genes is to use genes that enhance resistance by different mechanisms so that single mutations in the pathogen will not overcome both transgenes simultaneously. Then the hypothetical probability of the pathogen acquiring mutations to overcome the enhanced resistance is the product of the probabilities of overcoming each transgene individually, which is increasing small as the number of stacked genes increase. Ideally these stacked genes should be as closely linked as possible, so that in subsequent generations the genes segregate together. A multiple gene construct containing a *Trichoderma harzianum* endochitinase, Ech42 (Lorito 1998; Lorito *et al.*, 1998), a TEV-NIa self-cleaving proteinase (Bodman *et al.*, 1995; Carrington and Dougherty, 1988; Ceriani *et al.*, 1998; Dasgupta *et al.*, 1998) and a wheat oxalate oxidase (Lane, 2002), in which one large coding region was driven by a single promoter was developed by W.A. Powell, H. Gao and H. Liang (Liang *et al.*, 2005). The large peptide string is then cleaved by the NIa proteinase into active Ech42 and OxO enzymes. This construct has been shown to cleave properly in *Arabidopsis* and retain expected enzymatic activity (Liang *et al.*, 2005). Similar constructs with constitutive and wound-inducible promoters are currently being used to transform American chestnut. Future constructs could combine any combination of gene products, including possible combinations of antimicrobial peptides (Powell *et al.*, 1995, 2000; Liang *et al.*, 2002) or other available resistance-enhancing polypeptides and enzymes (Powell *et al.*, 2006).

3.2 Public Perceptions

European chestnut is used for both wood products and as an edible nut crop with the latter use probably dominating. Although a blight-resistant European chestnut would be welcomed by many nurserymen and orchardists, a combination of public acceptance concerns, regulatory roadblocks and reasonably successful alternative techniques to control the blight in commercial orchards will probably keep transgenic European chestnut trees confined to the laboratory and greenhouse for the immediate future.

The situation is different for the American chestnut. Over the last decade C. Maynard, W. Powell and S. Merkle have presented “The American Chestnut Story”, which includes information on using genetic engineering to achieve blight resistance, to dozens of groups ranging from the African Violet Society to the national convention of the Society of American Foresters. Reactions have been almost universally positive. Public acceptance for genetically modified crops in general is higher in the United States than in Europe (Lusk and Rozan, 2005; Byrne, 2006). But what we have observed for blight-resistant American chestnut goes well beyond acceptance and might be better described as eager anticipation. The typical question asked of us has been how soon will they be available.

3.3 Deployment of Blight-Resistant American Chestnuts

3.3.1 Clonal deployment: micropropagation, nutgrafting and somatic seedlings

As discussed in Sections 2.2.4.2 and 2.2.4.3, micropropagation and nutgrafting will allow clones that are recalcitrant to somatic seedling production to be regenerated into whole plants. The primary disadvantage of both micropropagation and nutgrafting is the high labor inputs and, therefore, high production costs. Both of these propagation tools may be used to produce limited numbers of expensive plants. They may be marketed through the horticulture industry as street trees, but costs will probably be prohibitive for commercial reforestation. They may also be

of use as planting stock for seed orchards to be described in Section 3.2.2.

Somatic embryo or somatic seedling technology will provide the most efficient route for mass propagation of blight-resistant American chestnut for commercial reforestation if further improvements can be made in germination and conversion rates, and the process can be scaled up. Production costs are speculative, but they will be considerably lower than micropropagated plants or nutgrafts, but probably higher than bare-root seedlings.

3.3.2 Seedling deployment: producing a blight-resistant and genetically variable population

Chestnut trees flower early, but may take a decade or longer before they reach sufficient size to produce large crops of nuts. Seed orchards must, therefore, be considered a long-term delivery method. Seedlings do, however, have two advantages over the clonal propagation methods described in Section 3.2.1. They are usually less expensive to produce than any of the clonal propagation methods, and if the transgenic plant can be crossed with wild-type trees and still express resistance, the resulting population can contain considerable genetic diversity. In annual crop species, the problem of introducing genetic diversity is dealt with by using a transgenic line as the nonrecurrent parent in a backcross breeding program (Mumm and Walters, 2001). In cotton, it can take as little as five years to move a transgene of interest from a single transformation event into a dozen or more elite varieties (Meredith, 2006). As described previously, the final step in classical backcross breeding is several generations of selfing to “fix” the gene in each variety. *C. dentata* is an obligate outcrossing species (Rutter *et al.*, 1990), making it impossible to use self-pollination to produce varieties that breed true for blight resistance. However, such uniformity may be both undesirable and unnecessary. Foresters practicing traditional long-rotation forestry (defined here as at least 25 years from planting to harvest) are accustomed to dealing with highly genetically variable populations of trees.

One approach to producing a genetically diverse seedling population with a high proportion of blight resistant trees is a seed orchard of

somewhat novel design. The New York State Chapter of the American Chestnut Foundation has approximately a dozen germplasm archives containing open-pollinated progeny from more than 50 *C. dentata* parent trees. Most were planted at wide spacing and are suffering considerable mortality from chestnut blight and deer damage (Macfee, 2001). These gaps will allow room for interplanting with transgenic trees. Because the small transgenic trees would be planted among established wild-type trees, bulk collection of open-pollinated seed from the whole orchard would contain only a small proportion that carry the transgenes of interest. However, approximately 50% of the nuts collected from the transgenic trees would inherit the resistance gene and the linked *bar* gene. The selectable marker gene *bar*, in combination with the herbicide Finale, has proven to be effective in selecting for transformation events *in vitro* (Polin *et al.*, 2006; Rothrock *et al.*, 2007). The *bar*–Finale combination may also prove useful in identifying seedlings containing the transgenes by mass screening in nursery beds for herbicide tolerance. Such a screened seedling population would undoubtedly contain escapes, but this would be in exchange for the enhanced genetic diversity provided by the wild-type pollen parents and would probably be acceptable for reforestation.

Traditional long-rotation silviculture usually includes several intermediate cuts before plantations reach final harvest age. Considerable natural mortality is also expected (Nyland, 2002). Given these two assumptions, 50% or even 75% blight susceptible escapes should be acceptable in commercial planting stock. Susceptible wild-type trees would be removed by natural mortality and intermediate cuts, and the final harvest would be essentially the same as if the entire stand had been planted with blight resistant seedlings.

3.4 Conserving the Genetic Diversity of Remnant American Chestnut Populations

In the emotionally charged climate that surrounds the deployment of transgenic plants, the authors were hesitant to discuss introgression of transgenes into remnant populations, however, the ultimate goal of the American Chestnut Foundation is to

restore the species throughout its former range. Once the APHIS-BRS and the Environmental Protection Agency (EPA) regulatory requirements for “full deregulated status” have been met, the transgenes have been clearly shown to provide resistance to chestnut blight, and the transgene products have been proven to be safe for human and animal consumption, it is the intent of the NY Chapter of the American Chestnut Foundation to encourage its members to plant blight-resistant seedlings near flowering wild-type trees to *encourage* introgression into these remnant populations. Such purposeful introduction of transgenes for blight resistance into natural populations may meet with some opposition. We argue, however, that such introgression is a highly desirable outcome. The long-term survival of the American chestnut will be enhanced if resistance genes are allowed to introgress into and revitalize these remnant populations. This will help recreate a vigorous and self-sustaining species. Powell *et al.* (2005) and Merkle *et al.* (2007) made a strong case that the reintroduction of blight resistant chestnut trees will counteract the century-long disruption to the environment caused by the original introduction of chestnut blight.

3.5 Cutting-Edge Technologies

3.5.1 Cotransformation

Green fluorescent protein has proven to be a highly effective scorable marker in American chestnut (Polin *et al.*, 2006) and in many other species (see for review, Stewart, 2006). This marker allows nondestructive detection of transformed cells and their development into transgenic somatic embryos. It is not uncommon when using selectable marker genes such as BAR with PPT selection, that a significant number of escapes (nontransgenic cells or embryos that survive the selection) are obtained (Lebedev *et al.*, 2002; Reustle *et al.*, 2003; Dolgov and Skryabin, 2004). Using GFP screening in combination with BAR selection greatly reduces escapes (Tain *et al.*, 1997).

Although *gfp* is considered one of the environmentally safest marker genes (Jaiwal *et al.*, 2002), there still is public resistance to its use because of the confusion between “glowing” and “fluorescence”. “Glowing” brings to mind

something unnatural, while “fluorescence” is natural and is often seen in everyday objects. Until there is general public acceptance for GFP, the best strategy is to take advantage of GFP while keeping the option open for removing it later through breeding. One way to accomplish this is to perform a co-transformation (Brasileiro and Aragao, 2001) in which the GFP marker gene is on a separate vector construct from the resistance-enhancing gene. In this method, two or more *Agrobacterium* cultures, each containing a different vector construct, are mixed and used to co-inoculate the plant tissue. As many as four vector constructs have been used to co-transform aspen, resulting in 35%, 27%, 19%, and 19% of transgenic events containing one, two, three, or four constructs, respectively (Li *et al.*, 2003). In preliminary experiments with American chestnut somatic embryos, using selection for only one of the two vectors, approximately 40% of the transformation events contained the nonselected vector. The advantage of using co-transformation is that the transgenes will often be incorporated in different chromosomes of the plant. Therefore, the *GFP* marker gene can be used to identify the first generation of transgenic plants, but if needed, the *GFP* gene could be removed by breeding and selection for offspring that retain the resistance-enhancing gene but not the *GFP* marker. The method can be used for any combination of transgenes that need to be segregated at a later date.

3.5.2 The OxO gene and VspB promoter

The first transgenic American chestnuts to reach field tests contain an oxalate oxidase transgene that comes from wheat and is expressed by a vascular promoter that comes from soybean. Oxalate oxidase catalyzes the degradation of oxalic acid (also called oxalate) into H_2O_2 and CO_2 . The OxO enzyme is of interest because *C. parasitica*, the chestnut blight fungus, produces large amounts of oxalate at the canker margin, which helps lower the pH to toxic levels and binds calcium (Roane *et al.*, 1986). Oxalic acid has also been associated with pathogenesis in other fungi (Noyes and Hancock, 1981; Marciano *et al.*, 1983; Cessna *et al.*, 2000).

The *OxO* gene has been shown to enhance pathogen resistance in several transgenic crop species including soybean, peanut, and sunflower (Donaldson *et al.*, 2001; Liang *et al.*, 2001; Burke and Rieseberg, 2003; Cober *et al.*, 2003; Heritage, 2003; Livingstone *et al.*, 2005). Liang *et al.* (2001) demonstrated that constitutive expression of *OxO* in hybrid poplar enhanced resistance to the fungal pathogen *Septoria musiva*, another oxalic-acid-producing pathogen. Welch *et al.* (2007) showed that the *OxO* transgene protects lignin formation in transgenic chestnut callus tissues exposed to oxalic acid. Lignin biosynthesis is a critical step in forming a barrier zone around developing canker margins, which, in turn, is an important feature in chestnut blight resistance (Hebard *et al.*, 1984). The *OxO* gene was also selected because it comes from wheat, a familiar crop species that is consumed by most people every day. It, therefore, brings with it a sense of familiarity.

Driving the expression of the *OxO* transgene is a regulated promoter that can control which plant tissues express the gene. In the pTACF3 vector construct, the promoter from the *VSPB* gene was chosen because its expression pattern is primarily in stems and wound sites, the tissues that are the primary infection sites of the chestnut blight, but it is not expected to be produced in significant amounts in the nuts. Vascular expression of this transgene is intended to minimize effects on nontarget organisms without sacrificing effectiveness against *C. parasitica*.

3.5.3 Establishment of somatic embryo cell lines from leaf tissue

Most somatic cell lines are established using immature zygotic embryos as explants. Corredoira *et al.* (2003, 2006) were able to establish somatic cell lines from recently unfurled leaves from micro-propagated shoot cultures of European chestnut. These shoot cultures had been established from parent trees two years of age. If this technique can be extended to mature trees of any age, it will be possible to transform blight resistance genes or other genes of interest into clonally propagated commercially accepted varieties. An alternative procedure for obtaining somatic embryogenesis from material collected from trees was reported in the related species *Quercus robur* (Toribio *et al.*,

2004; Valladares *et al.*, 2006). In this case, somatic embryos were induced in expanding leaves of epicormic shoots forced from branch segments taken from mature (over 100 years old), selected trees. Attempts will be made to obtain a similar somatic embryogenesis system for mature chestnut trees.

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Birches

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The birch family (Betulaceae) consists of 6 genera (*Betula*, *Alnus*, *Carpinus*, *Corylus*, *Ostrya*, and *Ostryopsis*) and about 150 species (Furrow, 1990). On the basis of extensive leaf and pollen fossil records, the Betulaceae is believed to have originated during the late Cretaceous period. The pollen records show that *Betula* and *Alnus* were differentiated already in the Santonian (80 Mya (million years ago); Crane and Stockey, 1987). Although earlier fossil records of *Betula* from the Paleocene and early Eocene exist, the oldest and most complete specimens of *Betula* fossils are from the middle Eocene (45 Mya) and comprise several leaf remains as well as infructescences, staminate inflorescences, and fruits (Crane and Stockey, 1987; Crane, 1989). The fossils are assigned to the extinct species *Betula leopoldae*. Although the fossils of *B. leopoldae* are not precisely similar to any living species, they resemble the species of the subgenus *Betulenta*, such as *Betula alleghaniensis* (Crane, 1989).

In Europe, pollen of the genus *Betula* is well known from late Tertiary (Dickson, 1984). Also the recolonization of Europe by forest tree species after the last glaciation is well documented in the pollen fossil records (Huntley and Birks, 1983). Pollen data, as well as macrofossils, indicate that

birch was present in central Europe during the full glacial (Huntley and Birks, 1983; Willis *et al.*, 2000). Birch populations were not limited only to a few southern refugia, but were locally present in a belt that ran eastward into Russia, and also on the northern European plains. Furthermore, birch pollen was widely present in parts of the central and northern Europe during the late-glacial period (Willis *et al.*, 2000). When the ice started to retreat, silver birch, as a pioneer species, occupied suitable habitats and quickly spread northward. Studies on chloroplast genome have proved that after the glaciation Europe was reoccupied by two main waves of recolonization: one from east and one from west (Palmé *et al.*, 2003). Following these two main waves of recolonization, today's silver birches in Europe can be classified into two main chloroplast haplogroups, of which one is dominant in the northwest and the other in the southeast and east. To Fennoscandia birch migrated about 10 000 years ago from refugia located to southwest, south, and southeast from Finland (Huntley and Birks, 1983; Hyvärinen, 1987; Willis *et al.*, 2000). The recolonization of North America by forest tree species after the last glaciation has recently been studied by Williams *et al.* (2001, 2004).

The modern birches are common trees and shrubs of boreal and north temperate zones of the northern hemisphere (Furrow, 1990) (Figure 1). The greatest species diversity occurs in the mixed mesophytic forests of eastern Asia, but the family



Figure 1 A Finnish birch forest

is ecologically important throughout middle and high latitudes where they range in habit from dwarf shrubs of arctic tundra to large trees of subtropical forests. The northern white birches (e.g., *Betula pendula* Roth, *Betula pubescens* Ehrh., and *Betula*

papyrifera Marsh) are relatively fast-growing and short-lived pioneer species requiring high light intensity, “colonizers par excellence” (Ashburner, 1993a), whereas the birches of more southern mixed mesophytic forests (e.g., *B. alleghaniensis*

Britt., *Betula grossa* Sieb. and Zucc., and *Betula maximowicziana* Reg.) are longer lived, less light-demanding, often dark-barked species that are not so easily shaded out, and maintain their place in canopy as occasional individuals (Schilling, 1989; Perala and Alm, 1990; Ashburner, 1993b).

1.2 Taxonomy, Genome Size, and Cytological Features

The genus comprises approximately 30–35 species (Furrow, 1990; de Jong, 1993), but the range of accepted species by different authors ranges from 30 to over 150 (de Jong, 1993), and considerable controversy still exists regarding the systematics of the genus. The uncertainty of the number of species and their phylogenetic relationships are mostly due to the high polymorphism in morphology. Furthermore, hybridization is very frequent, and for this reason, introgression, the transfer of genes between species, may have played an important part in the evolution of the genus (Alam and Grant, 1972; Furrow, 1990; Atkinson, 1992).

Taxonomically, the birches are a difficult group, not only because of their high vegetative variability and frequent hybridization, but also partly because of the confusions related to the binomial nomenclature. Many birch species have at least two different commonly used Latin names (i.e., *B. pendula*/*Betula verrucosa*/*Betula alba*, *B. pubescens*/*B. alba*) and these names are used in parallel with each other. Furthermore, variation in common names is even greater. For example, *B. pendula* is called as a silver birch, weeping birch, and European white birch.

Different attempts to identify sections or subgenera, and the relationships among the *Betula* species have been made on the basis of morphology, biochemical characters, and/or chromosomal numbers (Regel, 1865; Winkler, 1904; Nakai, 1915; Komarov, 1936; Pawlowska, 1983; de Jong, 1993; Keinänen *et al.*, 1999a). Regel (1865), the original monographer of *Betula*, divided birches into two main sections, *Eubetula* and *Betulaster*. The *Eubetula* section was further divided into three subsections, *Albae* (white birches), *Costatae* (yellow birches), and *Nanae* (dwarf birches). The section *Betulaster* contained only few Asian birches in the subsection *Acuminatae*. Since then this division has been

revised numerous times by number of authors into different subsections or subgenera (summarized in Furrow, 1990; de Jong, 1993).

Phylogenetic relationships within the genus *Betula* (Betulaceae) have been studied using two flower-specific silver birch genes, *BpMADS2* and *BpFULL1* (Järvinen, 2004), the *ADH* (alcohol dehydrogenase) gene (Järvinen *et al.*, 2004), nuclear ribosomal DNA (nrDNA) ITS (internal transcribed spacer) sequences (Li *et al.*, 2005), and the chloroplast *matK* (maturase K) gene and parts of its upstream and downstream flanking regions (Järvinen *et al.*, 2004). In general, the results obtained from the nuclear *ADH*, *BpMADS2*, and *BpFULL1* genes fit rather well with the infrageneric classifications proposed for birches (e.g., Regel, 1865; Winkler, 1904; de Jong, 1993), except for *Betula schmidtii* and *Betula ermanii* (Järvinen, 2004; Järvinen *et al.*, 2004). In all phylogenetic trees *B. schmidtii* (subgenus *Neurobetula*) grouped with the species in the subgenus *Betula* (including *B. pendula* and all other white birches), and *B. ermanii* (subgenus *Neurobetula*) grouped with the species in subgenus *Chamaebetula* (including *Betula humilis* and *Betula fruticosa*). The chloroplast *matK* data, with only five parsimony informative characters, divided the *Betula* species into two groups: one containing the American species *B. papyrifera*, *B. lenta*, and *B. alleghaniensis*, and the other including all the other species studied (Järvinen *et al.*, 2004).

Living species of *Betula* are all $n = 14$ or higher, but there is no unanimity on the base number of the genus (see Furrow, 1990). The base chromosome number 14 is commonly accepted, but Brown and Al-Dawoody (1979) found that meiotic behavior in hybrid birches ($2n = 42$) suggests that these trees are actually hexaploids, not triploids, which leads to a base chromosome number of 7. Furthermore, in meiosis the chromosomes in the $2n = 28$ and $2n = 56$ plants tend to lie in groups of 7 and for this reason the original basic chromosome numbers of birches is thought to be 7 rather than 14 (Eriksson and Jonsson, 1986). The small number of quadrivalents during the meiosis (multivalent chromosomes, which develop from four chromosomes during the meiosis) has also been thought to support the base number of 7. The DNA density for *B. papyrifera* with 84 chromosomes was equivalent to plants that would

possess 63 somatic chromosomes (Grant, 1969). Since a DNA value equivalent to 63 chromosomes is a multiple of 7 and not for 14, this suggests that the genomic number of *Betula* is $n = 7$. Also, the latest studies with molecular markers offer evidence for a base number of 7 in *Betula* (Williams and Arnold, 2001). Stebbins (1971) estimated that approximately one-third of the angiosperm plants possess more than two complete genomes (i.e., multiplied sets of the diploid chromosome number of the genus), and it is probable that also the present basic chromosome number of the genus *Betula* is of ancient polyploid origin. Various researchers have also postulated that the original basic number for the woody angiosperms, including birches, as well as for the angiosperms as a whole is $n = 7$ (Raven and Kyhos, 1965; Ehrendorfer *et al.*, 1968; Raven, 1975).

Polyploidy is a prominent process in plants (e.g., Wendel, 2000; Adams and Wendel, 2005). Species of *Betula* form a polyploid series, with chromosome numbers of $2n = 28, 56, 70, 84$, and 112, and additional numbers in some hybrids (reviewed by Furlow, 1990). From the evolutionary point of view, the genus *Betula* is still young and probably still evolving (Jäger, 1980; de Jong, 1993), which partly explains the polyploid nature of the genus and the occurrence of various ploidy levels (Särkilähti and Valanne, 1990). Furthermore, the differences in ploidy levels among the different subsections/subgenera indicate that several independent polyploidizations have occurred within the genus.

The DNA amount in the unreplicated haploid nucleus of an organism is known as its C-value (Swift, 1950). C-value is irrespective of the ploidy level of the taxon and it equals to genome size in diploid species, but always exceeds genome size in polyploid species. Nuclear DNA C-values differ about 1000-fold among angiosperms, ranging from about 0.1 pg (picogram) to about 125 pg per cell, and tend to be characteristic for a taxon (Bennett *et al.*, 2000). No published estimate of the genome size of *B. pendula* exists, but for another diploid representative of white birches, *Betula populifolia*, the C-value is 0.20 pg per cell (Olszewska and Osiecka, 1984; <http://www.rbgekew.org.uk/cvalues>). The closely related tetraploid *B. pubescens* has a genome size of C DNA = 0.75 pg per cell (Mowforth, 1986; <http://www.rbgekew.org.uk/cvalues>). When

all else is equal, the DNA amounts for polyploids are expected to increase in direct proportion to ploidy level. This means that tetraploids (such as *B. pubescens*) are expected to show double the mean C-value for diploids (such as *B. pendula* and *B. populifolia* (Bennett *et al.*, 2000)). However, the reported C-value for tetraploid *B. pubescens* (0.75 pg per cell) is almost four times higher than that for diploid *B. populifolia* (0.20 pg per cell). One interpretive factor could be intron size differences (e.g., Wendel *et al.*, 2002) caused by insertions/deletions between diploids and tetraploids or higher species, such as seen in *ADH* gene in *B. pubescens* and *B. papyrifera* compared to diploid *B. pendula* and *B. populifolia* (Järvinen *et al.*, 2004). At present, however, there is only little information on the correlation between genome and intron sizes in plants (Wendel *et al.*, 2002).

1.3 Habit and Habitat

Economically, the most important species is the diploid ($2n = 28$) silver birch (*B. pendula* Roth), which belongs to the variable white birch complex (*B. alba* L. *sensu lato*), sometimes considered geographic races of a single species, or as an aggregation of more or less closely related different species, subspecies, and hybrids (Furlow, 1990). Silver birch and the polyploid downy, or hairy birch, (*B. pubescens*) are distributed throughout the northern Eurasia; but silver birch extends further south than downy birch, whereas downy birch is more northerly and easterly in distribution (Atkinson, 1992). The southern limit of silver birch appears to be limited by the average July rainfall of 10 mm, and the northern limit by protection from cold northeasterly winds (Atkinson, 1992). In general, silver birch is restricted to drier and lighter soils, whereas downy birch grows in wetter soils. Silver birch grows up to 30 m high, usually with one stem (Atkinson, 1992). The bark of silver birch is smooth and silvery white and exfoliates in long, thin strands. In young trees (up to 10 years old) the bark is light brown, while in old trees the basal part of the trunk is black and vertically fissured. Young branches are spreading or ascending, whereas older branches are often pendulous. Silver birch is a wind-pollinated, outcrossing species with monoecious and a diclinous flowers. The male flowers consist of a minute perianth and two

stamens, whereas the female flowers consist only of a pistil, having no perianth.

1.4 Traditional and Molecular Breeding: Promises and Constraints

Silver birch is one of the three most important forest tree species, and the most important deciduous tree species in Nordic countries, and used, for example, in plywood, pulp, and furniture production. Breeding of silver birch can be considered to have started in late 1940s by selection of so-called “plus trees” on the basis of the growth and stem characteristics of the tree. The objective of the selection of plus trees was to use them in crossing experiments and establish field trials in order to screen genotypes with superior, heritable growth characters. The superior genotypes were selected for seed orchards that were established in polythene tunnels for the production of genetically improved birch seed for forest cultivation. Nowadays almost all birch seed (90%) used in nurseries in Finland originates from the birch seed orchards (Haapanen and Mikola, 2004). From a biological point of view, silver birch has several advantages in comparison to the breeding of boreal conifers, Scots pine, and spruce (for a review, see Koski and Rousi, 2005). The large genetic variation of silver birch (e.g., Howland *et al.*, 1995; Keinänen *et al.*, 1999b; Laitinen *et al.*, 2000; Rusanen *et al.*, 2003; Pritinen *et al.*, 2003) offers a thankful starting point for selection and breeding. Flowering at an early age allows a faster breeding cycle, and occurrence of interspecific hybridization and natural polyploidy offer optional breeding methods besides conventional selection. Remarkable genetic gain has already been achieved by using conventional breeding; for example, the stem volume of silver birch has been increased about 30% (Hagqvist and Hahl, 1998).

Despite the fact that silver birch is the main deciduous species of conventional tree breeding in Finland (Haapanen and Mikola, 2004), it has still undergone relatively little breeding. This is mainly because conventional breeding of woody plants is very slow compared to annual plants due to long generation cycle, large size of the trees, and long time needed for testing of many economically important properties. Also the phenotypic variation of many commercially

important and desired traits of forest trees is complex, and regulated by many loci (Neale and Savolainen, 2004). To speed up breeding of silver birch, new biotechnological approaches have been introduced, including marker-assisted breeding, micropropagation, and gene transfer. Several research groups are now studying the possibilities to improve wood quality, ozone, and drought and freezing tolerance, and insect and pathogen resistance of trees by using these technologies. However, for the time being, molecular breeding and use of genetically modified (GM) plants in silviculture are strictly controlled in the European Union (EU), and the possible use of transgenic plants has inflicted strong public concern about the putative environmental risks associated with GM plantations. For a review of regulatory considerations for transgenic trees, see Nehra *et al.* (2005).

2. DEVELOPMENT OF TRANSGENIC BIRCHES

2.1 Micropropagation and Gene Transformation

Silver birch micropropagation has been reported by many researchers, e.g., by Chalupa (1981), Ryyänen and Ryyänen (1986), Särkilähti (1988), Törmälä (1990), and Jokinen *et al.* (1991), and reviewed by Meier-Dinkel (1992). Micropropagation is relatively straightforward and succeeds routinely also from mature trees, with few exceptions (K. Keinonen, personal observation). During establishment of the shoot culture, a low level of cytokinin ($0.4\text{--}0.6\text{ mg l}^{-1}$) and a very low level of auxin ($0.001\text{--}0.05\text{ mg l}^{-1}$) have been used. Micropropagation of related species, such as *Betula platyphylla* var. *szechuanica* (McCown and Amos, 1979), *Betula celtiberica* (Perez and Postigo, 1989), *B. papyrifera* (Minocha *et al.*, 1986), and *B. pubescens* (Kauppi *et al.*, 1999) has also been described.

Induction of adventitious shoot buds, which are needed for plant regeneration from, for example, transgenic cells, has been reported to occur in *B. pendula* via callus (Huhtinen and Yahyaoglu, 1974; Simola, 1985; Srivastava *et al.*, 1985; Welander, 1988), through direct organogenesis from leaf discs (Piagnani *et al.*, 1990; Leege and

Tripepi, 1993), and from stem segments (Chalupa, 1981). Somatic embryogenesis has been used in large-scale propagation with several woody plants (Jain *et al.*, 1996), and embryogenic cultures have been used in transformation both with biolistic and *Agrobacterium*-mediated methods. With silver birch, somatic embryogenesis has been described by Kurtén *et al.* (1990), but its usage in transformation of birch has not been reported.

Micropropagated and seed-born silver birches have been compared in field tests. Viherä-Aarnio and Velling (2001) found no differences between them in survival, in height growth, or in biotic damage. Instead, differences among birch clones were very large, indicating that careful selection and field testing are needed before wide-scale practical forest cultivation using micropropagated plants can take place. In a 7-year field experiment, Jones *et al.* (1996) compared the growth of trees produced from nodal stem sections or callus tissue with that of seedlings. Micropropagated trees were found to grow at a similar rate compared to trees originating from seeds.

Transgenic birches have been successfully produced both with biolistic and *Agrobacterium*-mediated gene transfer (see Tables 1 and 2). With biolistic method, the gene transfer frequency was reported to be 6% (Valjakka *et al.*, 2000). Similar or lower numbers in frequency have usually been achieved with *Agrobacterium*-mediated transformation with most of the birch clones. One reason for the low success is the pathogenicity of *Agrobacterium*. Infection of birch tissue leads usually to severe hypersensitive response (HR). The tissue becomes necrotic or transparent in a few days after the beginning of co-culture. The strength of HR can be limited in several well-known ways: (1) by reducing compounds such as thiosulfate and cysteine, (2) with antioxidants,

(3) with polyvinylpyrrolidone or charcole, which adsorb phenolic compounds, (4) with inhibition of ethylene synthesis or action, and (5) by incubation of cultures under low light and at lowered temperature. The use of a liquid co-culture medium instead of a solid medium has proved to be especially effective in limiting the HR (Keinonen, 1999).

In flowering-related studies in the University of Joensuu, Finland, early-flowering birch clones have been used (Lemmettyinen *et al.*, 1998). Optimization of transformation protocol for these clones increased the transformation frequency up to about 90%. With 25 gene constructs, over 1000 transgenic lines have been produced during the recent years. In flowering-related transformations, it is necessary to produce about 20 transgenic lines with every construct in order to create at least a few strong transgenic phenotypes. With some exceptions, plants of only about one-third or one-fourth of the transgenic lines have proved to have proper phenotypes for further studies.

For the long-term storage of valuable germplasm, whether transgenic or in conventional breeding, a reliable method of cryopreservation for silver birch is now available. After cryopreservation, regeneration percentage is about 40% for the *in vitro* material (Ryynänen, 1998), and over 70% for the *in vivo* material (Ryynänen *et al.*, 2002). Furthermore, the stability and the function of the transferred genes of the regenerated plants were found to be maintained (Ryynänen *et al.*, 2002).

2.2 Flowering

In contrast to the great variation in their vegetative parts, birches are rather uniform in their reproductive organs, including separate male and

Table 1 Biolistic gene transfer in silver birch (*B. pendula*)

Gene construct	Trait	Preculture ^(a)	Culture ^(a)	Selection (mg l ⁻¹) ^(a)	Reference
CaMV 35S::RbcS	Altered carbon metabolism	CIM 6–7 days	CIM 6 months, SIM 4–7 months	Km 50–200	Valjakka <i>et al.</i> , 2000
CaMV 35S::PtCOMT Ubb1::PtCOMT	Altered lignin	CIM 6 days	NM	NM	Aronen <i>et al.</i> , 2003
<i>Agrobacterium aux</i> and <i>rol</i> genes	Altered wood	CIM 6 days	NM	NM	Piispanen <i>et al.</i> , 2003

^(a)CIM, callus-inducing medium; SIM, shoot-inducing medium; NM, not mentioned; Km = kanamycin

Table 2 *Agrobacterium*-mediated gene transfer in birch

Betula	Strain ^(a)	Gene construct	Trait	Preculture ^(a)	Co-culture ^(a)	Selection (mg l ⁻¹) ^(a)	<i>Agrobacterium</i> elimination	Reference
<i>B. pendula</i>	LBA4404	pin2::uidA	Wound-inducible promoter, reporter gene	–	SIM, solid 3–5 days	Km 50	Claforan	Keinonen-Mettälä <i>et al.</i> , 1998
<i>B. pendula</i>	LBA4404	CaMV 140S::chitinase4	Fungal resistance gene	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan, carbenicillin	Keinonen, 1999
<i>B. pendula</i>	C58C1	CaMV 35S::uidAINT	Reporter gene	CIM 5 days	SIM, liquid 3 days	Km 150	Claforan	Lemmetyinen <i>et al.</i> , 1998
<i>B. pendula</i>	C58C1	CaMV 35S::BpMADS1	Flowering related genes	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan	Lemmetyinen <i>et al.</i> , 2004a
<i>B. pendula</i>	C58C1	CaMV 35S::BpMADS6	Flowering related genes	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan	Lemmetyinen <i>et al.</i> , 2004b
<i>B. platyphylla</i>	N.m.	Spider peptide gene, C peptide sequence of Bt gene	Insecticidal gene	NM	NM 2 days	NM	NM	Zhan <i>et al.</i> , 2003
<i>B. pendula</i>	C58C1	BpFUL1::BARNASE	Flowering related promoter, cytotoxic gene	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan	Länneppää <i>et al.</i> , 2005b
<i>B. pendula</i>	C58C1	BpMADS2::BARNASE, BpMADS8::BARNASE	Flowering related promoters, cytotoxic gene	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan	Länneppää <i>et al.</i> , 2005a
<i>B. pendula</i>	C58C1	etr-1	Mutated etr-1 promoter, ethylene receptor gene	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan	Vahala <i>et al.</i> , 2003
<i>B. pendula</i>	C58C1	CaMV 35S::4CLantisenase	Change in lignin	CIM 5 days	SIM, liquid 4 days	Km 75	Claforan	Seppänen <i>et al.</i> , 2006

^(a)NM, not mentioned; CIM, callus inducing medium; SIM, shoot inducing medium; Km, kanamycin



Figure 2 Silver birch: (a) male and female inflorescences and (b) germinating pollen grains in stigmas of female flower

female catkins. The male inflorescences develop at the ends of long shoots, and the female inflorescences at the ends of short side shoots (MacDonald and Mothersill, 1987; Atkinson, 1992). The flowers are in groups of three in the axils of three fused scales. The female flowers consist of a bicarpellate ovary with one anatropous ovule in each locule (Furlow, 1990; de Jong, 1993). The male flowers consist of a reduced perianth with one to four reduced tepals and one to four bifid stamens. The female inflorescences are produced in axillary buds and start to develop in mid summer. They overwinter inside buds, emerge usually in early May, and pollination takes place thereafter (Figures 2a, b). The male inflorescences develop inside the terminal buds and emerge in mid summer, overwintering about 30–40 mm long. The anthesis usually takes place in early May.

In general, trees are characterized by a long-lasting juvenile stage. In birch, it takes around 5–10 years until they start to flower in the field. In greenhouse conditions, this time can be reduced to 3 years (Viherä-Aarnio and Rynänen, 1994). The long juvenile period makes the breeding of birches slow and, therefore, accelerated induction

of flowering would be beneficial for breeding purposes. On the other hand, the prevention of flowering is necessary for the cultivation of transgenic trees.

The long juvenile period also makes the research of birch flower development tedious. In addition, the production of transgenic birch lines itself takes about 1 year (Keinonen, 1999). Fortunately, there is a lot of variation in flowering times of birches. By means of conventional breeding, the juvenile, nonflowering period has been reduced to few months (Stern, 1961). The clones originated from these early-flowering birches have proved to be very useful in flowering research (e.g., Lemmetyinen *et al.*, 1998; Lemmetyinen and Sopanen, 2004).

2.2.1 Genes controlling flower development in birch

The genetic regulation of birch flowering has been studied especially in University of Joensuu, Finland, where Tuomas Sopanen's group has been developing methods to prevent the spread of

transgenes in birch by making them nonflowering. The group has isolated several genes involved in the regulation flower development in birch. The group has concentrated mainly on MADS-box genes, which form a large group of transcription factor genes regulating different processes in plant development. In *Arabidopsis*, about 107 different MADS-box genes have been identified (Parenicová *et al.*, 2003), and a large number of the characterized MADS genes are related to flowering. In silver birch, the Sönanen group has characterized eight different MADS-box genes (Lemmettyinen and Sönanen, 2004).

BpMADS1 is a birch ortholog for *SEPALLATA3* (*SEP3*) (Lemmettyinen *et al.*, 2004a). In *Arabidopsis*, *SEPALLATA* genes (*SEP1–4*) are necessary for flower development. Without the activity of these *SEPALLATA* genes the flower organs (sepals, petals, stamens, and carpels) are converted into leaflike organs (Ditta *et al.*, 2004). *BpMADS1* is expressed in the inflorescence meristem for a relatively short period. The expression reappears later in developing flowers, especially in stamens and carpels. The ectopic expression of *BpMADS1* under the control of cauliflower mosaic virus (CaMV) 35S promoter accelerated flowering in tobacco and resulted in changes in the number and identity of floral organs in *Arabidopsis*. The suppression of the expression of *BpMADS1* in birch resulted, in some cases, in the transformation of male inflorescence growth into vegetative growth. As an inflorescence-specific gene, the promoter of *BpMADS1* has been used in the prevention of flowering (see below).

BpMADS2 and *BpMADS8* are birch homologs for *Arabidopsis* B-function genes *PISTILLATA* (*PI*) and *APETALA3* (*AP3*), respectively (Järvinen *et al.*, 2003; Länneppää *et al.*, 2005a). The B-function is necessary for the development of petals and stamens (Jack *et al.*, 1992; Goto and Meyerowitz, 1994). *BpMADS2* has been used in studying the nucleotide variation of birch populations as well as the phylogeny of the genus *Betula* (Järvinen *et al.*, 2003; Järvinen, 2004).

Three of the birch genes isolated, *BpMADS3*, *BpMADS4*, and *BpFULL1* (FUL-like 1, formerly *BpMADS5*), belong to the *API/SQUA* group of plant MADS-box genes (Elo *et al.*, 2001). *BpMADS3* is most similar in sequence to *APETALA1* (*API*) and *SQUAMOSA* (*SQUA*)

while *BpMADS4* and *BpFULL1* are more closely related to *FRUITFULL* (*FUL*). *API* is an A-function gene necessary for the development of sepals and petals in *Arabidopsis*. All these three birch genes are expressed in both male and female inflorescences during the development, but *BpMADS4* is also expressed in leaves and roots. Overexpression of any of these genes in tobacco under the control of CaMV 35S promoter resulted in very early flowering. This suggests that these genes are likely to be involved in the determination of the identity of the inflorescence or floral meristem. They can also be used in biotechnological applications for acceleration of flowering.

Although the ectopic expression of *BpMADS3*, *BpMADS4*, or *BpFULL1* resulted in very early flowering in tobacco, there was a considerable difference between the flowering time of *BpMADS4* and *BpMADS3* sense plants in birch. While there seemed to be a critical size or age required for flowering in *BpMADS3* sense lines, the *BpMADS4* sense lines could make inflorescences extremely early (Elo *et al.*, 2007; Lemmettyinen *et al.*, unpublished results). Silencing of the *BpMADS4* expression using the antisense or RNA interference (RNAi) techniques delayed inflorescence development in transgenic lines (Elo *et al.*, 2007; Lemmettyinen *et al.*, unpublished results). Taking together, these results suggest that *BpMADS4* is involved in the regulation of the juvenility in birch. This finding is of special interest because the regulation of juvenility in trees is very poorly understood.

BpMADS6 is a birch ortholog for *Arabidopsis* *AGAMOUS* (*AG*) and is expressed in birch stamens and carpels (Lemmettyinen *et al.*, 2004a). The ectopic expression of *BpMADS6* resulted in early flowering in tobacco and *Arabidopsis* and also had some effects on the flower structure. Suppression of the expression of *BpMADS6* in birch resulted in the formation of male inflorescences without stamens or caused other abnormalities in the inflorescences. The putative birch homolog to *AGL11*, *BpMADS7*, is expressed in male and female inflorescences (Järvinen, unpublished results).

Two other non-MADS-box flowering-related genes have also been isolated: a birch homolog of *Arabidopsis* *LEAFY*, *BpFLO* (Hurmerinta and

Elo, unpublished results), and a birch SBP-box gene, *BpSPL1* (Lännempää *et al.*, 2004).

2.2.2 Generation of nonflowering birch

The main limitation for the cultivation of transgenic trees in the field is the risk of spreading of various transgenes into natural populations (Bhalerao *et al.*, 2003). The ecological consequences of the potential crossing of transgenic trees with native ones are hard to predict. Furthermore, governmental regulatory guidelines restrict the use of GM plants in EU and elsewhere. Thus, the long-term testing and cultivation of transgenic trees in the field is impossible unless the risk of spreading of the transgenes is eliminated. The most adequate way to ensure this is to prevent the spreading of transgenes via pollen and seeds by blocking the flower development completely. The use of nonflowering trees as recipients of economically important transgenes would be one effective way to prevent the spreading of transgenes into natural tree populations.

The amount of inflorescences produced per tree varies, but in general, adult birches produce great amounts of inflorescences. Silver birch is wind pollinated and the gene flow through pollen is very efficient (Atkinson, 1992). Furthermore, birch produces considerable amount of seeds (up to 100 kg ha⁻¹, or 150 000 seeds/m²) every year and the dispersal of seeds is also by wind. It is unclear, how much the allocation of resources to inflorescences reduces the vegetative growth. However, one could expect that the prevention of flowering would increase the growth, even substantially. Therefore, the inability to flower would itself be greatly beneficial. In addition, the birch pollen is highly allergenic, and an estimated 100 million individuals suffer from birch pollen allergy (Vrtala *et al.*, 2001). Depending on the amount of nonflowering birches planted, the quantity of allergenic pollen in the air could be, at least, locally reduced.

The flowering can be prevented by several different techniques, for example, by silencing the necessary gene(s) for flowering by using antisense or RNAi techniques, or by using tissue-specific ablation by using flower-specific promoter-cytotoxin gene construct (Figure 3). The regulation of flowering is however, highly complicated.

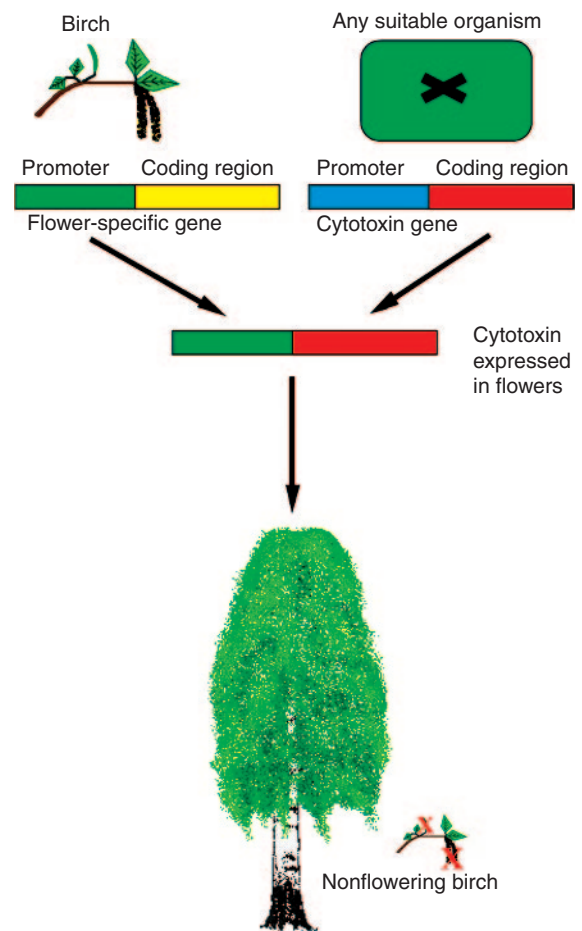


Figure 3 Strategy for the prevention of flowering using the tissue-specific ablation method

Single developmental processes are regulated by several genes that may also compensate each other's functions. Therefore, several genes must probably be silenced to prevent flowering, and the identification of a single gene, absolutely necessary for flowering, is difficult, or even impossible. However, in birch, *BpMADS4* is one of the central genes regulating flowering and can be used either to accelerate or prevent flowering (Elo *et al.*, 2007). The most reliable method to prevent flowering, however, has been the tissue-specific ablation.

The birch flowering was prevented, for the first time, by utilizing the promoter of *SEP3* ortholog *BpMADS1* (Lemmetyinen *et al.*, 2001, 2004a, b). The expression of *BpMADS1* is restricted to inflorescences, and therefore, its promoter is

suitable to regulate the expression of cytotoxic gene, *barnase*. The barnase is a ribonuclease isolated from *Bacillus amyloliquefaciens* (Hartley, 1989). The original barnase protein contains a signal peptide targeting the protein out of the cell. By deleting this sequence the gene efficiently kills the cell it is expressed in. The construct was first tested in tobacco and *Arabidopsis*, resulting in plants with nonflowering phenotype (Lemmetyinen *et al.*, 2001). Importantly, this study showed that the prevention of flowering is able to enhance vegetative growth. This can be explained at least partly by the change in growth habit. In wild-type tobacco, there was a cessation in vegetative growth during the development of terminal inflorescence and seed ripening. In nonflowering tobacco plants, the growth continued without cessation, or the cessation period was short during the time the plant tried to form inflorescence. In *Arabidopsis*, the construct prevented flower formation, and usually stalk formation as well. At times, some

shoots grew from many-leaf rosettes and formed rosettelike structure on the top, which was in turn, a base for the new shoot forming.

The development of birch inflorescences was also successfully prevented using the same *BpMADS1::BARNASE* construct (Figure 4a) (Lemmetyinen *et al.*, 2004b). However, in many transgenic lines the prevention of inflorescence formation was not complete, and many lines showed some disturbance in growth (Figure 4b). Although analyses of GUS (β -glucuronidase) construct lines showed that the isolated promoter fragment of *BpMADS1* lead to intense flower-specific expression in tobacco and *Arabidopsis*, it is possible that some important regulatory elements are missing. It is known that some regulatory elements of the promoters of MADS-box genes are located in introns (Sieburth and Meyerowitz, 1997; Hong *et al.*, 2003).

According to its sequence homology to *Arabidopsis FUL*, birch *BpFULL1* was predicted to be expressed early in the inflorescence development

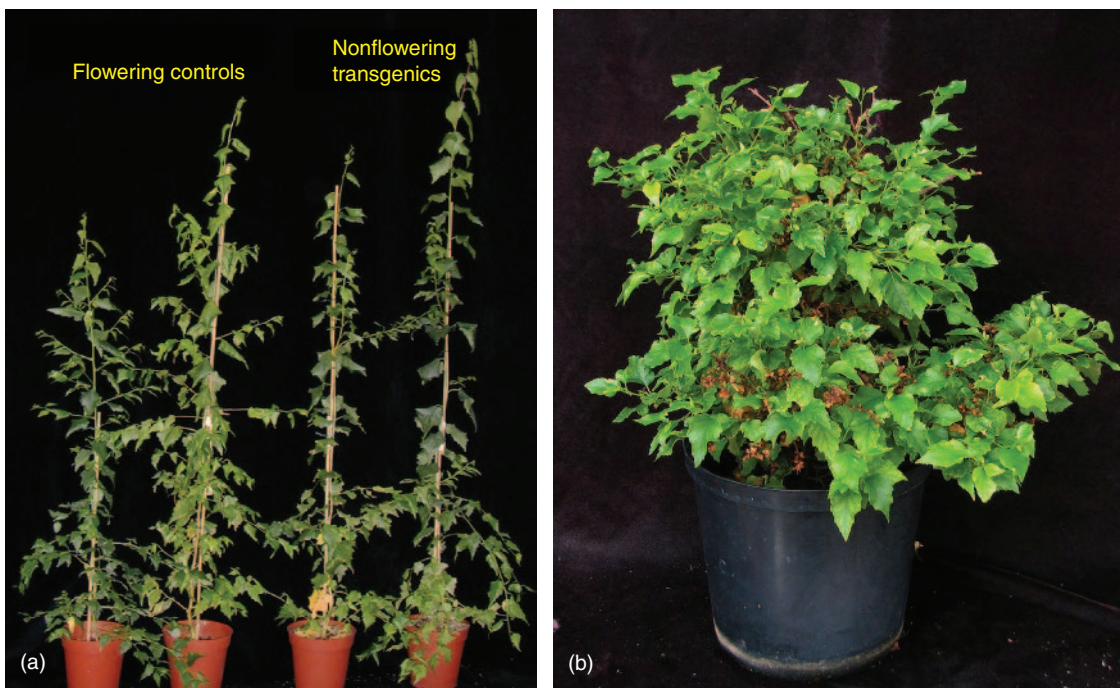


Figure 4 The nonflowering birches generated by introducing the *BpMADS1::BARNASE* gene construct into the early-flowering birch clone: (a) the two birches on the left are wild-type control plants forming inflorescences, the two birches on the right are transgenic plants, which do not form any inflorescences; (b) in some clones, the barnase construct has resulted in growth disturbances and “bonsai”-like phenotype

and function as a genetic switch between the shoot and floral development. The promoter-GUS analyses and *in situ* hybridizations further supported this hypothesis (Länneppää *et al.*, 2005b). The barnase construct with the 2.5-kb-long fragment of *BpFULL1* promoter prevented the development of inflorescences in transgenic *Arabidopsis*, resulting in a dramatic increase in the number of rosette leaves. In transgenic tobacco, the construct efficiently prevented the formation of inflorescences. Nonflowering birch lines with vegetative growth comparable to that of the wild-type birches were also generated. Artificial overwintering treatment demonstrated that the development of both male and female inflorescences was blocked. These results indicate that the *BpFULL1::BARNASE* construct is a very promising gene construct in preventing the flower development in various plant species and is likely to be the best construct available at the moment. The stability of the nonflowering phenotype in birch obtained using the *BpFULL1::BARNASE* construct is currently being tested in a long-time field cultivation experiment (see below).

The promoters of birch B-function genes, *BpMADS2* and *BpMADS8*, have been tested in the prevention of flowering (Länneppää *et al.*, 2005a). The birch gene *BpMADS2*, homologous to *Arabidopsis PI*, had been isolated previously (Järvinen *et al.*, 2003). *In situ* hybridization revealed that *BpMADS2* was expressed in male inflorescence meristems, stamen primordia, and later in stamens as well as in the carpel primordia (Länneppää *et al.*, 2005a). According to promoter-GUS analysis, the 3-kb fragment of *BpMADS2* promoter showed to be inflorescence-specific in *Arabidopsis* (Länneppää *et al.*, 2005a). The *BpMADS2::BARNASE*, in turn, resulted in malformed stamens and petals. Furthermore, flower development was totally prevented in several lines: the inflorescence stems grew and elongated continuously and produced cauline leaves. In tobacco, *BpMADS2::BARNASE* resulted in inflorescence stems lacking flowers. In birch, *BpMADS2::BARNASE* resulted in degenerated stamens after the overwintering treatment as well as malformed female inflorescences and reduced number of carpels in some of the lines.

BpMADS8 was shown to be homologous to *Antirrhinum DEFICIENS* (*DEF*) and *Arabidopsis AP3* (Länneppää *et al.*, 2005a). *BpMADS8*

was expressed in the birch male inflorescence meristems and the expression became stronger in the developing stamens. No expression was detected in tepals and bracts or in the female inflorescences. The promoter-GUS construct driven by a 2.4-kb-long promoter fragment of *BpMADS8* was shown to have specific expression in *Arabidopsis* stamens (Länneppää *et al.*, 2005a). The *BpMADS8::BARNASE* construct resulted in male sterility in *Arabidopsis*, but the carpels developed normally. In tobacco, empty buds or malformed floral organlike structures were produced. Most of the transgenic birches carrying *BpMADS8::BARNASE* construct grew normally, and produced normal female inflorescences after an artificial overwintering treatment. However, the male inflorescences were shorter and the stamens were malformed. No pollen was released from the inflorescences, or seen on the surface of the malformed anthers.

In most of the transgenic birch lines obtained, *BpMADS2::BARNASE* and *BpMADS8::BARNASE* constructs did not result in vegetative disorders. It has been reported that several research groups have attempted to produce sterile plants using B- and C-function gene promoters fused to cytotoxin genes (Van Der Linden *et al.*, 2002). However, these promoters have not been silent in vegetative tissues leading to severe development and growth impairment. Therefore, the *BpMADS2* and *BpMADS8* promoters seem to be very promising from the point of view of biotechnological applications. The corresponding *BARNASE* constructs could be used to induce male sterility or total prevention of flower development. The highly stamen-specific promoter elements of *BpMADS8* should be studied in more detail. When fully characterized, these elements could be used to engineer artificial promoters, which could be used to target transgene expression in the late stamen development.

2.2.3 Acceleration of flowering

The shortening of the juvenile phase of birches is possible by means of conventional breeding (Stern, 1961), or by altering conditions of culturing (see e.g., Meilan, 1997). The problem of conventional breeding is that the desired properties do not necessarily accompany the short juvenile period.

For example, the early-flowering birches bred by Stern (1961) were hardly suitable starting material for the breeding program for improving of birches because of their undesirable, shrubby growth habit. However, for the flowering studies, the early-flowering clones are valuable model plants.

The alteration of environmental conditions to accelerate flowering is a valuable tool in acceleration of birch breeding. Even the cultivation in the ordinary greenhouse shortens the juvenile period up to about 2 years (Viherä-Aarnio and Ryynänen, 1995), depending on the clone. By growing birches in continuous light and elevated CO₂ concentration the juvenile period has been reduced to few months (Holopainen and Pirttilä, 1978). However, the amount of acceleration of flowering is highly dependent on the genotype, which restricts the selection of different good genotypes.

The use of genetic modification has enabled the acceleration of flowering, at least in principle, regardless of genetic background. In trees, the target genes for genetic modification of flowering have been studied in many species, for example, in poplars, citrus, birch, eucalyptus, spruces, and pines (for a review, see Lemmetyinen and Sopanen, 2004).

The first demonstration of the transgenic acceleration of flowering in trees was achieved by the overexpression of *Arabidopsis* gene, *LEAFY* (*LFY*), which caused flower formation in hybrid aspen only after a few months (Weigel and Nilsson, 1995). In this case, however, the plants did not form normal inflorescences (catkins), but formed single male flowers. These flowers were sterile because the anthers did not open. In other poplars, the overexpression of *PTLF*, the poplar homologue of *LFY*, resulted in only a limited effect on flowering time (Rottmann *et al.*, 2000). In birch, the effect of overexpression of birch homologue of *LFY*, *BpFLO*, was dependent on birch clone (Lemmetyinen *et al.*, unpublished results).

The overexpression of *API* in citrus showed that the expression of this gene is sufficient to lead to the formation of normal, fertile inflorescences (Peña *et al.*, 2001). In birch, there are three characterized genes belonging *API/FUL* group, *BpMADS3*, *BpMADS4*, and *BpFULL1* (Elo *et al.*, 2001). They all have been effective in acceleration of flowering, and the overexpression of any of them resulted in early flowering in tobacco. The most

effective birch gene in this group was *BpMADS4*. On the basis of sequence comparison, the most similar gene in *Arabidopsis* is, however, a root-specific gene, *AGL79*, the function of which is still unknown (Parenicová *et al.*, 2003). The overexpression of *BpMADS4* in tobacco resulted in very early flowering, and the structure of whole plant resembled inflorescence.

Similarly, the overexpression either of *BpMADS3* or *BpMADS4* resulted in early flowering in birch (Figure 5) (Elo *et al.*, 2007; Lemmetyinen *et al.*, unpublished results). While the plants overexpressing *BpMADS3* required a certain size before they started abundant flowering, the plants overexpressing *BpMADS4* started flowering even just after they had been transferred onto soil. Therefore, it seems that *BpMADS4* is involved in regulating the transition from juvenile phase to adult phase. The use of genes regulating the development of floral structures may, however, be problematic in the acceleration of flowering, because they may also cause some degree of sterility. This was also detected in the birches overexpressing *BpMADS3* or *BpMADS4* (Elo *et al.*, 2007; Lemmetyinen, unpublished results). Therefore, the use of the genes regulating flowering time exclusively might be more appropriate. Recently, promising results have been obtained



Figure 5 Early-flowering birch: early flowering was obtained by overexpressing *BpMADS4* in normally flowering birch clone (arrow indicates the male inflorescence)

from poplar by the overexpression of poplar homolog of flowering time gene *FLOWERING LOCUS T* (*FT*), *PtFT1*, which resulted in very early formation of inflorescences (Böhlenius *et al.*, 2006).

2.3 Resistance against Fungal Diseases

In general, birch is susceptible to detrimental pathogens only when the growth starts to decline at the age of 45–60 years. Silver birch is mainly rotted by *Polyporaceae* fungi, which start the rotting of timber in the heartwood and then proceed to the living sapwood (Uotila, 1987). Birch leaf rust, caused by the fungus *Melampsorium betulinum*, is the most important leaf disease both in plantations and in natural birch forests in Fennoscandia (Poteri and Rousi, 1996). Leaf rust causes yellowing and premature falling of the leaves, but it does not affect the wood. However, if leaf rust epidemics appear in several sequential years, the growth of young seedlings may decrease because of the shortened assimilation period (Uotila, 1987; Vuorinen, 1992). Some pathogenic fungi may cause stem lesions mainly in young birch trees, especially when the bark is damaged by forestry work or other agents or in fringe-area plantations. In young cultivated birch stands, firm rot around the pith is a serious cause of concern (Uotila, 1987). Several different pathogens (both fungi and bacteria) are responsible for this defect. Stem spotting disease caused by a group of fungi (e.g., *Fusarium avenaceum*, *Botrytis cinerea*, *Godronia multispora*, *Cylindrocarpon* spp., and *Alternaria* spp.) is common in nurseries and can be very harmful to young birch seedlings.

Advances in the research on the molecular mechanisms of disease resistance in crop plants has opened up possibilities for testing biotechnological applications for increasing disease resistance also in forest trees. These applications include introduction of genes encoding antimicrobial recombinant proteins of plant or nonplant origin into trees (e.g., Peña and Séquin, 2001). The possibilities to improve fungal disease resistance in silver birch have been tested by constructing and studying transgenic birch lines carrying a chitinase IV gene from sugar beet. Chitinases catalyze the hydrolysis of chitin, a β -1,4-linked polymer

of N-acetyl-D-glucosamine, which is the major component of the cell wall of most filamentous fungi (Bartnicki-Garcia, 1968). Hydrolysis of the chitin, found in the fungal hypha, by chitinases kills the fungus without causing damage to the plant cells. Sugar beet chitinase IV is a basic endochitinase belonging to class IV chitinases that are secreted extracellularly and deposited in the apoplast (Neuhaus *et al.*, 1991; Mikkelsen *et al.*, 1992). Sugar beet chitinase IV has been shown to inhibit the growth of the conifer pathogen *Heterobasidion annosum* *in vitro* (Susi *et al.*, 1995). In other studies, class I and V chitinases have been shown to inhibit the growth of various fungal species *in vitro* (e.g., Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988; Arlorio *et al.*, 1992; Melchers *et al.*, 1994; Vierheilig *et al.*, 2001). Overexpression of chitinase genes has increased fungal disease resistance in many plant species (e.g., Broglie *et al.*, 1991; Grison *et al.*, 1996; Asao *et al.*, 1997; Lorito *et al.*, 1998), but so far the approaches employing transgenic chitinases in trees are in early stages of development.

Sugar beet chitinase IV gene with the enhanced (4 \times) CaMV 35S was transferred to silver birch through *Agrobacterium*-mediated gene transfer as described in Keinonen-Mettälä *et al.* (1998). The resistance of chitinase transgenic silver birch lines against fungal diseases was tested in a greenhouse and in a field trial. In the greenhouse experiment, the chitinase transgenic lines with high sugar beet chitinase IV expression showed improved resistance to the leaf spot fungus *Pyrenopeziza betulicola* (Fuckel) (Pappinen *et al.*, 2002). However, in a 3-year field trial, the chitinase transgenic birch lines did not differ from the control, or were more susceptible to the leaf spot fungus than the wild-type control trees (Pasonen *et al.*, 2004). The contradictory results from the greenhouse and the field trial may be due to the fact that only a high inoculum of one *P. betulicola* isolate was used to infect the plants in the greenhouse, while natural infection in the field consisted of various genotypes of the same pathogen. Also, several biotic and abiotic factors to which the plants were exposed in the field trial, but not in the greenhouse, have most likely influenced the fungal disease resistance of the studied birch lines. However, the results from the field trial revealed that some resistance against

the birch rust fungus *M. betulinum* was achieved (Pasonen *et al.*, 2004).

2.4 Insect Herbivory and Mammalian Herbivores

Silver birch is one of the most important food plants for insects in Finland (Annala, 1987). Of geometrid defoliators using silver birch as a resource, *Operophtera brumata* is among the most voracious species (Tikkanen *et al.*, 2000). The damage may be severe after the insect outbreak, but usually climatic conditions enable birch to compensate the damage by regrowth. Beetles may damage trunks of mature trees and thereby reduce the economical value of the timber (Raulo, 1981). For instance, *Hylecoetus dermestoides* and *Trypodendron signatum* bore tunnels in logs and timber. *H. dermestoides* can also attack living trees and a simultaneous infection by rot fungi can be fatal to the tree. Larvae of the birch cambium fly, *Phytobia betulae*, mines in the birch wood near the cambium layer and cause 1–4-mm-wide brown streaks in the wood (Annala, 1987). The streaks decrease the value of birch wood used in plywood and furniture industry, but the damage is purely aesthetic; the mechanical strength of the wood is not affected.

Birches are important winter food for many herbivorous animals, such as moose (*Alces alces*), hares (*Lepus*) and voles (*Microtus*, *Clethrionomys*) (Rousi *et al.*, 1989, 1990; Tahvanainen *et al.*, 1991). Voles cause considerable damage to forest plantations, especially during the peak years of density fluctuations, every third or fourth year. Voles destroy birch seedlings under snow cover and they can eat the bark of the seedlings until the basal diameter reaches 4 cm (when the seedling is about 5 years old). Hares feed on the upper branches of young birch seedlings and especially in a winter time the damage can be fatal for the seedlings. Moose browsing on young birch seedlings occurs throughout the year and can cause serious damage to birch plantations.

Although herbivory is clearly an important biotic stress factor causing reduction of growth and economical value of timber, the only biotechnological application reported so far appears to be the transformation of spider insecticidal

peptide gene into *B. platyphylla* in China (Zhan *et al.*, 2003). Insect feeding tests with *Lymantria dispar* showed that the development of the larvae was significantly retarded, but no information of possible field trials has been reported.

2.5 Wood Quality

Lignin is an undesirable wood component in pulp and paper industry, and manipulations of the content and subunit composition of lignin are among the most intensively studied biotechnological applications in forest trees. The extensive use of birch roundwood for chemical pulp emphasizes the need for understanding the wood properties and characteristics of lignin biosynthesis in birch (Aronen *et al.*, 2003). Lignin precursor biosynthesis involves a complex array of *O*-methyltransferases controlling the production of differentially methylated monolignols, which give rise to hydroxyphenol (H), guaiacyl (G), and syringyl (S) subunits of lignin (Boudet, 2000). Lignin accumulation and subunit composition can be biotechnologically altered by regulation of relevant enzymes by antisense, sense, or sense co-suppression techniques (Li *et al.*, 2003). The current knowledge emphasizes the essential role of 4-coumarate:coenzyme A ligase (4CL) (Hu *et al.*, 1999; Harding *et al.*, 2002) and caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) (Zhong *et al.*, 2000) in lignin biosynthesis.

Aronen *et al.* (2003) have studied the role of caffeate/5-hydroxyferulate *O*-methyltransferase (COMT) in lignin biosynthesis in birch. The constructs pRT99/35S-PtCOMT and pRT99/UbB1-PtCOMT carrying a bispecific caffeate/5-hydroxyferulate *O*-methyltransferase gene from *Populus tremuloides* were transferred into birch by using a biolistic microprojectile bombardment (Valjakka *et al.*, 2000). The aim of the transformation was to increase COMT activity and thus the amount of more easily degradable S units of lignin. COMT has also been reported to have 5-hydroxy-coniferaldehyde *O*-methyltransferase activity catalyzing the diversion of guaiacyl monolignol intermediates into syringyl types (Li *et al.*, 2000). Two transgenic 35S CaMV-PtCOMT and UbB1 (ubiquitin promoter from sunflower)-PtCOMT lines were

regenerated. The Klason determination of the 2-year-old stem material revealed no considerable alterations in the lignin content in three transgenic lines when compared to the control plants but in two lines the S/G ratio was reduced by 69–72%. Alterations in the S/G ratio were not detected in either of the UbB1-PtCOMT lines, but in one line the Klason lignin content was decreased. Aronen *et al.* (2003) concluded that the introduction of the sense construct of the *PtCOMT* gene into birch did not lead to the expected alterations in lignin composition but the results provide an insight into possibilities how lignin biosynthesis in birch can be modified by affecting the functioning of individual genes.

Seppänen *et al.* (2006) isolated a birch *4CL1* gene fragment and used it for antisense transformation of silver birch. The objective was to study whether the lignin content could be lowered and the subunit composition could be modified by *4CL* antisense transformation. Birch *Bp4CL1* complementary DNA (cDNA) was cloned in antisense orientation downstream of a 35S CaMV promoter in the pBI121 binary vector containing the *nptII* (neomycin phosphotransferase II) gene for kanamycin selection. *Agrobacterium* strain C58C1 (pGV2260)-mediated gene transfer (according to Keinonen-Mettälä *et al.*, 1998) with a binary vector system was used to introduce the *Bp4CL1* gene into birch. The transformation yielded three polymerase chain reaction (PCR) and Southern blot positive lines, which were studied in more detail. Lignin content was determined according to TAPPI-T 222 method, which separates the acid-insoluble fraction of lignin (Klason lignin) and the acid-soluble lignin. Quantitative analysis of the four most important structural units of lignin, β -O-4, β -5, β - β , and dibenzodioxocin was done using two-dimensional NMR spectroscopy as described in Heikkinen *et al.* (2003). Chemical composition of the stem wood of two of the lines did not differ from that of the control plants in the view of total lignin and cellulose contents, and monosaccharide and polysaccharide compositions. One line contained slightly more lignin and less cellulose compared to the other transgenic lines and the control. This line showed also significantly slower growth rate compared to the control plants. Seppänen *et al.* (2006) concluded that the antisense transformation of birch with *4CL* gene did not lead to expected

changes in wood chemistry but, instead, resulted in changes in growth characteristics, especially in root biomass and morphology.

2.6 Phytoremediation

Phytoremediation has received increasing attention in recent years and the possibilities to use genetic engineering in the improvement of plants for phytoremediation have been studied (for a review, see e.g., Kärenlampi *et al.*, 2000; Eapen and D'Souza, 2005). In addition to large size, forest trees have an extensive root system, which enables an efficient uptake of pollutants from the soil. The falling of leaves of deciduous trees in autumn makes the collection of the pollutant concentrated from soil easy.

Birch is quite tolerant to some toxic compounds found in contaminated soil and therefore, its ability to accumulate heavy metals and its suitability in phytoremediation have been studied by many groups (e.g., Eltrop *et al.*, 1991; Österås *et al.*, 2000; Rosselli *et al.*, 2003). Although birch is not considered as an accumulator but as a metal-tolerant plant, it transfers reasonably high concentrations of metals to its above-ground parts (Rosselli *et al.*, 2003). Differences have been found between birch clones in Cu and Zn tolerances (Denny and Wilkins, 1987; Utriainen *et al.*, 1997), and for this reason birch clones have been screened for their suitability to phytoremediation (Kopponen *et al.*, 2001). So far there are no reported transgenic applications on phytoremediation in birch but the results of poplars (Che *et al.*, 2003) indicate that trees form a potential group of plants.

2.7 Other Traits

The growth, in general, and especially the productivity are economically important. The maximum growth is a sum of several factors. Nitrogen metabolism is considered to be one of the critical factors restricting growth. In nitrogen assimilation, nitrate reductase (NR) (and nitrite reductase, NiR) is the central gene. Its regulation has been studied in birch (Friemann *et al.*, 1992; Hachtel and Strater, 2000), and amino acid sequence of its protein has been modified

(Schöndorf and Hachtel, 1995), but there are only a few attempts to improve nitrogen assimilation in birch by genetic modification. A field experiment with transgenic birch altered for carbon and nitrogen metabolism (sense-RbcS and NR lines) was established in year 2000 by Hely Häggman at the Punkaharju Research Station of the Finnish Forest Research Institute, Finland, but unfortunately the field experiment was destroyed in 2004.

Birches are also a source of chemical compounds. Whiteness of *Betula* bark is due to the development of the white wax betulin (Ashburner, 1993b). Betulin is a naturally occurring triterpene found predominantly from the bark of birch trees and it can form up to 30% of the dry weight of the bark (Alakurtti *et al.*, 2006). The high availability of betulin from the birch bark and easiness of its isolation make it a potentially important raw material for some polymers. On the other hand, betulin can be easily converted to betulinic acid, which has been shown to possess a wide spectrum of biological (antifungal) and pharmacological (antimalarial, antitumor, and anti-inflammatory) activities. Thus, modification of the respective biosynthetic routes shows potential for production of promising chemicals for pharmacological applications, but no plans to achieve this have so far been reported.

2.8 Testing: A Need for Field Trials

As sessile organisms, higher plants exhibit a great degree of plasticity in response to environmental stimuli. The regulation of gene expression is the key process for adaptation to changes in environmental conditions, and thus for survival. High genetic diversity means ability to adapt to changing environmental conditions. This is important especially for long-lived species, such as forest trees, that are subject to environmental conditions varying greatly from year to year. GM plants are first tested in greenhouse, but the testing is largely done in controlled environmental conditions. In natural environments, however, GM plants are exposed to conditions that are highly variable and fluctuations are often both irregular and stochastic. Hence, long-term field trials in testing GM trees are highly important. The importance of field trials is also emphasized by the varying results from the greenhouse and

field trial studies as was reported by Pappinen *et al.* (2002) and Pasonen *et al.* (2004).

A 3-year field trial of transgenic silver birch was conducted in Helsinki (Finland) in 2000–2003 in order to produce information that could be utilized in assessing the risks of GM birch. The information collected from the field trial focused on the biological interactions between chitinase-transgenic birch and other organisms, i.e., pathogenic and mycorrhizal fungi, and herbivores. The expression of the sugar beet chitinase IV gene in transgenic birch was stable over the whole duration of the trial (Pasonen *et al.*, 2004). In the field trial, significant variation among the 15 transgenic lines was observed in the growth parameters as well as in parameters linked to general plant condition and leaf phenology. The variation among the lines was greatest in height, diameter, and relative growth rate. It was hypothesised that the differences among the lines could be explained by the differences in the level of sugar beet chitinase IV expression but only little support was found for that. The level of the transgene expression was not detected to have influence on the parameters related to growth or leaf phenology, but instead it had influence on the parameters that are associated with the stress status of the tree. The significant effect of the level of sugar beet chitinase IV expression on the color value and general condition of the trees, and the increased levels of red color and the lower value for general condition of the transgenic plants compared to the control plants may indicate ecophysiological stress among the transgenic trees (Pasonen *et al.*, 2008).

Long-term field and greenhouse experiments were also established in Joensuu (Finland) in 2005 in order to clarify putative environmental risks associated with GM birch (Figures 6a and b). In this field experiment, M. Keinänen and his research group have been studying potential risks related to the establishment of plantations of silver birch GM for prevention of flowering, using *Bp-FULL1::BARNASE* plants. The field performance of the plants, growth and architecture, resistance to herbivores (Figure 7), and other stresses, as well as consequences for the soil consumer community are monitored in a regular basis during the growing season. In addition, potential changes in chemical composition and expression of the antibiotic marker gene, *nptII*, are analyzed.

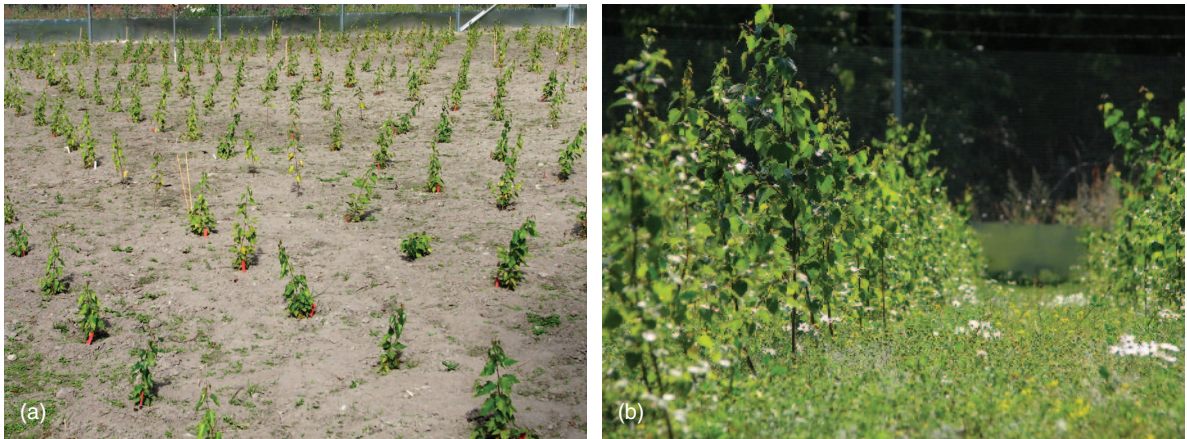


Figure 6 A field trial of nonflowering birches established in Joensuu (Finland) in 2005 (the field trial was established in order to study the stability and functionality of *BpFULL1::BARNASE* construct and environmental risks associated with genetic modification and nonflowering): (a) the field in summer 2005 and (b) in summer 2006

3. FUTURE ROAD MAP

3.1 Future Prospects

At the moment, research work on GM silver birch is for the most part basic, aiming to further understanding of the functioning of genes that regulate, for example, flowering, disease resistance, and lignin biosynthesis. A research consortium led by Tapio Palva at the University of Helsinki, Finland has recently established a large expressed sequence tag library from silver birch, which will be used as a basis for birch oligonucleotide array chips (M.K. Aalto and E.T. Palva, personal communication). This will undoubtedly enable further genomic studies on birch and enhance its potential as a deciduous woody model plant. In contrast to *Populus*, birch is monoecious and lines with lowered self-incompatibility have been found (Atkinson, 1992), which enables the production of homozygous lines.

Scenarios concerning the future use of GM trees vary according to region and prevailing forestry practices. In addition to the possible environmental risks and our limited ability to predict those risks, there are many other problems that will possibly hinder GM silver birches from large-scale forestry use in practice. Long rotation time of many forest tree species including silver birch pose considerable challenges for the confirmation of the long-term expression



Figure 7 In the field test of nonflowering birches, the herbivory is among the subjects studied. The larva of *Epirrita autumnata* eating a birch leaf

of introduced genes despite the fact that the expression of many transgenes would only be required in a specific tissue or at limited stage of the life cycle. Silver birch is well adapted to different latitudes and altitudes and has ecotypes that have different requirements for seasonal temperature variation. Taking the adaptation of silver birch especially to local thermal conditions into account, the use of GM silver birch in practical forestry would require a wide genetic background for the cultivated GM trees. In current research, transgenic lines are usually regenerated only from a couple of clones. Hence, it is not thoroughly known how much variation is there among the genotypes in their predisposition to genetic transformation. Furthermore, the sociological aspect of biotechnology should also be taken into account before considering the use of GM trees in forestry. It is reasonable to ask what are the main ethical views and concepts of the use of biotechnology and what kinds of public concerns, attitudes, and perceptions there are on the issue of using GM trees.

3.2 Addressing Risks and Concerns

Gene technology provides a new powerful tool for speeding up breeding of forest trees and solving problems connected to stress physiology, disease resistance, and yield and quality of the wood. On the other hand, the resolved problems may also create new problems that need to be solved before GM trees can become an integral part of our ecosystem. The potential problems associated with the use of GM birches include the changes in the consumer community caused by altered quality of the birch as a resource. Birches are important food plants for mammalian herbivores, such as moose, hare, and voles (Rousi *et al.*, 1989, 1990; Tahvanainen *et al.*, 1991), and numerous insect herbivores (Annala, 1987; Shaw, 1984; Pritinen *et al.*, 2003). Genetic modification of a plant may cause changes in the structure, function, and diversity of the community of organisms inhabiting the GM plantation. This may be due to changes in the palatability of the modified plant or, for example, due to the absence of flowers, pollen, and seeds in the transgenic plantation. Establishing plantations composed mostly or

entirely of GM trees can be expected to affect the abundance of these consumers and the species interacting with them. Furthermore, studies on life history trade-offs indicate costs of reproduction in various organisms (Roff, 1992). Thus, plants modified for prevention of reproduction can be expected to have additional resources for growth and defenses against environmental challenges, such as herbivory. Palatability of plant tissues is considered to be related with the quality and quantity of secondary metabolites in them (Hartley and Jones, 1997). Both growth rate and the level of defense may alter plant palatability for herbivores and furthermore, change the performance of herbivores and their population dynamics in a way that could cascade through the entire community. Changes in plant palatability may also affect leaf litter decomposition and nutrient mineralization in soil, thus potentially having far-reaching effects in the forest ecosystem. These aspects are currently being investigated in a field trial of young non-GM birch stands manipulated for the presence of seeds and inflorescences by the research group of M. Keinänen at the University of Joensuu, Finland. Both below- and above-ground consumer communities are sampled to reveal long-term effects cascading throughout the food webs.

3.2.1 Nontarget effects of chitinase transgenic birch

In nature, trees interact with a broad spectrum of organisms, and GM trees may have both direct and indirect effects on other species. In boreal soil ecosystems, forest trees form symbiotic associations with a number of ectomycorrhizal fungi that facilitate nutrient supply and provide protection against pathogens (Smith and Read, 1997). Moreover, beneficial mycorrhizal and saprophytic fungi contain chitin in their cell walls and may be highly sensitive to transgenic chitinases or overexpression of plants' own chitinases. The ability of transgenic birch to form ectomycorrhizae, when constitutively expressing sugar beet chitinase IV, was tested with the common ectomycorrhizal fungus, *Paxillus involutus* (Batsch) Fr. (Pasonen *et al.*, 2005). The results showed that all the transgenic birch lines were able to form normal ectomycorrhizae

containing distinctive mantles and Hartig nets, and the level of sugar beet chitinase IV expression had no influence on mycorrhizal colonization.

Below-ground food webs are composed of dynamic communities that are closely interlinked with the above-ground systems (Wardle *et al.*, 2004). While saprophytic microbes form the basis of soil decomposer food webs, soil fauna stimulate microbial activity by grazing on soil microbes and secreting N-rich excrements in the decomposing matter (Setälä, 2002). The decomposition process of the leaf litter from transgenic chitinase birch lines was studied in a field trial (Vauramo *et al.*, 2006). The mass loss of the litters was measured after 8 and 11 months of the establishment of the experiment. Mass loss of chitinase transgenic leaf litter did not differ from that of the control plants. Similarly, no differences in the fungal (litter ergosterol content) or total microbial biomass or activity between the transgenic and control plants were detected. A single transgenic line showing high chitinase expression differed significantly from the control in the mean number of nematodes. The nematode population in this litter showed distinct temporal dynamics compared to the controls, thus indirectly indicating microbial differences in the litter (Vauramo *et al.*, 2006). Kotilainen *et al.* (2005) reported a negative response of nematodes to chitinase transgenic leaf litter but a positive effect on the numbers of collembolans in a microcosm experiment.

The attractiveness of chitinase transgenic birch to two common insect herbivores, aphids (Homoptera: Aphididae) that feed on leaf fluids and to a cambium miner, *P. betulae* (Diptera: Agromyzidae) larvae of which cause an aesthetic defect to birch wood, were monitored in a field trial. In general, more aphids were found from the transgenic trees than from the control trees. A more detailed analysis of insect densities revealed that aphids were the most abundant group of insects in all trees, and the differences in the aphid densities corresponded to the differences in total insect densities (Vihervuori and Lyytikäinen-Saarenmaa, unpublished results). The variation in the occurrence of *Phytobia* larvae in the transgenic and control trees was not connected to the transgene expression but was mainly explained by plant size (Pasonen *et al.*, 2008).

3.2.2 Nontarget effects of birch genetically modified for lignin biosynthesis pathway

While genetic engineering may improve the quality or quantity of wood, lignin modifications can have potential ecological impacts by influencing the palatability of leaves to herbivores, the resistance of trees to pathogens, and the decomposition of plant residues by soil organisms (Pilate *et al.*, 2002). The effects of the modification of lignin biosynthesis pathway in birch on the feeding preferences of five common insect species that feed on birch leaves, larvae of the moths *Aethalura punctulata* (Denis and Schiffermüller), *Cleora cinctaria* (Denis and Schiffermüller), and *Trichopteryx carpinata* (Barkhausen) (Lepidoptera: Geometridae), and adults of the leaf beetles *Agelastica alni* (L.) (Coleoptera: Chrysomelidae) and *Phyllobius* spp. (Coleoptera: Curculionidae) were studied by Tiimonen *et al.* (2005). Transgenic birch lines showed decreased S/G ratio in stems and leaves. The results from the controlled feeding experiments revealed that *A. punctulata* and *C. cinctaria* showed some feeding preference to the leaves of some transgenic lines but the differences could not be directly associated to lignin modification. The other tested species did not show any preference to different types of the leaves and the relative growth rate of the tested herbivores did not vary between the transgenic and control leaves.

Fungi and bacteria that act as main primary consumers of decomposable materials in soil may be highly sensitive to changes in lignin content and composition of the decomposing plant material. The effects on decomposer subsystem can be far reaching because decomposers indirectly regulate plant growth by determining the supply of available nutrients (Wardle *et al.*, 2004). Seppänen *et al.* (2006) studied the effects of the *4CL* antisense transformation of silver birch on the interactions between transgenic birch and ectomycorrhizal fungus *P. involutus*, and the decomposition of the transgenic leaf litter. The transformation was reported to cause no marked changes in wood chemistry but to have pleiotropic effects on root biomass and morphology. Despite the disturbed root formation, the transgenic birch lines formed normal ectomycorrhizas with *P. involutus* and

were equally well colonized by the mycorrhizas *in vitro* when compared to the nontransgenic control plants. In a field trial, the decomposition of the transgenic and wild-type birch litter was equally rapid, and no marked differences were found between the transgenic lines and their controls in total microbial biomass or activity. The effects of the genetic transformation on the ergosterol content of the leaf litter were contradictory; the litter from one transgenic line had significantly higher ergosterol content, and the litters from two other transgenic lines had significantly lower ergosterol content than the litter from wild-type birch (Seppänen *et al.*, 2006).

3.2.3 Risk from gene flow

If fertile transgenic trees would be tested in long-term field trials or cultivated in plantations, transgenes could spread in an uncontrolled way via pollen and seeds to wild populations of the same or related species. For the present, ecological consequences of the spreading of transgenes are not well known. The possibility that transgenic plants would hybridize with wild-type plants of the same species, and finally introgress into native populations, is one of the most frequently mentioned threats from the use of GM plants (Conner *et al.*, 2003). Many ecological and genetic factors will finally determine how easily seeds or pollen migrate from one population to another and whether hybridization and genetic introgression occur. Gene flow and introgression from GM plants to wild-type plants have mainly been studied in *Brassica* (Rieger *et al.*, 2002; Warwick *et al.*, 2003) and other herbaceous plants (Watrud *et al.*, 2004). However, empirical data on the hybridization abilities or gene flow among GM trees is not available. Considering the relative ease of massive long-distance transport of pollen and seed of silver birch and other forest trees, broad geographic ranges and high levels of natural gene flow among populations, the release of pollen or seeds from GM trees could have further-reaching consequences than from crop plants. Furthermore, many agricultural crops are selfers while most forest trees, including silver birch, are outcrossers, and have relatively weak reproductive barriers making them relatively prone to genetic pollution.

Hence, prevention of flowering or induction of sterility would provide a solution to the problems caused by the risk of the spreading of transgenes into natural birch populations.

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Douglas Fir

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The conifers are a small but ancient group of seed plants. They originated over 200 million years ago (Mya) and were a dominant form of vegetation during the Mesozoic period and were represented by many families and an unknown number of species. Presently, they are represented by only 5 to 8 families, 68 genera, and 629 species (Farjon, 1998). The number of families varies because recently some families have been combined (Cupressaceae and Taxodiaceae into the Cupressaceae) and others have been split. The commonly recognized families now and their time of origin are as follows: Podocarpaceae with 18 genera and 184 species and originating about 223 Mya; Araucariaceae with 3 genera and 41 species and originating about 215 Mya; Pinaceae with 11 genera and over 200 species and originating about 200 Mya; Cupressaceae with 28 genera and 135 species and originating about 200 Mya; Taxaceae with only 5 genera and 22 species and originating about 200 Mya; and Cephalotaxaceae with but 1 genus and 10 species and originating about 160 Mya. Conifers are predominately north temperate in distribution with the Pinaceae being strictly in the northern hemisphere and the Araucariaceae being strictly in the southern hemisphere, while

the remaining families may occur in both hemispheres with Taxaceae, Cephalotaxaceae, and Cupressaceae predominately in the northern and Podocarpaceae predominantly in the southern (Farjon, 1998).

Douglas fir (*Pseudotsuga menziesii* Mirb. Franco) is in a small genus (*Pseudotsuga*) currently thought by some to have only four species and three varieties (Farjon, 1998), or more commonly six species (Orr-Ewing, 1966; Thomas and Ching, 1968), but some authors recognize more than 10 species (Martinez, 1963). Species are native to China, Taiwan, Japan, and western North America. *P. menziesii* (Figure 1) is the most familiar and important species, having an enormous north–south range of almost 5000 km, and extending from the Pacific Coast to the eastern slope of the Rocky Mountains (Fowells, 1965) (Figure 2). It is distributed throughout most of the southwestern part of British Columbia, throughout the Pacific Northwest in the United States, and extends southward along the Rocky Mountains and dry interior regions well into Mexico at higher elevations. In British Columbia and the Pacific Northwest, Douglas fir is commonly recognized as having coastal (var. *menziesii*) and interior (var. *glauca*) varieties. It is widely planted as a commercial forest species and especially as an ornamental in many other temperate regions of the world from Europe to New Zealand.



Figure 1 Douglas fir branch and mature seed cones

1.2 Botanical Description

Douglas fir, *P. menziesii* (Mirb.) Franco (Figure 1) is a member of the family Pinaceae, the largest family of conifers. Although, most conifers have

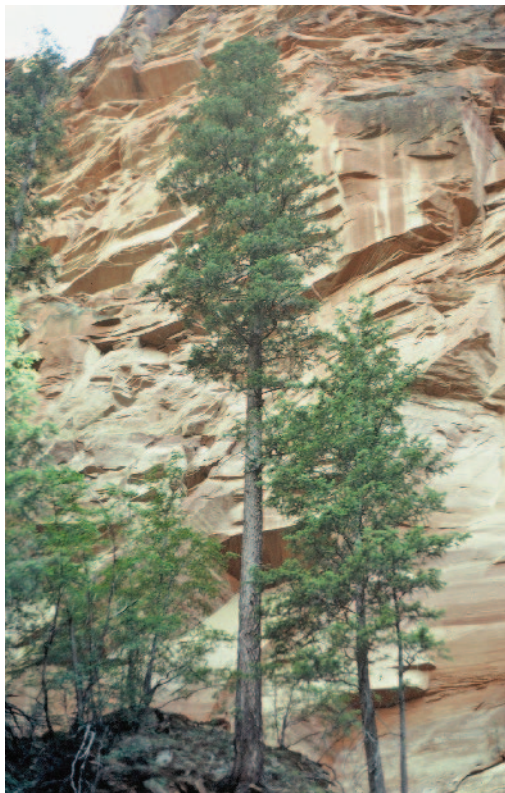


Figure 2 Douglas fir growing at high elevations in the Rocky Mountains of Arizona

24 chromosomes, Douglas fir, as well as other *Pseudotsuga* species, has a $2n$ number of 26 (Thomas and Ching, 1968).

Pseudotsuga macrocarpa (big-cone Douglas fir) is the closest relative and is found in southern California and northern Baja California. At least four additional species have been recognized in Mexico: *Pseudotsuga flahaulti*, *Pseudotsuga guinieri* Flous, *Pseudotsuga macrolepis* Flous, and *Pseudotsuga rehderi* Flous (Martinez, 1963). However, their status as species is questionable and they may only represent extremes in ecotypic variation of *P. menziesii*. Four species are commonly recognized from China, Taiwan, and Japan: *Pseudotsuga forrestii* Craib, *Pseudotsuga japonica* Beissner, *Pseudotsuga sinensis* Dode, and *Pseudotsuga wilsoniana* Hayata (Henry and Flood, 1920).

Douglas fir withstands wide temperature ranges from -30 to 43°C , with an average range of 7 – 13°C where it grows best. Douglas fir can be found in many parts of western North America and is utilized primarily for lumber. It grows rapidly on favorable sites and is very long lived; ages in excess of 500 years are common and some exceed even 1000 years. The older trees can become very large. The largest reported grew in Washington State and measured 4.8 m in diameter and 118 m in height. For satisfactory establishment it requires more light than many of its commonly associated species including western and mountain hemlocks, western red cedar, Sitka spruce, and grand and amabilis firs. Because it always occurs in association with these more tolerant species, it rarely maintains a climax position and is generally recognized as a subclimax species. Its widespread occurrence in even-aged stands results largely from stand destruction by periodic fires, clear cutting, and insect attack or a combination of these factors. If the stands are protected from these disturbances, they would gradually be replaced by the more tolerant western hemlocks, western red cedars, and the true firs. However, its thick bark makes older Douglas fir trees fairly resistant to fires. Common insect pests are the Douglas fir beetle (*Dendroctonus* sp.) and borers (*Melanophila* sp.) that attack trees from the large pole size to maturity killing trees in small patches to very large areas. Several trunk and root rots cause extensive loss and have prevented the replanting of Douglas fir in some areas. These include fungal

species of *Fomes*, *Polyporus*, and *Poria* (Fowells, 1965).

1.3 Economic Importance

The very wide distribution and high quality of lumber derived from Douglas fir make it one of the most important economic species in western North America. The high quality of the lumber from Douglas fir is recognized internationally, and many countries are choosing to grow their own plantations. Its wood is moderately light to heavy, hard, and strong. It is used extensively in construction as beams, studs, lumber, and plywood, as well as interior and exterior finishing. It is one of the best-known softwood timber trees in the world market (Hosie, 1979). Harvest of the species is extensive and in most areas regeneration is not by natural but by planting seedlings.

1.4 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

The two main objectives of Douglas fir breeding are improvement of economic value and maintenance of adaptability (Howe *et al.*, 2006). Adaptability refers to cold and drought tolerance and is usually selected for in the breeding zones. Stem volume is the main determinant of economic value, followed by stem quality and wood properties (Howe *et al.*, 2006).

Although cones are still collected from wild trees and the seed extracted and used to produce seedlings for reforestation, most Douglas fir seedlings are now derived from selected parent trees grown in clonal seed orchards. Over several decades, animal and plant breeding techniques have been adopted, modified, and applied to many forest trees. Compared to agricultural crops, forest tree breeders have had to consider unique ecological, population, and quantitative genetics issues in recurrent selection programs and deployment strategies. Tree breeders have worked with wild populations, requiring that factors such as geographic variation, seed transfer within environmentally similar zones of adaptation, and progeny testing to be taken into consideration (Yanchuk, 2003).

Genetic improvement in trees is a very slow process compared to most agricultural crops, mainly because of the long juvenile growth period, often 10–20 years for many conifers, plus the long reproductive cycle, which may take from 15 to 27 months from cone initiation to seed maturity in different species. Very briefly, the tree improvement process must begin with genetic surveys of natural populations (provenances), the collection of samples from recognized provenances to be planted in several environments to test performance of the local provenances versus others. Superior tree selection based on phenological traits in the wild from within the provenances is then done and scions are collected and grafted to suitable rootstocks. For some species, rooted cuttings may be used to bypass grafting problems that often occur. Once the breeder has assembled a large enough population of selections from the wild (usually several hundred), the progeny from these may be tested. In this way the mother tree's true genetic worth may be tested by evaluating how its offspring perform relative to other offspring from other parents. Progeny testing may have many designs and may be done across many environments and the offspring performance statistically tested. Growth, yield, and genetic gain trials are commonly used. First-generation seed orchards may be established from the originally grafted scions or advanced breeding and testing may be done from the offspring of these first-generation seed orchard trees to produce a second-generation seed orchard in which the genetic gain is higher (Yanchuk, 2003). The time required to reach this second stage may be 20 or more years, depending upon the time it takes for the species to reach reproductive maturity (Figure 3) and the length of the reproductive cycle of the species. Therefore, any techniques that may shorten this time—cultural, physiological, or molecular—would be very helpful to the breeder.

Seed orchards designed for the production of genetically improved seed for reforestation have many inherent problems. They are usually placed outside the natural range of the species to prevent or reduce pollen contamination in the wind-pollinated conifers but this may interfere with good cone or seed production. Pollen cones and seed cones may not be produced in abundance every year, some trees may be poor



Figure 3 Reproductively mature Douglas fir clonal seed orchard

cone producers most years and synchronization of pollen shedding and seed-cone receptivity may be poor. These problems have led to the development of techniques to enhance cone production; collect, extract, store, test, and apply pollen; enhance cone and seed development and survival; harvest, test, and store seeds; and germinate seeds and grow uniform healthy and hardy seedlings for reforestation.

Because reforestation requires a constant supply of high-quality economical seeds, tree improvement programs were begun in the region in the mid 1900s. Although there is a large stock of stored Douglas fir seed collected in the wild in coastal regions, this is gradually being used less. Presently about 50% of the Douglas fir seed used for reforestation comes from Class A (genetically selected and orchard-produced) seeds. As an example, in British Columbia, 32.5 million container-grown coastal Douglas fir seedlings were planted on crown land between 2002 and 2005. With a sowing factor (number of seeds sown to obtain one seedling) of 2 for high-quality seeds, this is about 20 million seeds per year. Similar requirements would be required for private and public lands in the Pacific Northwest. This would be about 200 kg of seed for each of the two regions. Seed production in Douglas fir seed orchards is generally good and there are no major problems, but management can always be improved in order to get more abundant flowering earlier and the highest possible seed-cone survival and filled seed per cone. For good forest genetics, breeding, and seed orchard management, it is important to have a

basic knowledge of the reproductive cycle and the reproductive biology of the species. This is equally important for those doing research in molecular biology, especially if they are directing or doing their own specimen collection and preparation. They must recognize various sporophyte and gametophyte structures and the various stages of their development.

Conventional seed orchards are managed to produce large amounts of superior seeds, they can remain productive over decades and are highly cost effective (Howe *et al.*, 2006). The disadvantages of seed orchards are that genetic gains and financial returns are delayed because there is a long lag (10–15 years) between orchard establishment and quantitative production of improved seed (Howe *et al.*, 2006). An alternative that offers rapid genetic gains and lower costs is the miniaturized seed orchard (MSO). MSOs require less land, can be subjected to controlled pollination, management costs are lower because the crowns are closer to the ground, and seed harvesting may be possible 3 years after grafting (Howe *et al.*, 2006). Vegetative propagation is the strategy of choice for significantly reducing time consumption.

The achievement of conventional orchards is that they are now producing surplus seed and companies in the Pacific Northwest are harvesting and deploying seed from only the best parents.

1.4.1 Reproductive cycle

The wide distribution of Douglas fir makes it difficult to describe the phenology of the reproductive cycle. The occurrence of particular events is strongly affected by temperature and may vary by more than a month in individuals at the extremes of the distribution and by a few weeks in the same individual in different years. Consequently, the phenology given (Figure 3) is that of coastal Douglas fir growing at moderate elevations in the center of its range (Figure 4). Even though the phenology will vary with geographical distribution, the sequence and details of reproductive development remain the same and a single general description of the reproductive cycle is valid (Allen and Owens, 1972).

The reproductive cycle of Douglas fir is similar to most non-*Pinus* members of the Pinaceae in

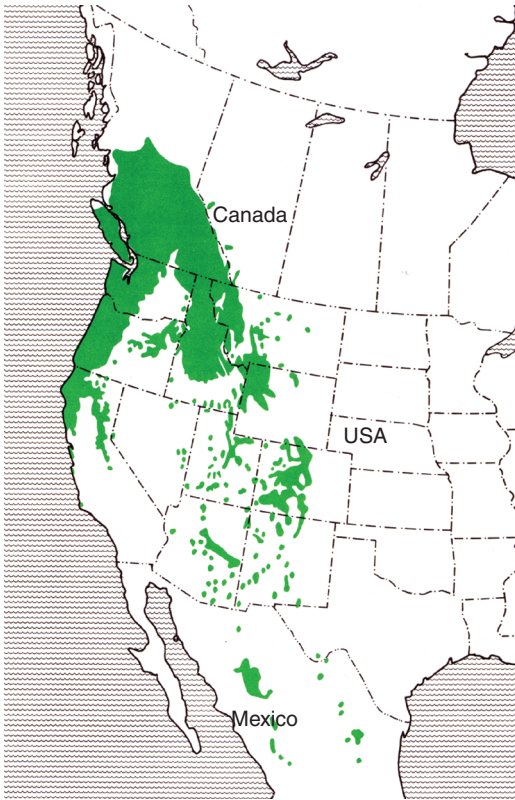


Figure 4 Distribution of Douglas fir

that it extends over about 17 months from cone-bud initiation and differentiation to seed cone and seed maturity. In some conifers such as *Pinus*, there is 1 year between pollination and fertilization, which increases the reproductive cycle to just over 2 years from cone initiation in the late summer and autumn to seed maturity (Allen and Owens, 1972).

1.4.2 Seasonal growth, bud initiation, and bud development

Seasonal growth in conifers is commonly thought to begin when vegetative buds burst in the spring, about mid May in coastal regions. However, considerable growth and development occur within the vegetative buds before bud burst (flushing). It is this growth and development that causes flushing. The vegetative buds of Douglas fir are completely preformed before winter dormancy

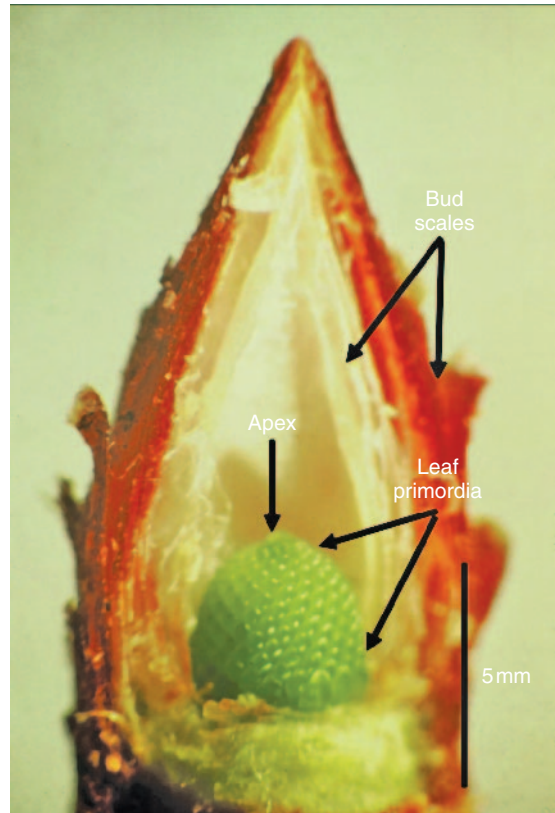


Figure 5 Dissected dormant vegetative bud of Douglas fir (the bud scales enclose the green bud bearing the leaf primordia)

and all leaf primordia for the next season's growth are present (Figure 5).

Vegetative buds resume growth about the last week of March. Cell divisions and cell elongation occurs in the shoot axis and leaf primordia causing the buds to swell. In early April, lateral bud apices are initiated in the axils of some of the leaf primordia. The axil is just distal (above) the point where the leaf joins the stem. These axillary or lateral bud apices initiate bud scales and for about 10 weeks during which time all lateral buds appear the same except for slight differences in size and in their position along the shoot (Figure 6). Potential pollen-cone buds are usually near the base of the shoot, whereas potential vegetative and seed-cone buds are more distal. The terminal buds remain vegetative (Figure 7). After about 10 weeks of bud-scale initiation, the lateral buds begin to differentiate into pollen-cone, seed-cone,

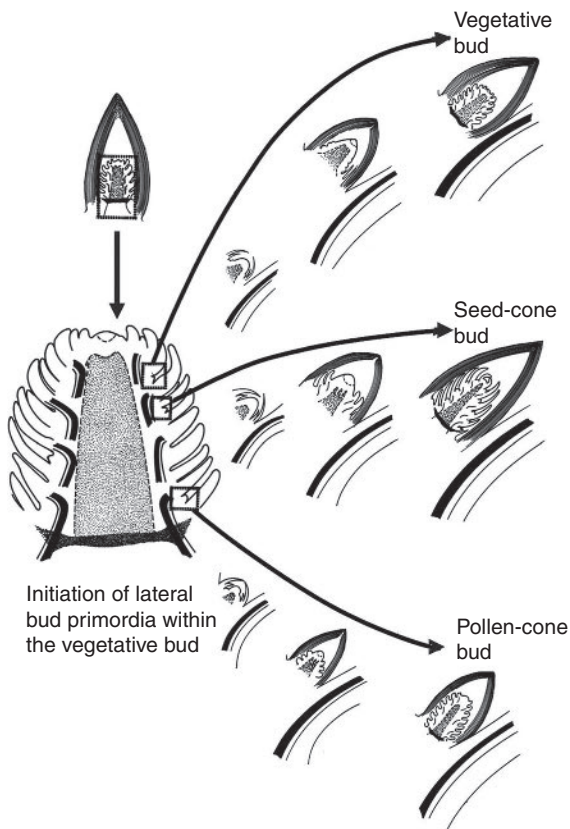


Figure 6 Diagram showing the initiation and pathways of axillary bud development in a vegetative bud on a lateral branch of a sexually mature Douglas fir

or vegetative buds. Pollen-cone buds initiate all of their microsporophylls, seed-cone buds all of their bracts and ovuliferous scales, and vegetative buds all of their leaf primordia before winter dormancy begins late in the fall of the year of lateral bud initiation. Some buds stop development after most bud scales have been initiated and remain as undeveloped latent buds (Figure 7). Vegetative and cone buds are completely preformed and recognizable before winter dormancy, which begins about November in coastal Douglas fir (Figure 7) (Owens and Smith, 1964; Owens, 1969).

Methods for cone induction in young trees in seed orchards have been developed in order to increase cone for breeding purposes and seed production for reforestation. Various cultural techniques have successfully been used including

root pruning, girdling, and fertilizer treatments often in combination with the application of gibberellin A4/7 (Ross *et al.*, 1985; Owens *et al.*, 1986).

1.4.3 Postdormancy cone development and pollination

After winter dormancy, pollen-cone and seed-cone buds resume growth usually early in March, a few weeks before vegetative buds on the same branch. Cone buds develop while still enclosed within the bud scales and pollen-cone buds form pollen and seed-cone buds form ovules. Meiosis occurs in pollen-cone buds late in March soon after they break dormancy and pollen is mature usually by mid April (Owens and Molder, 1971). Seed-cone buds break dormancy at about the same time as pollen-cone buds and form ovules, but meiosis within the ovules does not occur until the time of pollination. Pollen-cone and seed-cone buds burst usually in mid April and pollination occurs during about a 2-week period (Figure 8). At pollination, the pollen cones are pendant, they dry and the microsporangia split open releasing the pollen. At pollination, the seed-cone buds are erect with the long trident bracts are reflexed allowing pollen to enter the cone and pass down to the two ovules that develop on each ovuliferous scale (Figure 9) (Owens *et al.*, 1981).

There are some unusual features of Douglas fir reproduction that make it very different and sometimes more difficult for study and experiments than the pines. Douglas fir pollen is nonsaccate, meaning it has no air bladders, which are the floatation devices for pollen of many other conifers (i.e., pines, spruce, and true firs) (Figure 10) (Owens and Simpson, 1986). The pollen when shed contains less than 10% water content and appears indented due to the drying, and in this dry state it can be stored at -20°C in air-tight containers for many months or years. Mature pollen has five cells as shown with 4',6-diamidino-2-phenylindole (DAPI) staining (Figure 11), but contains no sperm cells—these form by division of the generative cell after pollen tube formation. In contrast to most conifers, when Douglas fir pollen germinates it does not immediately form a pollen tube; so, standard germination tests for viability cannot be used. Tests such as respiration

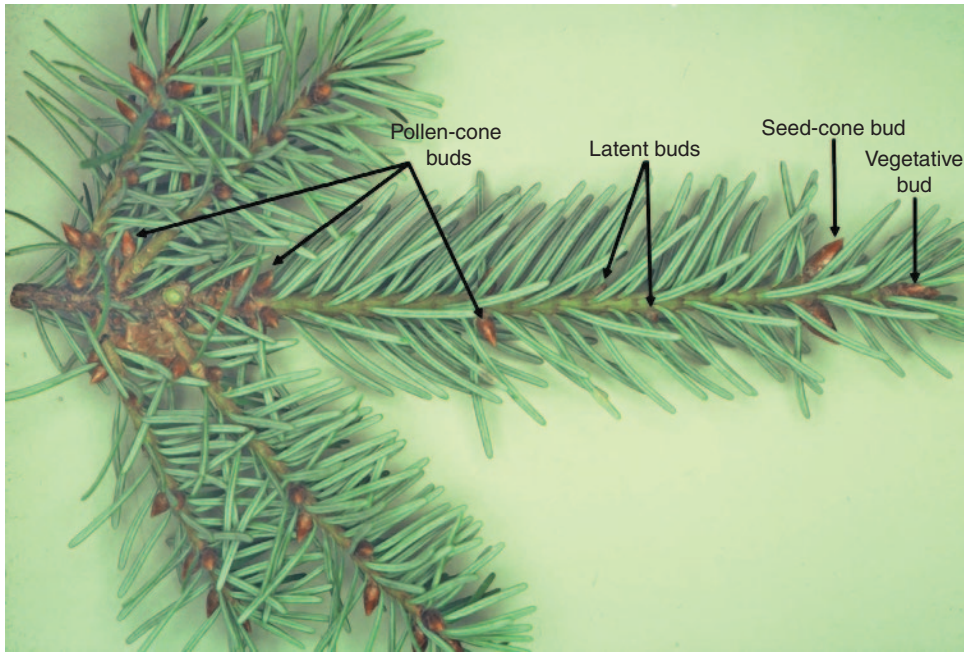


Figure 7 Branch from a sexually mature Douglas fir showing the usual position, size, color, and shape of vegetative lateral, pollen-cone, seed-cone, and latent buds

and electrolyte leakage must be used (Webber and Painter, 1996).

1.4.4 Pollination mechanisms

The pollination mechanism is related to the pollen and ovule structure and Douglas fir (Owens *et al.*, 1981, 1985) and larch (*Larix*) (Owens *et al.*, 1994) are unique in this regard among the conifers (Figure 12). Pollen carried by wind enters the erect and receptive seed cones and moves over the smooth cone surfaces down to the base of the ovuliferous scale, where the two ovules are located. The ovules have an inverted globose tip with a slitlike opening (the micropyle) and many stigmatic hairs to which the pollen adheres. The stigmatic tips capture pollen for several days; then the tip grows inward, engulfing the stigmatic hairs, and the attached pollen into the micropyle (Figure 13). Many other conifers, such as the pines and spruces, have ovules with an inverted two-pronged tip. A pollination drop is secreted by the ovule and emerges from a large oval-shaped micropyle between the two prongs. The

pollen grains, which are saccate, adhere to the sticky prongs and are picked up by the pollination drop. Because of the air bladders the pollen grains float up through the micropyle and into the micropylar canal of the ovule. Viable pollen grains taken into the ovules with the pollination drop germinate within a day or two and pollen tubes quickly form and grow into the nucellus at which time the sperms are formed. Because of this pollination-drop mechanism, pollen germination is a rather simple process in pines, spruce, and true firs (Webber and Painter, 1996). Five pollination mechanisms are recognized in the conifers (Owens *et al.*, 1998) and developmental and molecular studies have been made for conifer pollen tube growth and development (Fernando *et al.*, 2000).

1.4.5 Gamete structure, fertilization, and cytoplasmic inheritance

In Douglas fir, pollen grains that have been engulfed into the moist ovule germinate and elongate down the micropylar canal for about 9

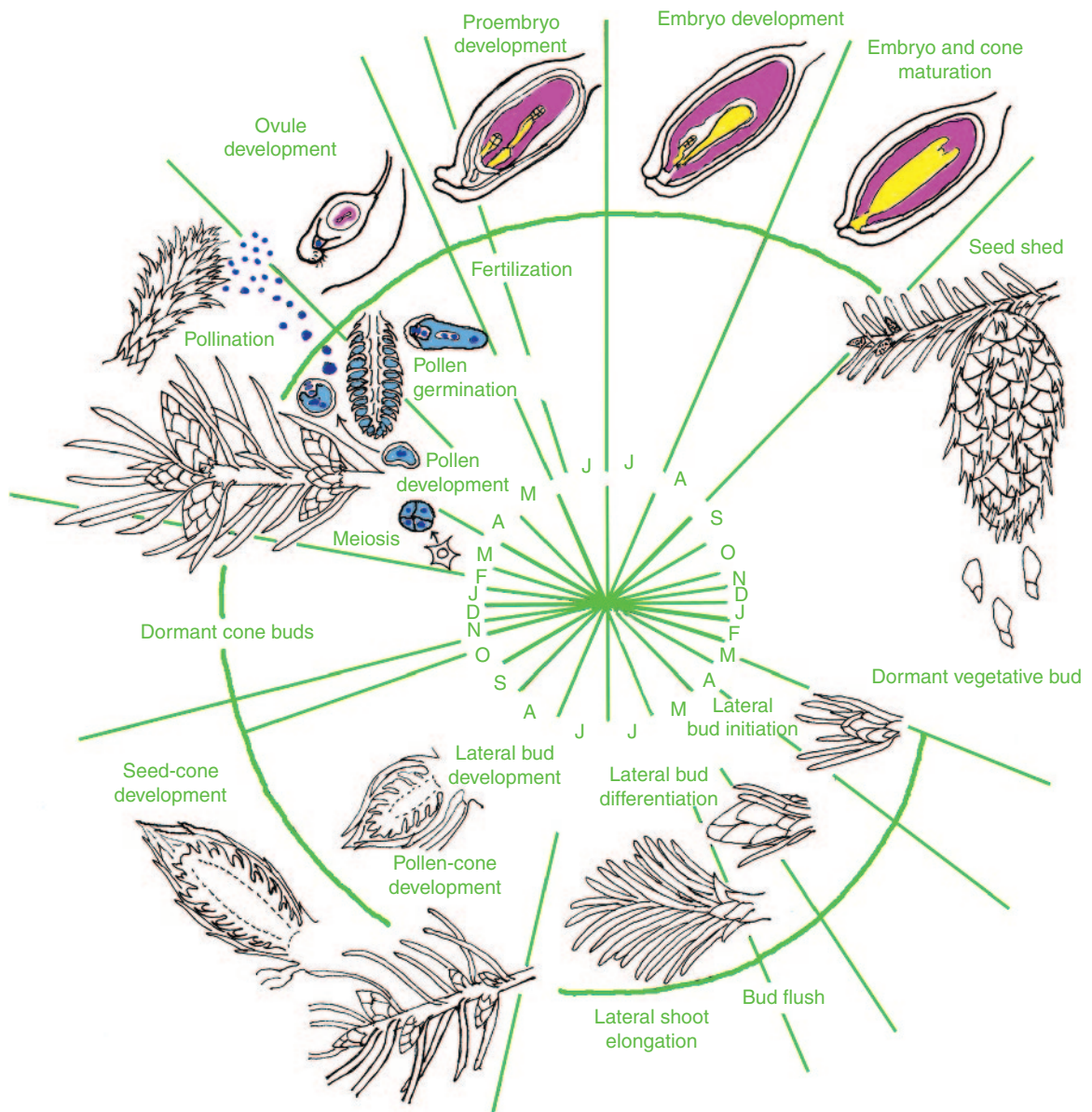


Figure 8 The reproductive cycle of coastal Douglas fir

weeks, until they reach the tip of the nucellus where the elongated pollen forms a narrow pollen tube that penetrates the nucellus (Takaso and Owens, 1994, 1996). During pollen tube growth, the generative cell within each pollen tube forms two equal-sized sperm nuclei that remain within the generative cell cytoplasm (Owens and Morris, 1990, 1991; Fernando *et al.*, 1997, 1998, 2001). The

development and molecular regulation of conifer pollen tube growth are reviewed by Fernando *et al.* (2001). During pollen elongation and pollen tube growth, the megagametophyte tissue in each ovule forms about 2000 cells, 2–6 of which are egg cells. The eggs are large complex cells in which mitochondria aggregate around the egg nucleus and plastids are transformed into large

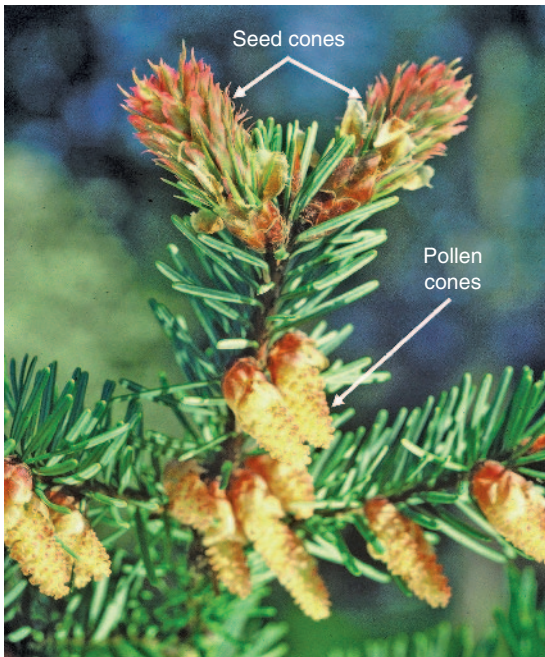


Figure 9 Douglas fir pollen cones and seed cones at pollination in April

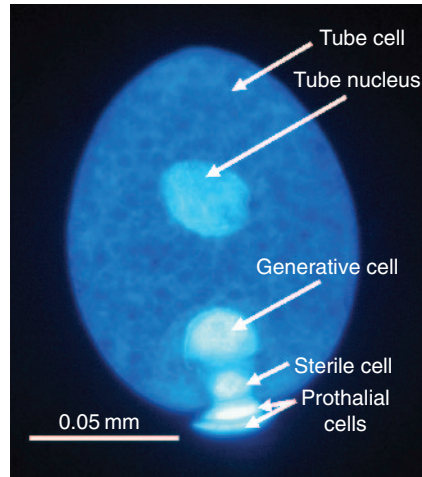


Figure 11 Germinated Douglas fir pollen stained with DAPI (five cells)

dysfunctional vesicular structures, often called large inclusions (Singh, 1978). Each egg is enclosed by an archegonial jacket layer with several neck cells at the tip facing the micropylar canal. Each pollen tube grows to a separate archegonium, penetrates the neck cells, and bursts into the egg cell releasing the two sperms, generative cell cytoplasm, and other pollen tube contents into the egg cell. Variable numbers of eggs may be fertilized in each ovule depending upon the number of pollen tubes growing through the nucellus (Figure 14).

The leading sperm moves toward the egg nucleus and the second sperm trails behind. Between the two sperms, the generative cell cytoplasm, with its abundant paternal mitochondria and plastids, also moves toward the egg nucleus. The leading sperm fuses with the egg nucleus and the trailing sperm degenerates. The paternal mitochondria and plastids intermingle with the abundant maternal mitochondria surrounding the egg nucleus forming the neocyttoplasm of the zygote. Thus, most zygote mitochondria (about 90%) and all plastids are paternal in origin. This type of cytoplasmic inheritance, in which mitochondria are biparentally inherited and plastids paternally inherited, is found in all of the Pinaceae that have been studied thus far (Owens and Morris, 1990, 1991; Morgensen, 1996). The paternal inheritance of chloroplast DNA for Douglas fir has been confirmed using restriction

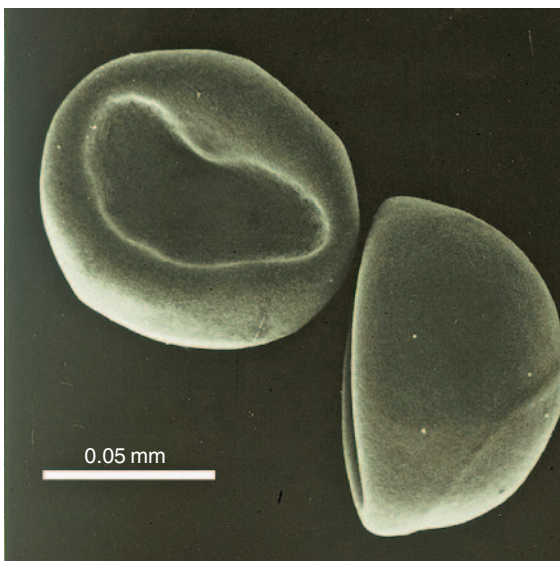


Figure 10 Scanning electron micrograph of mature, dry Douglas fir pollen

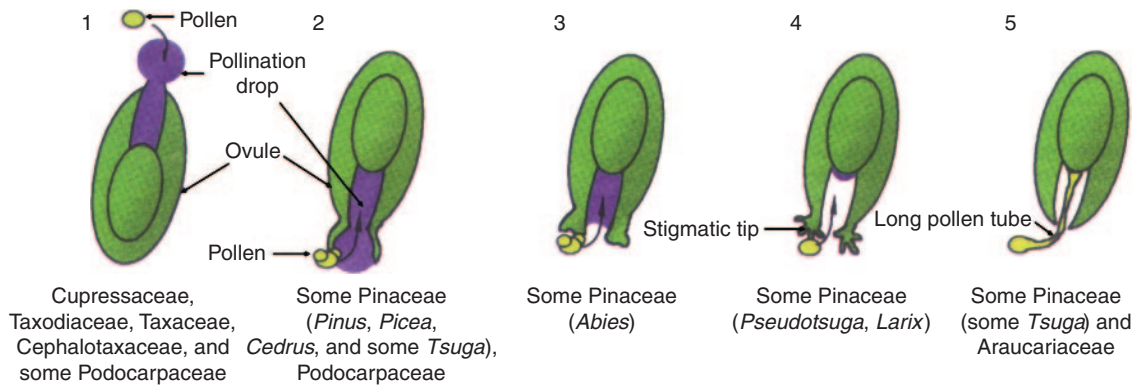


Figure 12 The five pollination mechanisms found in conifers

fragment length polymorphism techniques (Neale *et al.*, 1986). The type of cytoplasmic inheritance varies among conifers with members of the Podocarpaceae, Araucariaceae, and Taxaceae being similar to that of the Pinaceae but members of the Cupressaceae having paternal mitochondrial and plastid inheritance (Bruns and Owens, 2000). The type of cytoplasmic inheritance that occurs in a species can be traced back to the structure and development of pollen, sperms, archegonia, and eggs (Bruns and Owens, 2000), and the fate of the pollen tube and egg organelles at fertilization (Owens and Morris, 1990, 1991; Morgensen, 1996).

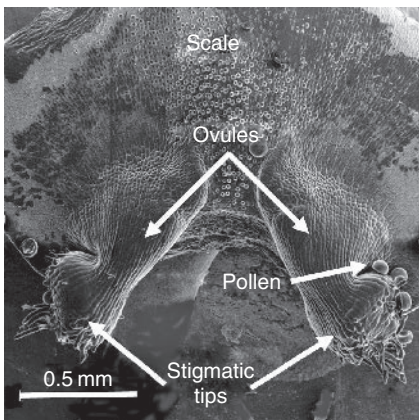


Figure 13 Scanning electron micrograph of the ovuliferous scale of Douglas fir at pollination showing the stigmatic tips with pollen attached

1.4.6 Embryo development

Embryo development begins immediately after fertilization and in conifers can be divided into four stages: proembryo, early embryo, mid embryo, and late embryo (Allen, 1943, 1946, 1947a, b; Allen and Owens, 1972; Owens *et al.*, 1993). The proembryo in most conifers includes the stages of development from the zygote (Figure 14) to the 16-cell proembryo (Figure 14), but in Douglas fir proembryo development stops at the 12-cell proembryo and there is no rosette (dysfunctional suspensor) tier. The early embryo begins when the primary suspensors elongate pushing the apical tier of cells through the archegonial jacket and into the megagametophyte tissue (Figure 14) and includes the elongation of the suspensor system and the formation of a multicellular, club-shaped embryo (Figure 14). The mid embryo includes the rapid enlargement of the embryo and the formation of the root or radicle, shoot, and cotyledons. The late embryo includes the completion of development of these structures and their maturation.

Most conifers, such as the pines, have two types of polyembryony, a process by which several embryos can develop within one ovule. Simple (or archegonial) polyembryony occurs when more than one egg is fertilized in an ovule and each fertilized egg may develop into an embryo. Douglas fir, with four to six eggs per megagametophyte, usually has simple polyembryony if more than one pollen grain enters the ovule. But the second type of polyembryony,

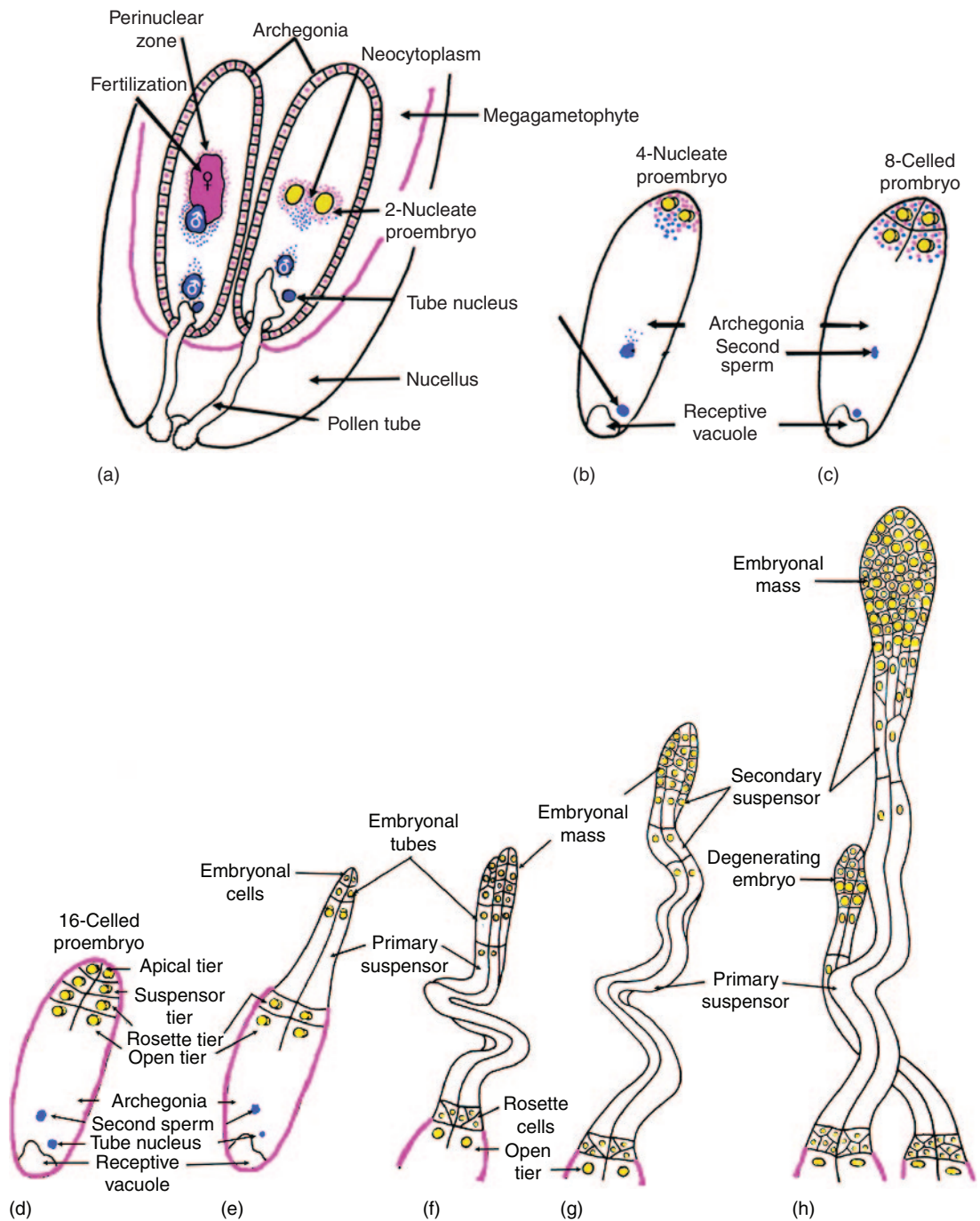


Figure 14 (a)–(h) Diagram showing fertilization, cytoplasmic inheritance, and the proembryo and early embryo stages of development in Douglas fir (sperms and paternal organelles are shown in blue, egg nucleus and maternal organelles are shown in red, and embryo cells are shown in yellow)

cleavage polyembryony, is absent in Douglas fir. In cleavage polyembryony, after the suspensors have pushed the apical tier into the megagametophyte, the apical tier separates into four files of cells and each file can develop into a separate early embryo. This multiplies the number of possible early embryos by four. In simple polyembryony, all of the embryos are genetically different leading to competition among the early embryos. In cleavage polyembryony, there may be several groups of embryos (equal to the number of fertilized eggs) each group with four genetically identical embryos. The advantage of simple polyembryony is obvious but that for cleavage polyembryony is not. Perhaps creating a larger number of genetically identical embryos in an ovule may simply increase the chance for one to survive. However, during early embryo development all but one of the embryos abort leaving usually only one embryo in the seed.

Although cleavage polyembryony does not occur in *Pseudotsuga* and *Picea*, cells in the four files may contribute equally to the embryo, or two files or sometimes only one file divide and elongate more than the others and grow over the others. But, unlike cleavage polyembryony these files do not separate. This has been called delayed, incomplete, or incipient cleavage and is an intermediate form of development between cleavage and no cleavage (Schopf, 1943). The causes for cleavage and incomplete cleavage are not known and the evolutionary relationship is unsettled.

In Douglas fir fertilization occurs at the beginning of June and proembryo development takes place during the next 1–2 weeks within the archegonia. This is followed by another 2 weeks of early embryo development then 2 weeks of mid embryo development. By mid July embryos are small but easily seen in dissected ovules, tissue regions can be recognized within the embryos, and storage products have started to form. Late embryo development begins about mid July and continues until the end of August when embryos are mature (Figures 8, 15, and 16). At first the late embryo fits tightly within the corrosion cavity of the megagametophyte (Figure 16), but as the embryo matures and dehydrates, space forms between them.

Storage products begin to accumulate in the megagametophyte at fertilization and in the embryo at about the mid embryo stage. The

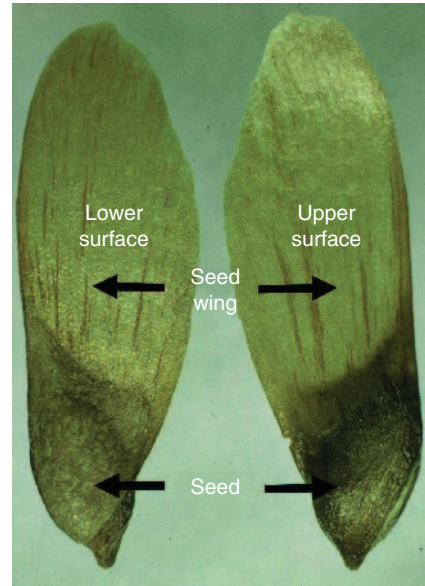


Figure 15 Mature Douglas fir seed

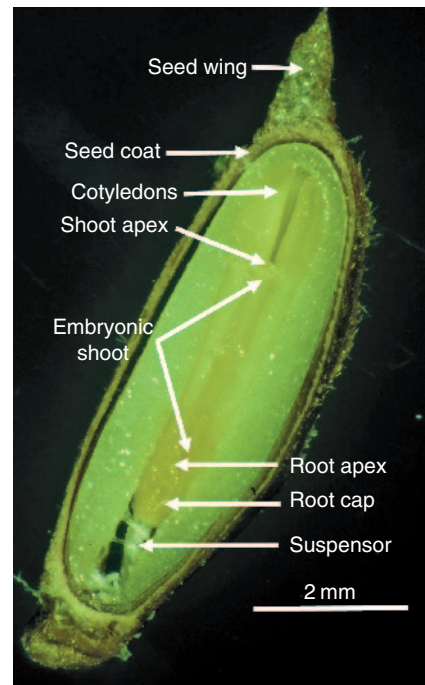


Figure 16 Mature seed from Douglas fir that has been sliced in half longitudinally showing a portion of the seed wing attached to the three-layered seed coat (the seed is hydrated so the diploid embryo fits tightly within the haploid megagametophyte tissue; cotyledons, embryonic root and shoot, shoot apex, and suspensor are visible)

megagametophyte is a maternally derived, haploid tissue. Megagametophyte cells are the primary food storage tissue supplying the developing embryo and at seed germination. Formation of the storage material is by conversion of soluble substances that are present in the megagametophyte at fertilization into complex insoluble storage products (mostly lipids and proteins) in the megagametophyte and embryo in the mature seed. At fertilization megagametophyte cells are small cells with thin walls and contain several large vacuoles, plastids with starch grains, and a few small protein bodies. During early embryo development, some of the starch is broken down, protein bodies enlarge, and become more abundant and many small lipid bodies form. During mid and late embryo development and embryo and seed maturation, protein bodies become very large and more abundant and lipid bodies almost fill the megagametophyte cells (Figure 17a). Most embryo cells also accumulate smaller protein and lipid bodies that fill most of the cells, but some cells of the embryonic root store starch and other specialized cells, such as secretory cells (Figure 17b), and meristematic cells of the shoot apex and procambium, have few storage products.

During embryo development there is a transfer of nutrients from the megagametophyte to the

embryo and a general conversion of soluble reserves to insoluble reserves, mostly as lipid and protein bodies. The dry weight of the megagametophytes more than doubles from the time of fertilization until embryo maturity, at which time embryo dry weight is only about 10% of megagametophyte dry weight. Soluble sugars decrease in megagametophytes and embryos during development. The small amounts of lipids present in the megagametophyte at fertilization become abundant by 6 weeks after fertilization and dominate the storage products in the mature seeds (Figure 17a). In mature Douglas fir seeds, 90% of the lipids are in the megagametophyte and 10% in the embryo. In the megagametophyte and embryo of mature seeds, lipids make up about 60% and 45% of the dry weight, respectively, and proteins make up about 16% and 11% of the dry weight, respectively. At seed maturity, soluble sugars make up only 2% and 3% of the dry weights of megagametophyte and embryos, respectively. These proportions of seed storage product and the abundance of lipid and protein bodies appear to be typical for the *Pinaceae*.

The seed coat begins to differentiate about the time of fertilization and is complete by late August. The seed wing develops from the ovuliferous scale but fuses with the seed coat (Figure 15). Seed cones begin to dry and turn brown by late August, then

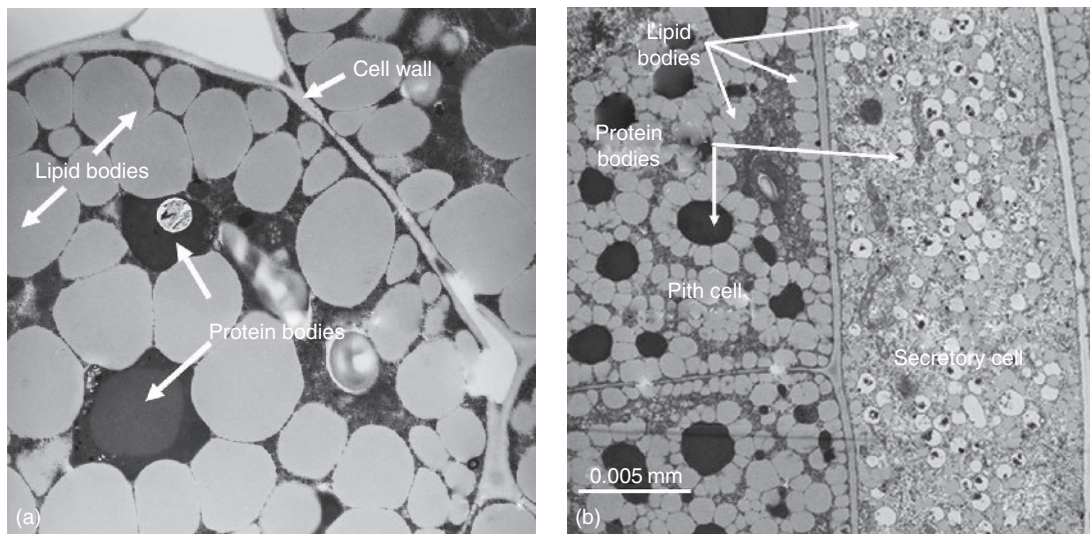


Figure 17 Electron micrographs of lipid and protein bodies as storage products in the mature megagametophyte (a) and mature embryo (b)

open and release seeds in September (Allen and Owens, 1972).

The entire reproductive cycle, from cone-bud differentiation until seed and cone maturity, takes about 17 months and includes one winter dormant period. The time from pollination to cone maturity takes only about 6 months. However, the entire reproductive cycle must be considered because many things can go wrong during the first 11 months that may reduce cone and seed production (Owens and Morris, 1991).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

The major limitation of conventional breeding is the length of the reproductive cycle. In addition, genetic gains per single trait are decreased as more traits are incorporated into the breeding program and very little is known about the genetics of real traits (Howe *et al.*, 2006). Somatic embryogenesis is more acceptable than transgenic breeding, because it eliminates the need for regulation and because public perception is less negative. It is also possible to capture multiple traits in a few individuals, and these elite individuals can be easily propagated via somatic embryogenesis. A limitation of somatic embryogenesis is that the most successful protocols require embryogenic tissues for induction and these tissues are only available for a limited time. New protocols involve callus induction from mature tissues and seedling regeneration via organogenesis. Another limitation is that once new hybrids are generated, field testing may take up to 20 years before the new genotypes are characterized. So, is there a need for transgenic Douglas fir? A few authors concur that genes conferring insect and fungal resistance, and reproductive sterility would be important candidates for transgenic approaches (Strauss *et al.*, 1995; Prudham, 2003; Howe *et al.*, 2006). The gypsy moth and sudden oak death, caused by *Phytophthora ramorum*, are notable future threats to Douglas fir (Howe *et al.*, 2006).

2. DEVELOPMENT OF TRANSGENIC DOUGLAS FIR

With Douglas fir, as with the majority of other conifers, transformation is still in its infancy, with

experiments focusing on optimizing protocols to obtain stable and numerous transformants. A sense of urgency for developing and deploying transgenics does not exist due to the severe public dissent and the success of somatic embryogenesis, which permits production of superior trees from the natural gene pool and results in multiple gains. Clonal forestry via somatic embryogenesis offers 40–60% genetic gain in volume, disease resistance, and wood quality and permits deployment of the next generation in 1.5 years (Sutton, 2002). In open-pollinated seed orchards, the genetic gain is only 13% and the next generation is deployable in 12 years (Sutton, 2002).

The initial view that conifers were not hosts of *Agrobacterium* (Ellis *et al.*, 1989) may have contributed to the lag in technology and caused the early investigators to concentrate their efforts on electroporation and particle bombardment for the production of transgenic conifers. Although tissues and cells have been transformed via *Agrobacterium*, electroporation, and particle bombardment, the single reference to a transgenic Douglas fir is a personal communication from Howe *et al.* (2006) stating that a transgenic Douglas fir was maintained up to 3 years of age. There are no examples of *in planta* transformation, that is, co-cultivation of germinating seed or female cones with *Agrobacterium*. The following is a compilation of all the work that has been done to introduce transgenes into Douglas fir tissues. It will serve as an informative guide and resource for those wishing to make advances in transforming and regenerating Douglas fir.

2.1 Donor Genes, Methods, Selection of Transformed Tissue, and Testing

Dandekar *et al.* (1987) were the first to transform Douglas fir tissue, using two strains of *Agrobacterium* to deliver kanamycin resistance to micropropagated shoots and seedlings. The bacterial aminoglycoside phosphotransferase (*APH(3')II*) gene, conferring kanamycin resistance, was co-integrated into the pTiA6 *Agrobacterium tumefaciens* plasmid by homologous recombination. The resulting strains were AtK12X562E, containing the pCGN562 cosmid with the (*APH(3')II*) gene under control of the octopine synthase promoter and 3'

polyadenylation site; and AtK12X167 containing the pCGN167 plasmid with the (*APH(3')II*) gene under control of the cauliflower mosaic virus (CaMV) 35S promoter. As a result of recombination, two copies of the (*APH(3')II*) chimeric gene were inserted in tandem in the pCGN562 cosmid with corresponding right and left borders. The micropropagated shoots and seedlings were inoculated with *Agrobacterium*. Tumors were observed within 5–6 weeks near the base of the shoots and within 9–10 weeks near the hypocotyls of seedlings. Tumor weight was significantly greater on seedlings than on shoots, and strain K12X562E caused 20% of shoots and 15% of seedlings to develop tumors, while strain K12X167 resulted in tumors on 5% of the shoots and 10% of the seedlings. This transformation efficiency may be partly explained by the activity of the two promoters (Dandekar *et al.*, 1987). Selection of transformed tissues was carried out by excising the tumors and culturing on phytohormone-free medium containing 500 $\mu\text{g ml}^{-1}$ carbenicillin. The tumors grew rapidly for 8–10 weeks, then growth decreased and the tumor deteriorated. Neomycin phosphotransferase activity was assayed by detection of radiolabeled phosphorylated kanamycin when ^{32}P -ATP was used as a substrate. A radiolabeled product corresponding, in size, to product from *Escherichia coli* carrying (*APH(3')II*) was detected in transformed tissues, but not in untransformed tissues. Octopine was detected in all tumors by high-voltage paper electrophoresis. To further test the stability of the transformation, Southern analysis was performed with an internal fragment of the transfer-DNA as a probe, and hybridization was seen with all tumor tissues but not in untransformed tissues.

In a further attempt to identify *Agrobacterium* strains that transform conifers at high frequencies, Ellis *et al.* (1989) screened 36 *A. tumefaciens* strains and 1 *A. rhizogenes* strain. The initial screen on white spruce resulted in the selection of 14 strains that were later used to transform Douglas fir, Sitka spruce, and Engelmann spruce. Two binary vector systems were employed independently to assess the integration and expression of kanamycin resistance and luciferase activity. The vector pEND4K contained the *neomycin phosphotransferase* (*nptII*) gene under control of the nopaline synthase promoter and terminator sequences. The vector pLUX2 containing the

chimeric firefly luciferase gene from pDO432 was derived from pEND4K by replacing *nptII* with *LUX2*. *Agrobacterium* was inoculated into 6-month-old seedlings by piercing the stem five times with a 26-gauge hypodermic needle that was dipped in *Agrobacterium*, or by making a slice into the stem so that a flap of tissue remained attached, and placing *Agrobacterium* on the cut surface and pressing the flap against the stem. These procedures were repeated so that each seedling received 10 inoculations, 5 inoculations on stem tissue formed prior to flushing (old growth) and 5 inoculations on stem tissue formed during flushing (new growth). It was found that the slice method did not increase the frequency of infection. Gall formation was assessed visually and galls were removed for opine analysis 5 months after inoculation. *Agrobacterium* was eliminated from the galls by surface sterilizing the galls followed by incubation on hormone-free medium containing 250 $\mu\text{g ml}^{-1}$ cefotaxime and 500 $\mu\text{g ml}^{-1}$ carbenicillin for 2 months. Resistance to kanamycin was tested by transferring these galls to medium containing 50 $\mu\text{g ml}^{-1}$ kanamycin. Luciferase activity was assessed by placing the galls void of bacteria in assay buffer containing 1 mM D-luciferin and recording luminescence. Interestingly, it was found that galls on Douglas fir formed with equal frequency in old and new growth, while in Engelmann spruce and Sitka spruce less than 10% of galls were present on old growth. Also, Douglas fir galls were light brown and friable while spruce galls were green and nodular. The relative frequency of gall formation was visibly higher on Douglas fir than on the other species tested. This report shows that several different *Agrobacterium* strains are capable of infecting Douglas fir, therefore confirming that this method of transformation will be effective. The top five wildtype strains were C3/74, K6/75, B2/74, 40, and I10/75, while the disarmed strain A281 carrying the supervirulence plasmid pTiBo542 showed moderate efficiency of transformation. Luciferase activity was recorded in galls derived from Douglas fir and this indicates that the agropine synthase and nopaline synthase promoters are functional in this species.

Stomp *et al.* (1989) analyzed the effect of *Agrobacterium* strains from a different point of view. Four-month-old seedlings were inoculated by stabbing with a blade that had been dipped

overnight in *Agrobacterium*. The seedlings were assessed visually for gall formation, 8–12 weeks after inoculation, and opine production was confirmed. However, a year after inoculation, the researchers found that the galls had not enlarged but had dried up and sloughed off. Only 21 of 27 galls remained in Douglas fir, while 200 of 472 galls remained and 30 new galls appeared over all species tested. In further work with *Pinus* species, callus cultures could not be established from galls, and statistical analysis of the ability of various *Agrobacterium* species to infect hosts revealed that there is host-pathogen specificity. The extent of stem woodiness and plant age appeared to be inversely proportional to the frequency of gall formation. Stomp *et al.* (1989) also hypothesized that plant defense mechanisms or regulation of the phytohormones produced in the transformed tissues enabled pines to overcome infection and inhibit chronic gall formation.

In the single example involving electroporation, Gupta *et al.* (1988) established that embryogenic suspensor masses and then somatic embryos could be regenerated from Douglas fir protoplasts. They then transformed protoplasts with luciferase by electroporation. It was found that electroporation decreased plasmid viability from 90% to 45–55%. Luciferase activity was detectable up to 36 h postelectroporation. One inference that could be drawn from this study is that transformation of protoplasts followed by regeneration of plantlets via embryonal suspensor masses could be an alternative to *Agrobacterium*-mediated transformation.

To identify a system of transformation that would also permit regeneration in Douglas fir, Goldfarb *et al.* (1991) studied the effect of six variables on delivery and expression of foreign DNA using microprojectile bombardment. This method was chosen because it had resulted in stable transformation in a few angiosperms, and there were no reports of stably transformed conifer plants via *Agrobacterium* or electroporation. Goldfarb *et al.* (1991) germinated Douglas fir seeds in vermiculite in open trays and collected cotyledons when they emerged from the seed coat and began expanding but before the first true leaves started to elongate. The cotyledons were cut into 10-mm slices and placed on callus-forming medium (2.5 μ M 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 μ M benzyladenine (BA)) for 7–9 days. The plasmid PTVBTGUS containing the

GUS (β -glucuronidase) gene under control of the CaMV 35S promoter and the *NOS* terminator was precipitated onto gold particles that were bombarded onto the prepared cotyledons. Transient gene expression was assessed 1- or 2-day postbombardment by incubating the cotyledons overnight in GUS histochemical substrate buffer, and measuring the number of blue loci. This number gave the minimum estimate of cells with activity. When the time on induction medium was modulated between 3 and 14 days, it was found that the number of GUS-expressing loci was highest when the cotyledons were cultured at least 7 days prior to bombardment. For example, an average of 0.6 loci per cotyledon were observed after 3 days, but after 7 days an average of 5.9 loci per cotyledon were observed. It was also noted that after 7, 9, or 12 days, 90% of cotyledons had at least one locus, while only 40% of cotyledons had one locus after 3 days. Another variable tested was cytokinin pulse, which is known to induce adventitious bud formation. Cotyledonary whorls were immersed in 400 μ M BA for 2 h and then placed on phytohormone-free callus-forming medium. Bombardment after 7–14 days of pulsing resulted in loci in more than 99% of cotyledons. Multiple bombardments, seedling variability, and bombardment variability did not have a significant effect on the number of loci. To estimate the efficiency of transient expression, the number of loci on each cotyledon was counted, the cotyledon was sectioned on a freezing microtome and the number of gold particles was counted microscopically. It was found that only 0.006% of particles reaching the cotyledon were expressed. Exposing the tissues to auxin and cytokinin prior to treatment was critical for increasing the number of cells expressing the transgene and this could be attributed to the stimulation of cell division and the stabilizing effect it has on stable incorporation of foreign DNA (Goldfarb *et al.*, 1991). In Douglas fir, cell division is induced approximately 4 days after exposure to cytokinin, or auxin and cytokinin (Goldfarb *et al.*, 1991). It was concluded that biolistics is a potential technology for producing transgenic plantlets, because cotyledons can form multiple adventitious buds and shoots.

A further improvement in transgene expression may be achieved by using endogenous promoters. After studying various promoters and transgene expression in *Populus*, Ahuja (2000) concluded

that promoters cloned from plants or trees are less prone to methylation and silencing. Chatthai *et al.* (2004a) compared the ability of the CaMV 35S promoter with the Douglas fir metallothioneinlike gene promoter (pMTP) to drive the expression of GUS in Douglas fir tissues. In the Clontech pBI221 vector employed in this work, the *uidA* (β -glucuronidase) gene was under control of the CaMV 35S promoter and the NOS terminator. To create the pMTP0.9-GUS construct, the CaMV 35S promoter was excised out of the pBI221 vector and the Douglas fir pMTP was cloned in its place. These vectors were precipitated onto gold particles and the DNA was delivered to Douglas fir megagametophytes, immature zygotic embryos, and somatic embryos. GUS activity was measured 2-day postbombardment by counting the number of blue loci after incubation in GUS histochemical buffer. Megagametophytes and zygotic embryos bombarded with pMTP0.9-GUS showed two to three times more loci than those bombarded with pBI221, and there was no difference in expression levels in somatic embryos. Constructs with a series of promoter deletions revealed that expression was reduced by 50% when more than half of the promoter sequence was deleted, and this was attributed to the loss of the three ethylene-responsive elements present in the intact promoter.

In similar effort to the one described above, Forward *et al.* (2002) compared GUS activity when either the CaMV 35S promoter or the Douglas fir BiP promoter controlled GUS expression. The same pBI221 vector was utilized and the PmBiP (*P. menziesii* luminal binding protein) promoter replaced the CaMV 35S promoter region to make the test construct. Biolistic transformation of germinating Douglas fir zygotic embryos and counting the number of blue loci resulted in five- to sixfold higher expression when the Douglas fir BiP promoter was used. An unusual effect observed when *Arabidopsis* was transformed, was that expression was significantly higher when approximately 1000 bp were deleted from the PmBiP promoter sequence. Expression was increased in the entire plant and it was additionally observed in vascular tissue, lateral roots, and root tips (Forward *et al.*, 2002). This was attributed to the elimination of a putative negative regulatory region. The work with *Arabidopsis* also showed that this promoter is inducible by wounding.

A variation to the theme of endogenous promoters is tissue specificity. Chatthai *et al.* (2004b) showed that the Douglas fir 2S seed storage protein promoter (p2SSP) is capable of driving GUS expression in developing Douglas fir megagametophytes and mid-cotyledonary zygotic embryos. Because stable transformation in conifers was not feasible at the time, the p2SSP-GUS construct was transformed into tobacco seeds via *Agrobacterium*. GUS expression was observed in embryos and endosperm but not in the embryogenic radicle or root tip. When 2-week-old T₁ seedlings were analyzed, no GUS activity was detected in leaves, stems, or roots although stable integration was confirmed by polymerase chain reaction (PCR) and Southern blot analysis. These studies indicate that gene-regulating mechanisms are conserved among species that are evolutionarily distant to conifers, e.g., angiosperms. The angiosperm species serves as a useful model system for analyzing the function and regulatory mechanisms of Douglas fir genes.

2.2 Specific Regulatory Methods

The planting of transgenic trees involves applying for permits from the appropriate agency, and obtaining deregulated status. In the United States, the Animal and Plant Health Inspection Service (APHIS) regulates transgenic plants that have the potential to be plant pests, while the Environmental Protection Agency (EPA) regulates transgenic plants that produce a pesticidal substance (Strauss *et al.*, 1995). In Canada, the Canadian Food Inspection Agency regulates transgenic trees and confinement conditions are always necessary. Internationally, the Organization of Economic Cooperation and Development includes member countries Belgium, Canada, Finland, France, New Zealand, Norway, Portugal, Spain, and Sweden. In most other countries, regulation occurs at the national level.

3. FUTURE ROAD MAP

3.1 Expected Products

The benefits of deploying transgenic trees must greatly outweigh the costs of production (van

Frankenhuyzen and Beardmore, 2004; Howe *et al.*, 2006). It is evident that Douglas fir has enough genetic diversity to withstand the pests and diseases it has encountered. A niche for transgenic breeding includes resistance to Swiss needle cast, *Rhabdocline* needle cast, *Armillaria* root rot, *Phellinus* root rot; and, the exotics, gypsy moth (*Lymantria dispar*), and sudden oak death fungus, *P. ramorum* (Howe *et al.*, 2006). The insect toxin gene of *Bacillus thuringiensis* is of interest for general insect resistance, and the use of synthetic *cry* genes with host-specific codon bias was shown to increase the effectiveness of resistance (van Frankenhuyzen and Beardmore, 2004). Broad-spectrum antimicrobial peptides have been used to successfully engineer resistance to a range of fungal and bacterial diseases in angiosperm species (Osusky *et al.*, 2004). The benefit of engineering trees for resistance against a multitude of pathogens is that rare or elite genotypes will not be exterminated by disease. The Ponericins of the ant *Pachycondyla goeldii* were shown to combine antibacterial and insecticidal activities. Identification of a peptide with antifungal and insecticidal properties that are pertinent to Douglas fir would find applications in tree improvement.

Because tree value is predominantly determined by stem volume (Howe *et al.*, 2006), the identification of a gene or set of genes responsible for rapid, dense growth would classify these as candidates for genetic engineering.

Many authors agree that reproductive sterility is the best solution for the containment of transgenes (Strauss *et al.*, 1995; Ahuja, 2000; Tang and Newton, 2003; Poupin and Arce-Johnson, 2005). Research is still needed on the methods of cloning sterility and the identification of naturally occurring sterile trees that may be cloned. A unique and potentially beneficial aspect of sterile trees is that wood production will increase when no energy or nutrients are invested in cone and pollen production (Strauss *et al.*, 1995).

3.2 Risks and Concerns

The major risks associated with transgenic forest trees are invasive escape and vertical gene flow, horizontal gene transfer and undesirable effects on nontarget organisms and ecosystem processes,

and detrimental effects on transgenic trees due to the instability of transgene expression (van Frankenhuyzen and Beardmore, 2004). Invasive escape and vertical gene flow (transfer to wild relatives) are thought to result in weediness of a transgenic species over endogenous or native species. A greater risk of spread exists with transgenic trees than with crops because there is a higher likelihood that the trees will be more closely related to wild trees (Prudham, 2003; Poupin and Arce-Johnson, 2005). Finally, public concern about the release of transgenics is a major impediment. These concerns can be alleviated with time as more research and data will increase our understanding of risks. A variation to the theme of endogenous promoters is tissue specificity.

3.3 Expected Technologies

Somatic embryogenesis of conifers is currently the most efficient and advanced technology for producing superior trees. Additionally, it is suited to transgenics because rapidly dividing cells of embryogenic tissue are most easily transformed and sufficient material is available (Wenck *et al.*, 1999; Klimaszewska *et al.*, 2001). Wenck *et al.* (1999) increased the efficiency of transformation by adding additional copies of the virulence genes to the *Agrobacterium*. *Agrobacterium*-mediated transformation and regeneration of plantlets from rapidly dividing somatic embryos were successful with larch, pine, and several species of spruce (Wenck *et al.*, 1999; Klimaszewska *et al.*, 2001). The combination of elite genotypes that are being produced by somatic embryogenesis and transformation with desirable traits is a promising alternative for Douglas fir. Further experimentation is necessary with Douglas fir to establish transformation and tree regeneration as common techniques and then to start producing and testing transgenic trees of commercial value. Identification of endogenous promoters that will improve expression and stability of transgenes will make transgenic tree breeding a more realistic goal.

Another option for producing transgenic trees is via *in vitro* fertilization with transgenic pollen. Pollen transformation is a proven technology in alfalfa and the process will be identical in conifers. Labor and protocol optimization will not be

required and this will lead to significant savings of time and resources.

It is also expected that methods will be sought to achieve controlled gene expression, i.e., herbicide tolerance is only necessary during the first 2 years while plantlets establish themselves (Ahuja, 2000). Again, endogenous and inducible promoters will have a key role.

One potential application (Table 1) of transient expression, as seen with particle bombardment, is the transient induction of somatic embryogenesis from the mature tissues of rare or elite genotypes. Initiation of somatic embryogenesis is still challenging and alternative technologies are necessary to keep up with the increasing demand for wood products. The Douglas fir *LEAFY COTYLEDON1* (*LEC1*) gene (Vetrici and

Misra, 2005) is homologous to the *Arabidopsis thaliana* *LEC1* gene. AtLEC1 is a transcription factor that activates the transcription of genes required for normal embryo development. It specifies embryonic organ identity, suppresses the embryogenic potential of the suspensor during early embryogenesis and inhibits premature germination (Lotan *et al.*, 1998). Ectopic expression of AtLEC1 in *Arabidopsis* resulted in the spontaneous formation of somatic embryos from mature tissues (Lotan *et al.*, 1998). A *LEC1* gene was isolated from Douglas fir (*PmLEC1*, *P. menziesii* *LEAFY COTYLEDON1*) using reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of complementary DNA ends PCR (RACE-PCR), and Northern analyses indicate that its expression is embryo

Table 1 Stage-specific genes isolated from Douglas fir

Gene	Function ^(a)	Expression ^(a)	Promoter	Application to biotechnology	Reference
<i>PmLEC1</i>	Initiates embryo morphogenesis, prevents precocious germination	EE ^(b) ME ^(c)	Yes	Potential to induce somatic embryogenesis from rare or mature tissues, marker of early embryogenesis	Vetrici and Misra, 2005
<i>LYR/LVR</i>	Catalytic NADH oxidoreductase	EE ^(c)	Not yet isolated	Marker of early embryogenesis	Ramachandran and Misra, 2006
<i>PM18.2</i>	Low molecular weight HSP	EE ME LE G ^(c) S	Not yet isolated	Enhances stress tolerance, developmental regulation	Kaukinen <i>et al.</i> , 1996
<i>PM2.1</i>	Metallothioneinlike genebinds metals	ME ^(c) LE G S ^(c)	Yes	Promoter shown to drive transient and stable expression of GUS	Chatthai <i>et al.</i> , 2004a
<i>PM2S1</i>	2S seed storage protein	EE ME ^(c) LE	Yes	Promoter shown to drive transgene expression in a tissue-specific manner	Chatthai <i>et al.</i> , 2004b
<i>PmBiP</i>	Luminal binding protein Molecular chaperone	EE ^(b) ME LE G ^(c) S	Yes	Promoter shown to drive transgene expression, developmental and wound-inducible regulation	Forward <i>et al.</i> , 2002
<i>DF14</i> (<i>CPR</i>)	NADPH-cytochrome P450 reductase	LE G ^(c) S ^(b)	Not yet isolated	Seedling-specific expression	Tranbarger <i>et al.</i> , 2000
<i>PmCysP</i>	Cysteine protease	LE G ^(b) S	Not yet isolated	Germination-specific expression	Tranbarger and Misra, 1996

^(a)EE, early embryogenesis; ME, mid embryogenesis; NADH, nicotinamide adenine dinucleotide; HSP, heat shock protein LE, late embryogenesis; G, germination stage; S, seedling; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase

^(b)Maximal peak of expression

^(c)Peak of expression

specific, with transcript levels being highest during early embryogenesis (Vetrici and Misra, 2005). *PmLEC1* was cloned under control of the high-level CaMV 35S-35S-AMV (alfalfa mosaic virus) promoter and the NOS terminator in the pBI221 vector (Vetrici and Misra, 2005). *PmLEC1* rescued the *Arabidopsis lecl-1* null mutant and work is in progress to assess the spontaneous formation of somatic embryos (Vetrici and Misra, unpublished data). Transient expression of *PmLEC1* just to the point that somatic embryogenesis is induced from mature or rare tissues will save significant amounts of time because elite genotypes could be cloned directly and the field-testing phase would be eliminated. This will also serve to increase biodiversity.

Several other tissue-specific and developmentally regulated genes have been identified in Douglas fir (Table 1). The majority of these genes were isolated from differential screening or immunoscreening of stage-specific cDNA (complementary DNA) libraries. The corresponding regulatory sequences of these genes provide a repertoire of promoters for regulated transgene expression in Douglas fir and other conifers.

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FURTHER READING

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American Elm

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1. INTRODUCTION

1.1 History, Origin, and Distribution

In the late 18th century, French botanist Andre Michaux called the American elm (*Ulmus americana* L.) “nature’s noblest vegetable”. Many landscapers and homeowners felt the same way, and by the early 20th century, American elm trees were so popular that they were planted almost exclusively along streets in many US cities. Their tolerance of drought, pollution, and compacted soil made them ideal landscape trees, and they were a significant part of forest ecosystems as well. People admired their graceful form, whether rows of American elms were arching over a city street or a single huge tree was spreading majestically in a rural field (Figure 1). The popularity of elm trees is evidenced by the fact that “Elm Street” is one of the most generic and ubiquitous street name in the United States; it is even used in the media to refer to any residential street in any town in America. At the end of the 20th century, however, a few scattered individuals were all that remained of this once-dominant species.

Dutch-elm disease (DED) was first introduced to Europe about 1915 and the United States in 1930 (May, 1930; Heybroek *et al.*, 1982). It has since swept through each continent twice in separate pandemics (Brasier, 1991), killing most

mature trees within one or two growing seasons. DED is a vascular wilt disease caused by the introduced fungus *Ophiostoma ulmi* (Buisman) Nannf. or *Ophiostoma novo-ulmi* Brasier (hereafter referred to only as *O. novo-ulmi*, the cause of the more recent pandemics). Spores or hyphae of *O. novo-ulmi* can travel directly through root grafts of adjacent trees, but more commonly the disease cycle is completed through an insect vector that also spreads spores between trees. Elm bark beetles (either native North American *Hylurgopinus rufipes* or the European *Scolytus multistriatus*) feed on twig crotches of healthy adult elm trees, and then lay eggs under the bark of dying or dead elm trees, where *O. novo-ulmi* grows (Anderson and Holliday, 2003). After the eggs hatch, the larvae mature, and the adult beetles leave the dead tree, they carry *O. novo-ulmi* spores to healthy trees and complete the cycle. When *O. novo-ulmi* spores are deposited in the xylem vessels of a healthy, susceptible elm tree, they can spread passively through the plant’s vascular system. After spores germinate, hyphae can grow actively through pit membranes and infect other vessels (Hubbes, 1999). One of the plant’s responses to this invasion is to form bubblelike structures called tyloses, which typically swell and block the vessels in order to contain a pathogen. However, in the case of DED, the fungus generally spreads through and between vessels faster than the tyloses can



Figure 1 American elm in Poland, NY showing characteristic graceful vase shape [Photo courtesy of Fred N. Lampert]

contain it, so they only serve to intensify the wilting response.

Vessel size (both length and diameter) has also been correlated with DED resistance in some elm species. First, elm trees are most susceptible to DED in the spring, when their vessels are largest and transporting the most water (Hubbes, 1999). Smaller vessels would also be blocked more quickly by tyloses, which would increase the chances of effectively blocking the spread of *O. novo-ulmi*. Several experiments have reinforced these hypotheses. DED-resistant *Ulmus pumila* trees were shown to have lower conductivity and shorter or narrower vessels than susceptible *U. americana* trees (Elgersma, 1970). Vessel diameter and proportion of large vessels were correlated with susceptibility in *Ulmus minor* clones by Solla and Gil (2002), who concluded that resistance is proportional to speed of vessel compartmentalization. More recently, however, the same research group (Solla *et al.*, 2005) added that vessel size is not the only factor responsible for DED resistance, since the DED-tolerant *U. pumila* typically has larger vessels than the more susceptible *U. minor*.

Another significant response by the DED host is the production of phytoalexins called mansonones, which are produced in response to

elicitor molecules on the cell walls of the fungus (Yang *et al.*, 1989). Mansonones have been found in greater concentrations in DED-resistant than in susceptible elm species (Sticklen *et al.*, 1991), and in susceptible species when inoculated with less-aggressive forms of *O. ulmi* (Duchesne *et al.*, 1985), which indicates that they are probably a significant disease response in susceptible trees. When isolated from infected trees, mansonones have also been shown to inhibit the growth of *O. ulmi in vitro* (Sticklen *et al.*, 1991).

1.2 Botanical Description

Elms (*Ulmus* spp.) are in the strongly monophyletic family Ulmaceae, which together with Rosaceae, Cannabaceae, Urticaceae, Moraceae, and three or more other families, make up order Rosales (Sytsma *et al.*, 2002). The genus *Ulmus* consists of between 30 (Conde *et al.*, 2004) and 45 (Wiegrefe *et al.*, 1993) species, all of which are native to the northern hemisphere. Of these, a few are especially popular as plantation species. The English elm (*Ulmus procera*) has been planted in Great Britain for thousands of years. *U. procera* is almost entirely derived from a single clone that was introduced to England from Rome around 2000 years ago to be used for grapevine supports (Gil *et al.*, 2004). In The Netherlands, *Ulmus hollandica* (primarily clone “Belgica”) was planted extensively for landscaping, especially along the coast where they served as effective windbreaks and were very tolerant of salt spray (Karnosky, 1979). Both of these species are highly susceptible to DED, and any potential natural resistance from genetic diversity is reduced due to the fact that they are clonal.

In North America, the American elm (*U. americana* L.) was originally found throughout the eastern United States and southeastern Canada, and has since been planted throughout temperate areas of the continent due to its pleasing landscape characteristics and environmental tolerances. It can grow to be more than 500 years old and 42 m tall, typically with a spreading vase shape and a broad crown that can be wider than it is tall (Line, 1997).

Outside of intentional cultivation, the American elm is most commonly found in moist bottomland areas or near streams; mixed with ash, maple,

sugarberry, or sycamore in temperate hardwood forests (Bey, 1990). American elms, where they remain, are an important part of forest ecosystems, providing shelter and food for a variety of organisms. Squirrels, mice, birds, and deer eat the flowers and seeds, while bats and other small animals take shelter in the deeply grooved bark. American elm leaves contribute significantly to nutrient cycling and can improve soils, as they are high in calcium and other nutrients. In addition, they decompose relatively quickly compared to leaves from oaks, maples, and other common forest trees (Bey, 1990).

1.3 Economic Importance

People have cultivated elm trees (*Ulmus* spp.) for thousands of years. Long before they became popular for landscaping, elms were grown for grapevine supports in Italy and Spain, and their leaves were used for cattle feed when forage crops were insufficient (Karnosky and Mickler, 1986). Elm wood was historically used for timber, bows, wagon wheel hubs, and water pipes, and the bark was used by Native Americans for making rope and covering canoes (Smalley and Guries, 1993; Line, 1997). During World War II, elms' wide canopies and thick foliage were especially desirable for concealing factories and fortifications (Smucker, 1944). Elms are still grown for use as windbreaks, harvested for lumber, and of course planted for landscaping. The unique interlocking grain makes elm lumber resistant to splitting, and therefore ideal for bent or curved wood products, such as furniture, veneer, boats, and hockey sticks (Bey, 1990; Corchete *et al.*, 1997).

A street lined with mature American elm trees has an intrinsic aesthetic value that is difficult to quantify compared to timber or other commodity products. Left untreated, nearly all American elm trees that are exposed to DED will die within a few weeks to a few years, while trees grown to replace them take many decades to mature. In urban landscaping situations, this not only reduces aesthetic beauty and property value, but incurs significant additional expenses when dead trees have to be removed and disposed of (Cannon Jr and Worley, 1980). Therefore, treatment and prevention of DED are economically, aesthetically, and ecologically important.

1.4 Traditional Breeding and Treatment Options

Dr. Christine Buismann of The Netherlands, one of the first researchers to study DED in the early 20th century, also started a search for disease-resistant elms through selection and breeding programs (Holmes and Heybrook, 1990). One of the cultivars produced by this program, *U. hollandica* cv. "Commelin" is considered moderately resistant and is still being planted (Karnosky, 1979; Ben Jouira *et al.*, 1998). This program, along with various other selection, breeding, and testing programs, are still being maintained today.

Large-scale plantings for selection and breeding in the United States were started soon after the disease reached this continent. The United States Department of Agriculture (USDA) started a screening program in New Jersey in 1937, and had screened over 35 000 trees by 1944, at which point only one tree had lived through three successive inoculations with *O. ulmi* (Smucker, 1944). This program was interrupted by World War II, but restarted in 1970 in Ohio, and moved to Maryland in 1984, where it is still active today (Townsend and Douglass, 2001). Several commercially available DED-tolerant elm cultivars are products of this program, including "Valley Forge", "New Harmony", and some new cultivars that have not yet been released for general use, but show promise in screening experiments (Townsend *et al.*, 2005). Large-scale screening programs were also started at Cornell University in 1933 and the University of Wisconsin in 1958. The Wisconsin program incorporated breeding in 1969, and is also still active (Smalley and Guries, 1993). The popular "Liberty Elm" (actually a collection of six *U. americana* clones) is a product of the Wisconsin program, as well as "Independence", "Sapporo Autumn Gold", and several other cultivars that have been released for use by the nursery industry or the general public (see Sanatmour and Bentz, 1995, for a more thorough list of elm cultivars, common names, and hybrid histories). Despite its popularity, the Liberty Elm showed limited resistance to DED in more recent inoculation tests (Townsend and Douglass, 2001). Also, no DED-tolerant American elm clone has been reported to have resistance to elm yellows, a separate elm disease caused

by phytoplasma infections of phloem vessels (see Section 3.2). Of six Eurasian cultivars tested for elm yellows susceptibility by grafting infected bark patches, only one hybrid (cv. "Homestead") showed potential elm yellows resistance (Sinclair *et al.*, 2000). Between the programs mentioned above and other smaller breeding and selection programs, hundreds of thousands of American elm trees have been planted, grown, inoculated with *O. ulmi* and *novo-ulmi*, and observed for disease resistance.

Elms present unique difficulties to breeders as the popular species (American *U. americana*, English *U. procera*, and most clones of Dutch *U. hollandica*) are very susceptible to DED, but disease-resistant Asian species do not have the shape, growth characteristics, or environmental tolerances that make western species popular (Karnosky and Mickler, 1986). American elms are especially difficult to breed because they are tetraploid ($2n = 56$), instead of diploid ($2n = 28$) like other elms. Despite this restriction, early breeding programs (and later tissue culture experiments) tried for many years to hybridize American and Siberian elm (Redenbaugh *et al.*, 1981; Karnosky and Mickler, 1986). Of more than 20 000 controlled crosses performed between these two species, only about 100 seeds were obtained, and only a few of those germinated (Smucker, 1944). Even the most promising of these crosses did not have the leaf size or growth characteristics of full American elm.

At least one individual triploid elm was reported (purportedly with one American and one Asian parent, and many distinct American elm characteristics), and cuttings from this "hybrid" were shown to leaf out earlier, have intermediate leaf shape and size, and be disease resistant when compared to full American elms also grown from cuttings (Sherald *et al.*, 1994). This individual is a unique and valuable part of the American elm planting on the National Mall in Washington DC. This cultivar was initially designated NPS 3-487 and later named the "Jefferson Elm" (Sanatmour and Bentz, 1995). However, further tests revealed that NPS 3-487 is indeed a tetraploid full-American elm, albeit one with unusually high inherent resistance to DED (Townsend *et al.*, 2005).

Landowners and landscapers have tried a variety of preventative measures and treatments of DED. Given the fact that DED is spread by

beetles that breed in dead elm wood, the easiest and most effective DED control method is to remove and destroy (chip or burn) all dead elm wood. Sanitation programs are not 100 percent effective alone, but they are an essential part of all other DED prevention and control programs (Haugen and Stennes, 1999). One seemingly obvious preventative DED treatment involves using an insecticide to control the beetle vector. Spraying insecticides such as DDT has been performed with fair success in the past (Heybroek *et al.*, 1982), but the use of DDT was discontinued due to environmental concerns. Other insecticides have been used, but if the insecticide is applied by spraying, the entire crown of each tree must be treated, and multiple applications each season are usually necessary, which makes the process expensive and laborious, and likely to affect nontarget organisms. Beetle traps baited with pheromones have been widely used and can successfully attract large numbers of beetles (millions per year across the United States), but the ratio of beetles caught compared to the total population is still very small (Heybroek *et al.*, 1982).

A different strategy for controlling and preventing DED involves injecting fungicide or biological control agents directly into trees to kill or suppress *O. novo-ulmi*. Of course treatments of this type can only be applied to one tree at a time, making them expensive and impractical for widespread use outside of valuable landscape trees. Systemic fungicides can be effective in suppressing the fungus and all symptoms during the year of injection (Lanier, 1988), but they must be applied yearly for continued effectiveness, and if newly infected portions are not pruned out or retreated, symptoms from a single *O. novo-ulmi* inoculation can recur up to 4 years later (Campana, 1977; Sherald and Gregory, 1980). Various microbes have been tested *in vivo* for control of DED fungi and beetle vectors since the early 1980s, including *Trichoderma viride* (Ricard, 1983), *Pseudomonas syringae* (Myers and Strobel, 1983), *Streptomyces* spp. (O'Brien *et al.*, 1984), and *Phaeothea dimorphospora* (Bernier *et al.*, 1996). Some of these tests showed that the biological control agent induced resistance in the host when used preventatively, but none worked consistently as treatments against aggressive strains of DED. *Verticillium dahliae* was tested more recently as a preventative treatment against DED, and isolate

Vd-48 was found to significantly reduce wilting symptoms when inoculated 15–30 days before *O. novo-ulmi* inoculations, but *V. dahliae* could not be isolated outside the inoculation site as would be expected if it were systemic, and at high concentrations, it can cause its own disease symptoms (Solla and Gil, 2003).

Biological control of the disease vector has also been explored. *Bacillus thuringiensis*, which is commonly used to protect food crops from insect damage, as well as two *Trichoderma* species and *Scytalidium lignicola*, were shown by Jassim *et al.* (1990a, b) to increase mortality of both species of elm bark beetle when cultured on artificial media. Other *Bacillus* species tested in the same experiments gave mixed results, and no *in vivo* tests or techniques were published.

Nonaggressive strains of *O. ulmi* have been tested as preventative inoculations, and some caused increased host resistance to concurrent or subsequent inoculations with more aggressive strains (Scheffer *et al.*, 1980; Hubbes and Jeng, 1981). Increased DED resistance after exposure to less aggressive pathogens or other microbes may be partly due to physical blocking of vessels or lignin deposition, but is more likely due to an elicitorlike induction of plant disease responses (Solla and Gil, 2003) including mansonones as described below.

Other natural *O. novo-ulmi* control possibilities have been found in natural populations of the fungus. These viruslike agents, called d-factors, have been shown to reduce production of the *O. novo-ulmi* toxin cerato-ulmin (Sutherland and Brasier, 1995), and to reduce infections *in vivo* on both the susceptible *U. procera* and the more DED-tolerant *U. hollandica* “cv. Commelin” (Sutherland and Brasier, 1997). However, widespread success of d-factors as biological controls is unlikely because they would not spread between sexually or vegetatively incompatible populations of *O. novo-ulmi* (Hubbes, 1999), and would not spread at all if they did not increase the fitness of the organism.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Historically, the only way to genetically modify a plant to eliminate disease susceptibility or add new characteristics was through breeding and selecting desirable traits. Nearly all modern food crops

have been extensively modified through selective breeding, often to the point where they do not even resemble their wild progenitors. However, breeding is a lengthy process, especially in the case of slow-maturing trees, and it is impossible to determine ahead of time which parental traits will be passed on and expressed in progeny.

Genetic engineering, on the other hand, theoretically allows researchers to start with almost any plant source they choose, and add only one or a few genes that are necessary for resistance to a particular disease. The use of regulated promoters can refine the process even further, by directing where, when, and how much a given gene is expressed in the plant. Thus the process of producing a disease-resistant tree can potentially be faster, more controllable, and more predictable than traditional breeding (Giri *et al.*, 2004). Furthermore, in contrast to traditional breeding, parental explant sources for transgenesis can be selected for specific phenotypes, which would be far more likely to be maintained in all progeny. It is also easier to maintain genetic diversity in transgenic plants than traditionally bred selections by transforming a number of diverse genotypes. Genetic diversity is important for any plant population or restoration project, and has been specifically recommended for new elm releases (Karnosky, 1979). As global transport and the resulting inevitable spread of phytopathogens continues to accelerate, production of disease-resistant plants will become more urgent. Genetic engineering has the unique potential to accomplish this relatively quickly and effectively (Adams *et al.*, 2002).

A variety of methods have been employed to treat infected elm trees and prevent the spread of the disease, but many of these have been either ineffective, expensive, time-consuming, or detrimental to the environment (see descriptions of insecticides and fungicides in Section 1.4). Selection programs to find and breed American elm trees with natural DED resistance have had some success in producing DED-tolerant American elms, but the growth and selection process is very slow and not controllable. Also, any attempts to “breed in” resistance to American elm from other elm species are difficult due to ploidy differences between American and all other elms, and may result in trees without the desirable aesthetic characteristics of classic American elms. Finally, if trees are selected or bred only for DED

resistance, it is likely they would be susceptible to other known elm pathogens such as elm yellows, and they would not have the genetic diversity necessary to tolerate new diseases or environmental pressures.

Transformation of American elm trees with genes for DED resistance, under the control of regulated promoters, has the potential to produce trees that are truly resistant to DED. These resistant transgenic trees can be from a variety of genetic backgrounds, and have all the popular growth characteristics of American elms. Preliminary evidence as presented in this study suggests that genes encoding small antimicrobial peptides in particular show great promise for DED resistance, since they are naturally active against

microbes but harmless to plants and animals, and they can be synthesized to enhance these or other characteristics. Promoters that express these genes specifically in the vascular tissue, or only in response to fungal invasion, could mitigate concerns of reduced growth effects or interference with mutualistic soil microbes.

2. DEVELOPMENT OF TRANSGENIC ELM

2.1 Donor Gene

The plasmid construct initially used to transform American elms in this project was designated pSE39 (Figure 2). Starting at the right border

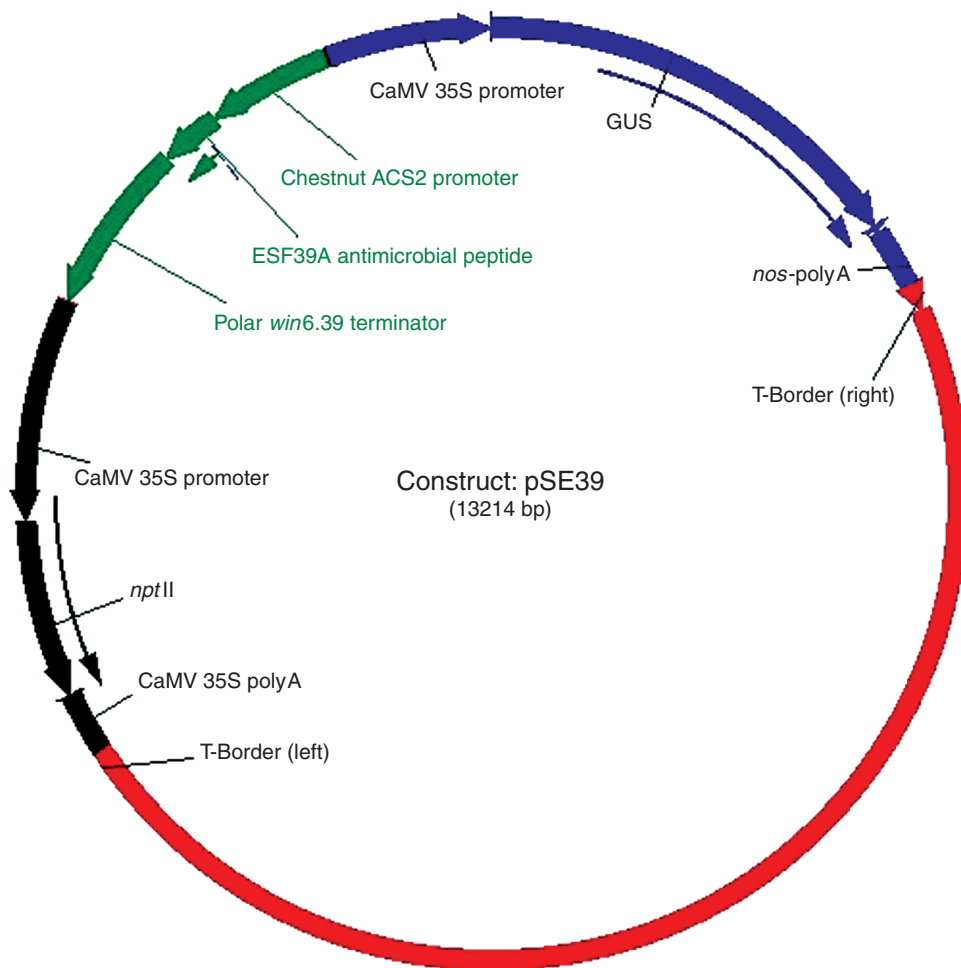


Figure 2 Plasmid pSE39 showing the GUS marker gene, the ESF39A antimicrobial peptide, and the *nptII* selectable marker

of the transfer-DNA (T-DNA), the first gene in this construct is *uidA* (codes for GUS, or β -glucuronidase). The constitutive cauliflower mosaic virus (CaMV) 35S promoter regulates this gene's expression, so all tissues in a transformed plant should express the GUS marker gene. It is followed by a nopaline synthase (*nos*-polyA) terminator.

The second gene, *ESF39a*, encodes a 39-amino acid protein that will be processed by the cell into antimicrobial peptide ESF22B, along with an N1a protease recognition sequence (Powell *et al.*, 2000). This protein has a unique amino acid sequence, but was designed to have the same α -helical, amphipathic structure as magainin-type antimicrobial peptides (Zasloff, 1987, see below), with enhanced antifungal and reduced hemolytic activities (Powell *et al.*, 1995, 2000). Proteinase recognition sites were also included to facilitate breakdown in vertebrate digestive systems. This gene is controlled by the *ACS2* promoter, which was cloned from American chestnut (*Castanea dentata*) and has been shown to express specifically in vascular tissue (Connors *et al.*, 2002). It is followed by the terminator from the wound-inducible chitinase gene, *win6.39*, originally isolated from poplar (Clarke *et al.*, 1994).

The third and final gene in pSE39 is the selectable marker *nptII* (neomycin phosphotransferase II), driven by a separate, oppositely oriented CaMV 35S promoter, for resistance to neomycin-class antibiotics including kanamycin. It is followed by a CaMV 35S polyA terminator. This allows early selection of potentially transformed shoots, as nontransformed tissue culture shoots should not survive on media with kanamycin. The selectable marker was intentionally placed farthest from the right border, to prevent survival of partially transformed shoots if the T-DNA failed to insert completely.

Cationic antimicrobial peptides are short, amphipathic proteins that inhibit growth of a variety of microbes. Epithelial surfaces, blood, and mucosal secretions from humans have been known to have bactericidal properties for almost a century (Skarnes and Watson, 1957; Ganz and Lehrer, 1999; Brogden, 2005). Some of these early researchers correctly inferred that the factors responsible for these properties were specifically active against negatively charged membranes, and were inducible by microbes. By the mid-

20th century, antimicrobial peptides (AMPs) were studied and described in more detail after being extracted from phagocytotic granules (Hirsch, 1956). At this point, they were known to be proteins that increased permeability of microbial membranes. More detail was elucidated with the discovery and characterization of cecropin class AMPs from *Cecropia* moth pupae, after the realization that insects showed an immune response without the lymphocytes or immunoglobulins that characterize vertebrate immune systems (Steiner *et al.*, 1981).

A different class of AMPs was discovered after the observation that *Xenopus* (African clawed frogs) rarely contracted microbial diseases, even when lesions and contaminated environments provided ideal conditions for infection (Chen *et al.*, 1988). Researchers studied *Xenopus* skin and isolated some of these AMPs, designated magainins, which were found to be effective against bacteria, fungi, and protozoa, but again not harmful to higher eukaryotes (Zasloff, 1987).

Over 880 AMP sequences have been published as of 2005 (Brogden, 2005), and thousands more probably exist. Various types of AMPs are found in a wide variety of organisms, including bacteria (Kristiansen *et al.*, 2005), fungi (Reddy *et al.*, 2004), most plants and all plant organs (Broekaert *et al.*, 1997; Fujimura *et al.*, 2005), many invertebrates (Richman *et al.*, 1997; Lamberty *et al.*, 2001), and a variety of vertebrates (Hancock and Scott, 2000; DeGray *et al.*, 2001). Their broad activity and relatively rapid response make AMPs ideal pathogen-fighting agents where a complex or adaptive immune response is absent or reduced. For example, most insects' response to pathogens that penetrate the cuticle is primarily mediated by AMPs (Lamberty *et al.*, 2001). Increased expression of AMPs has been shown in human newborn skin before more advanced immune defenses develop (Dorschner *et al.*, 2003). Also, high levels of AMP expression have been shown in plant germination both before and immediately after the seed coat breaks, at a time when other plant defenses are not fully functional (Terras *et al.*, 1993, 1995; Broekaert *et al.*, 1997).

AMPs are also being tested as treatments for a variety of human maladies, including cystic fibrosis-related lung infections and associated swelling (Zhang *et al.*, 2005), some types of cancer (Jacob and Zasloff, 1994; Doyle *et al.*, 2003), and

sexually transmitted diseases (STDs) including HIV and Herpes simplex virus (Yedery and Reddy, 2005). More diverse uses include disinfecting drinking water (Suarez *et al.*, 2002), use as a spermicidal contraceptive (Reddy *et al.*, 2004), and immobilizing *E. coli* on microtiter plates for detection (Gregory and Mello, 2005).

The modes of action of AMPs are diverse and not completely understood, but most rely on the amphipathic structure and attraction of cationic peptides to anionic microbial membranes (Epand and Vogel, 1999). Most AMPs are positively charged, and this charge is attracted to negatively charged lipids on prokaryotic cell membranes (Chen *et al.*, 2001), but not to cholesterol-containing membranes in advanced eukaryotic cells (Maloy and Kari, 1995; Tytler *et al.*, 1995). Various specific mechanisms have been proposed (Brogden, 2005), which generally show AMPs binding to prokaryotic membranes and disrupting ion transport and membrane permeability, which leads to cell death (Matsuzaki *et al.*, 1997; Ibrahim *et al.*, 2000). AMPs have also been shown to inhibit nucleic acid or protein synthesis (Brogden, 2005), to degrade mitochondria in fungi (Kristyanne *et al.*, 1997), and to change hyphal morphology or interfere with cell wall formation in fungi (Rajasekaran *et al.*, 2001).

Activity of AMPs against plant pathogens has been well documented both *in vitro* and *in vivo*. Cecropins, magainins, and synthetic magaininlike peptides have previously been shown to inhibit several bacterial and fungal plant pathogens *in vitro*, including *O. ulmi* (Jacobi *et al.*, 2000; Alan and Earle, 2002). Rice blight (*Rhizoctonia solani*) was strongly inhibited by cecropins and synthetic peptides (Oard *et al.*, 2004). Several important fungal phytopathogens including *Cryphonectria parasitica*, *Fusarium oxysporum*, and *Septoria musiva* were inhibited by synthetic magainin-based peptides, while host plant pollen germination was not affected (Powell *et al.*, 1995, 2000). Other plant pathogens that have been shown to be inhibited by a synthetic AMP include *V. dahliae* and *Phytophthora parasitica* (Rajasekaran *et al.*, 2001).

The many benefits of AMPs, especially their very broad antipathogenic activity and ease of synthesis and modification, make their encoding nucleotide sequences ideal candidates for use as transgenes. Many plants and even some animals have been transformed with AMPs, and most of these

have shown increased resistance to a particular pathogen or disease. Tobacco (*Nicotiana*), a model plant that is relatively easy to transform and propagate, has been transformed with a variety of types of AMPs, and the resulting transgenic plants were shown to be resistant to a variety of pathogens. Cecropin analogs increased resistance of tobacco to *Pseudomonas* bacterial wilt (Jaynes *et al.*, 1993), synthetic peptide D4E1 increased resistance to fungal pathogens (Cary *et al.*, 2000), a modified magainin increased resistance to an oomycete and bacteria (Li *et al.*, 2001), synthetic magainin analog MSI-99 increased resistance to bacteria and fungi when expressed from either the nuclear or chloroplast genome (DeGray *et al.*, 2001; Chakrabarti *et al.*, 2003), and overexpressed natural AMPs isolated from *Pharbitis nil* plants increased resistance to fungi (Koo *et al.*, 2002). Other nonwoody plants that have shown resistance to fungal pathogens when transformed with genes encoding AMPs include geranium, which was resistant to *Botrytis* (fungal blight) when transformed with a natural AMP from onion (Bi *et al.*, 1999); banana, which was resistant to multiple fungal pathogens when transformed with MSI-99 (Chakrabarti *et al.*, 2003); and potato, which was resistant to *Phytophthora* in tissue culture when transformed with a Cecropin-melittin hybrid AMP (Osusky *et al.*, 2000). More recently, potato was transformed separately with two magainin-class AMPs, both of which provided broad-spectrum blight and rot resistance and significantly increased potato storage times (Osusky *et al.*, 2004; Osusky *et al.*, 2005).

In addition to agricultural plants, some tree species have been transformed with AMPs and have also shown increased resistance to phytopathogens. For example, hybrid poplar was transformed with two AMPs (synthetic ESF12 and an Ac-AMP1 analog), and leaf disks with each construct showed fewer necrotic spots than nontransgenic controls (Liang *et al.*, 2002). Another hybrid poplar transformation resulted in increased resistance to two bacterial pathogens (*Agrobacterium tumefaciens* and *Xanthomonas populi*), but no significant effect on a fungal pathogen (*Hypoxyylon mammatum*) (Mentag *et al.*, 2003). Mixed results were also obtained with transgenic pear and apple trees expressing AMPs for resistance to fire blight and scab, respectively (Reynold *et al.*, 1999; Faize *et al.*, 2004).

Another plasmid construct, currently being tested in American elm transformations, includes a defensin isolated from spruce (A. Seguin, personal communication). Plant defensins are a class of AMPs consisting of small, basic peptides found in more than 80 plant species (Broekaert *et al.*, 1997). Like other AMPs, they inhibit a broad range of fungi, yet are nontoxic to mammalian or plant cells. They are mainly expressed in the peripheral cell layer, which is consistent with a role in a first line of defense against pathogens (Thomma *et al.*, 2002). PgD1 (*Picea glauca* defensin 1), a defensin isolated from *P. glauca*, has exhibited strong antifungal activity *in vitro* (Pervieux *et al.*, 2004). PgD1 was reported to be up-regulated by wounding and jasmonic acid treatment, as well as during seed germination. This suggests it is important in both constitutive and induced defense responses. A recombinant protein expressed by PgD1 *in vivo* was shown to cause extensive growth inhibition of three

fungus pathogens (*Cylindrocladium floridanum*, *F. oxysporum*, and *Nectria galligena*) (Pervieux *et al.*, 2004). Therefore, PgD1 could potentially be used to inhibit growth of ascomycete pathogens including *O. novo-ulmi*. Other studies have shown that transgenic expression of plant defensins can protect vegetative tissues from pathogen attack as well. For example, constitutive expression of a radish defensin enhanced resistance to the fungal leaf pathogen *Alternaria longipes* (Terras *et al.*, 1995), and constitutive expression of an alfalfa defensin in potato provided resistance to *V. dahliae* under field conditions (Gao *et al.*, 2000). Research is currently underway to investigate constitutive and vascular expression of PgD1 in transgenic American elm. PgD1 will be expressed in the elm vascular system by the cinnamyl alcohol dehydrogenase promoter *EgCAD* from *Eucalyptus gunni* (Feuillet *et al.*, 1995), from plasmid pMJMegCADDEF as seen in Figure 3.

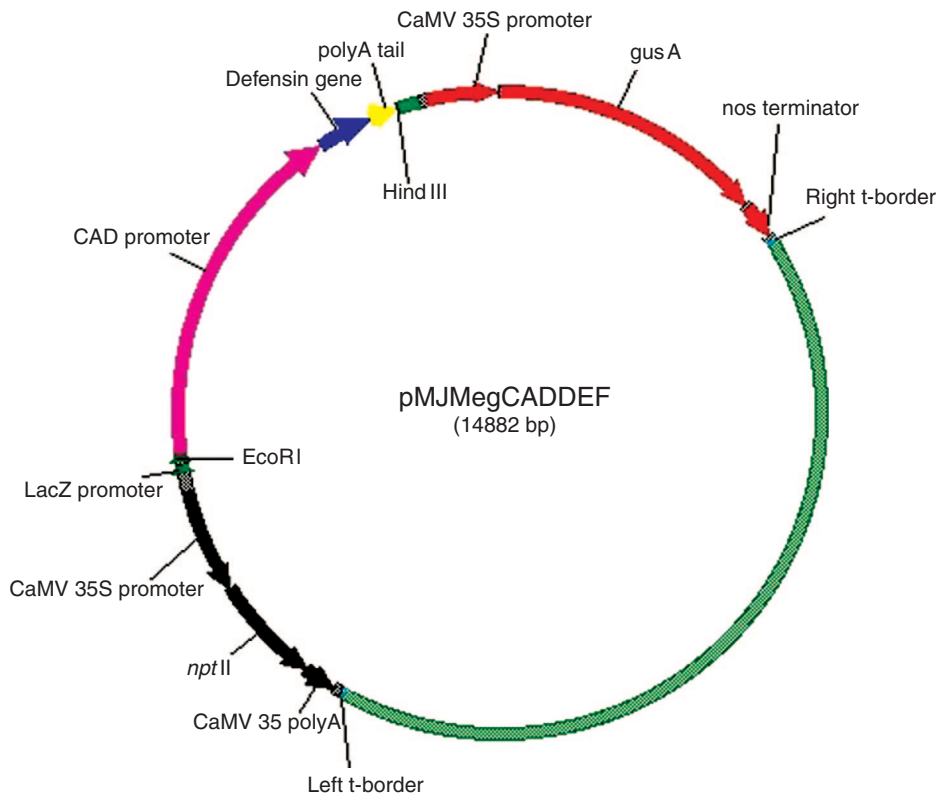


Figure 3 Plasmid pMJMegCADDEF [Source: A. Seguin, personal communication]

2.2 Methods Employed

American elm seed was purchased from F.W. Schmacher Co, Inc. (Sandwich, MA). American elm seedlings in pots and explants in tissue culture were grown on a light bench with a 16-h day, 8-h night cycle. For each transformation experiment, fully expanded young leaves were collected from seedlings, surface sterilized in 10% bleach for 10 min, rinsed, and sliced into approximately 1 cm² containing part of the midvein. Leaf pieces were co-cultivated with *A. tumefaciens* strain EHA105, containing the binary vector pSE39, in a minimal sucrose-based medium containing acetosyringone for *vir* gene induction (Gelvin and Habeck, 1990). After this liquid co-cultivation step, leaf pieces were placed on solid tissue culture media without selection for 3–5 days, rinsed with carbenicillin and cefotaxime, and then cultured on medium containing both of these antibiotics to ensure they were free of *Agrobacterium* (see Newhouse *et al.*, 2006 for a more detailed review of this transformation procedure and of tissue culture protocols and media).

2.3 Selection of Transformed Tissue

All putatively transformed shoots were regenerated under 100 µg ml⁻¹ kanamycin selection in tissue culture for at least 8–12 weeks (at least 3 transfers). When shoots were large enough to harvest a leaf tip (>9 mm²), they were screened with a GUS assay (see Section 2.1; Newhouse, 2005) to confirm the presence and expression of t-DNA in the plant genome. Each leaf tip was soaked in X-GLUC (5-bromo-4-chloro-3-indolyl β-D-glucuronide) overnight at 37 °C, and then soaked in 70% ethanol to remove chlorophyll from the tissue. Transformed tissue stained a dark blue color, while nontransformed tissue was colorless or light brown.

2.4 Regeneration of Whole Plants

Micropropagation or tissue culture of elm trees is essential for any genetic engineering program, and it would be beneficial for multiplying trees in traditional breeding or selection projects as well. At least two DED resistant European/Asian elm hybrids have been regenerated and multiplied in

tissue culture (Ben Jouira *et al.*, 1998; Mensuali-Sodi *et al.*, 1998). Furthermore, initial screenings for resistance to DED can be performed *in vitro*, which is easier and faster than inoculating trees in the field (Pijut *et al.*, 1990; Domir *et al.*, 1992; Krause *et al.*, 1996).

Elm trees were among the first woody plants to be successfully established in tissue culture (TC), from a cambial tissue explant of *Ulmus campestris* by R.J. Gautheret in 1940 (Karnosky and Mickler, 1986; Moore, 2003). Gautheret was not able to regenerate whole plants from his TC, however, and it was nearly 35 years before this was accomplished. *U. campestris* and *U. americana* were both successfully cultured and regenerated from stem section explants by two independent researchers around the same time (Chalupa, 1975; Durzan and Lopushanski, 1975). More recent elm TC efforts have focused on simplification and efficiency of the entire process. The use of leaf pieces as explants allows easier, less destructive explant collection, and more explants can be taken from each source plant. American elm leaf pieces were first used as explants in 1991 (Bolyard *et al.*, 1991b), and George and Tripepi (1994) studied American elm leaf explant TC conditions in more detail to improve regeneration efficiency.

Transformation of American elm for resistance to DED has been a goal almost as long as genetic engineering has been in existence (Sticklen *et al.*, 1991), but no report of *U. americana* transformation was published prior to the present study (Newhouse, 2005). The first elm to be transformed was the “Pioneer” hybrid (*Ulmus glabra* × *Ulmus carpinifolia*), for which internode stem sections were transformed separately with *Agrobacterium* and biolistics, each carrying the *GUS* gene (Bolyard *et al.*, 1991a). The hybrid elm “Commelin” ((*U. glabra* × *U. carpinifolia*) × *U. carpinifolia*) was cultured with an armed strain of *A. tumefaciens* and produced agropine-containing tumors, but no transgenesis with modified t-DNA was reported (Dorion *et al.*, 1995). English elm shoots have also been transformed with wild-type *Agrobacterium* (Fenning *et al.*, 1996), *Agrobacterium* carrying *GUS* and *nptII* genes (Gartland *et al.*, 2000), and more recently with *Agrobacterium* carrying a putative fungal resistance gene (Fenning *et al.*, 2005).

The TC protocol used in this experiment was originally inspired by an earlier publication showing regeneration of shoots from American

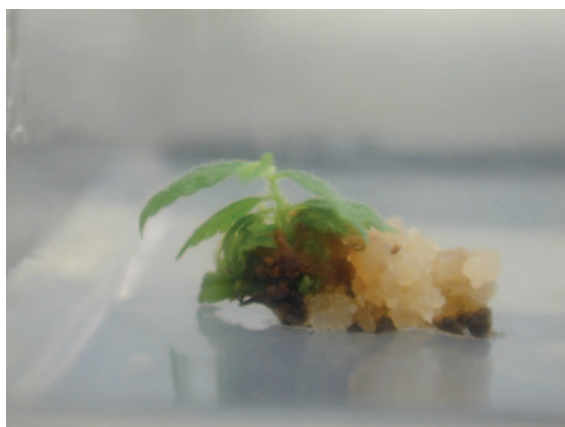


Figure 4 American elm shoot in tissue culture

elm leaf pieces (George and Tripepi, 1994), and is described in detail elsewhere (Newhouse, 2005; Newhouse *et al.*, 2006). Briefly, media consisted primarily of Driver and Kuniyuki Walnut medium (Driver and Kuniyuki, 1984) with 1X Nitsch and Nitsch vitamin powder (both from Phytotechnology Laboratories) supplemented with 22 μ M benzyl adenine (BA), 0.1 μ M indole-3 butyric acid (IBA), and 94 μ M AgNO_3 for shoot initiation; 5.5 μ M BA and 0.22 μ M IBA for shoot elongation; and 0.2% w/v activated charcoal for rooting. Leaves were placed face down (veins up) on Petri plates of shoot initiation medium until buds formed and just began to expand. Buds were transferred to MagentaTM cubes containing shoot elongation medium, where they multiplied and were subcultured every 3–4 weeks as new shoots formed (Figure 4). When shoots reached the top of the magenta cube or started to become woody, they were cut from the cluster of smaller shoots and callus, dipped in 1 mM naphthaleneacetic acid (NAA), and put in a tall MagentaTM cube of elongation medium. When roots became visible, the rooted shoot was carefully removed from the charcoal medium, potted in a peat-based potting mix, and slowly acclimated to ambient humidity.

2.5 Testing

2.5.1 Minimum inhibitory concentration (MIC) assay

The translated core of the ESF39A synthetic AMP (ESF22B) has been shown to inhibit growth



Figure 5 Pressure bomb apparatus

of multiple bacterial and fungal pathogens *in vitro*, and *S. musiva* specifically *in vivo* (Powell *et al.*, 2000; Liang *et al.*, 2002). However, the concentration at which this peptide inhibits growth of *O. novo-ulmi* had not previously been determined. Therefore, *O. novo-ulmi* was cultured with 0–100 μ M dilutions of ESF22B to determine the *in vitro* MIC. This procedure was based on the MIC assay by Catranis (1999).

ESF22B was suspended in sterile, distilled water, mixed with potato dextrose broth and low-melting point agarose, and diluted to a series of 12 concentrations ranging from 0 to 100 μ M in a 96-well culture plate. Approximately 100 *O. novo-ulmi* conidia (ATCC 34359) were added to each well. The plate was stored for 96 h in indirect light (16 h light, 8 h dark) at 23 °C. In five separate assays, 2.5–5.0 μ M ESF22B inhibited growth of *O. novo-ulmi*. However, when wild-type American elm sap was mixed with ESF22B and incubated at room temperature for 30 min prior to the assay, peptide concentrations of at least 42 μ M were required to inhibit *O. novo-ulmi*. A portable “pressure bomb” (PMS Instrument Company, Corvallis, Oregon) was used to extract total vascular sap from wild type and transgenic *U. americana* leaves (Figure 5). Two separate assays showed that the MIC of ESF22B, when exposed to wild-type American elm sap, was about 10 times greater than the MIC without sap (Newhouse *et al.*, 2007). Therefore, American elm sap or one of its constituents must have a moderately inhibitory effect on the activity of the ESF AMP. One possible cause

could be relatively high concentrations of cations such as Ca^{2+} in the elm sap. These cations could interfere with the specificity of AMPs to anionic membranes, which has been previously reported (Mills and Hammerschlag, 1993; Harrison *et al.*, 1997; Zhang *et al.*, 2005). This problem may be addressed by increasing expression levels of the peptide *in planta*, or by modifying the synthetic peptide so it is less sensitive to cations or other inhibitors in the sap.

2.5.2 Mycorrhizae

One question that should be raised when transforming plants to express antimicrobial compounds is whether beneficial microbes (i.e., mycorrhizal fungi) will be affected. Mycorrhizae are modified roots colonized with fungi in which, typically, the fungus supplies the plant with nutrients in exchange for energy in the form of photosynthate from the plant. Preliminary observations suggest that slower field growth of transgenic poplar constitutively expressing a wheat oxalate oxidase gene (Liang *et al.*, 2001) may be correlated with reduced mycorrhizal colonization (Horton and Powell, 2006). However, other research has shown that mycorrhizae may not abnormally affect or be affected by transgenic plants. For example, Turrini *et al.* (2004) concluded that aubergine (*Solanum melongena*) constitutively expressing a natural AMP isolated from *Dahlia* was resistant to pathogenic *Botrytis* and *Verticillium*, while symbioses with mycorrhizal *Glomus mosseae* were not significantly affected.

Mycorrhizae may have other effects on American elm pathogens as well, either directly or by contributing to the overall health of the plant. Several studies have explored possible relationships between mycorrhizal colonization and insect herbivores, which would be potentially applicable to the elm bark beetles that spread *O. novo-ulmi*. Conclusions of these studies varied widely, however, depending on the organisms studied and even on soil conditions (Borowicz, 1997; Rieske, 2001; Vicari *et al.*, 2002). Clearer results were seen when mycorrhizae were tested with some microbial pathogens. For example, mycorrhizal tomatoes infected with either fungal root pathogens or yellows-causing phytoplasmas showed less severe disease symptoms, and growth characteristics

more similar to healthy plants, when compared to nonmycorrhizal controls (Trotta *et al.*, 1996; Lingua *et al.*, 2002). This study is especially applicable to elm research, as the phytoplasma causing elm yellows is another significant threat to American elm trees (Sutherland *et al.*, 1997). Even if mycorrhizae do not have a direct effect on the DED pathogen or vector, healthy mycorrhizal relationships are important to consider when producing trees designed to have antimicrobial properties.

In order to quantify mycorrhizal colonization, roots were collected from healthy American elm trees with new growth approximately 3 months after field planting, from four wild-type seedlings and four transgenic plants. Staining was performed according to Brundrett *et al.* (1996). In brief, roots were rinsed over a 0.5 mm sieve and cleared by autoclaving at 121 °C for 15 min in 10% KOH (potassium hydroxide), fungal structures were stained by autoclaving again in Chlorazol Black E, and stained roots were stored in 50% glycerol at room temperature. Mycorrhizal colonization was quantified on 30 root samples from each plant using the “magnified intersections” technique (McGonigle *et al.*, 1990) with minor modifications. All individuals in both the transgenic and wild-type groups had formed extensive mycorrhizal associations within 3 months after field planting; differences in percent colonization between these groups were not significant ($p = 0.959$) in an unpaired *t*-test. Therefore, according to the data collected so far, mycorrhizal colonization of American elm trees is not affected by expression of the ESF39A AMP.

2.5.3 Molecular tests

DNA was extracted from wild type and transgenic elm leaf tissue using a modified hexadecyl trimethyl ammonium bromide (CTAB) procedure (Lodhi *et al.*, 1994). Polymerase chain reaction (PCR, Mullis and Faloona, 1987) with custom primers for the *ESF39a* and *GUS* genes confirmed T-DNA insertion into the elm genome (see Newhouse *et al.*, 2007 for more information on PCR primers and conditions). Southern hybridization was performed to distinguish between different transformation events (on buds originating from the same leaf piece) as well as to determine

transgene copy number. Genomic DNA was digested separately with two restriction enzymes, and a ^{32}P -labelled segment of the *GUS* gene was used as a probe. Single bands were produced in nine transgenic samples digested with two separate restriction enzymes. These nine individuals were determined to consist of multiple samples from only four transgenic events. Single bands provide evidence that each of these four lines contain only one copy of the T-DNA from the pSE39 construct. While it is unusual that all four tested events have only one copy of the t-DNA (Pappinen *et al.*, 2002; Polin *et al.*, 2006), *Agrobacterium* typically introduces fewer copies of transgenes than biolistics (Shou *et al.*, 2004), and there are several more elm transgenic lines that have not yet been tested for copy number.

2.5.4 Inoculation tests

Transgenic and nontransgenic trees were inoculated with *O. novo-ulmi* conidia to determine *in vivo* effectiveness of *ESF39a*. *O. novo-ulmi* (ATCC, 34359) was cultured on potato dextrose agar (PDA) for 1–3 weeks. Sterile water was washed over the fungal colony and diluted if necessary to a final concentration of about 1×10^8 conidia/ml. Trees at least two growing seasons old were inoculated with 20 μl of this spore solution, applied via a pipette into a vertical scalpel wound (Figure 6). Inoculated trees were kept in a growth chamber



Figure 6 Inoculation of young *U. americana* with *O. novo-ulmi*

or greenhouse at room temperature and observed for 11–15 weeks. Leaf color, presence of wilting, and any loss of leaves was observed. Wild-type seedlings were inoculated with sterile water to determine whether the inoculation procedure was harmful to the tree, and no staining or wilting was observed in these control trees.

After the inoculation observation period, inoculated trees were once again collected in the lab. Each tree was felled with a large scalpel or utility knife at least 2 cm below the inoculation point, leaving at least 2 cm of stump above the soil level. Each removed shoot was cut at an angle to observe any staining at the inoculation point and sectioned every 2 cm above and below the inoculation point. Sections of stained stem tissue were surface sterilized, cut into wedges, and placed on PDA media. *O. novo-ulmi* was successfully recultured from all stained tissue cultured in this manner, but not from unstained tissue or from noninoculated trees.

Some symptomatic differences were observed after an *O. novo-ulmi* inoculation experiment in which six nontransformed TC-derived trees were compared to six transgenic trees from each of two lines (designated FS5 and AN1). Eleven weeks after inoculation, nontransformed trees retained an average of 9% of their leaves, FS5 trees retained 41%, and AN1 trees retained 67%. These numbers decreased to 7%, 18%, and 52%, respectively, after 13 weeks, and were proportionally similar after 14 weeks. Despite the consistent trends, none of these differences in leaf retention between clones were statistically significant ($p > 0.05$).

Staining was present to some extent in all trees from this inoculation experiment, but the maximum distance at which the staining was observed below the inoculation point (Scala *et al.*, 1997) varied significantly 14 weeks after inoculation (Newhouse *et al.*, 2007). ANOVA and Tukey's Honestly Significantly Different (HSD) test revealed that in AN1 trees, staining spread a significantly shorter distance below the inoculation point than in nontransgenic control trees ($p < 0.05$), but spread of staining in FS5 trees was not significantly different from the other groups. Additionally, stem tissue above the inoculation point was dried and dead in four of the six nontransgenic controls and four of the six FS5 trees, but the entire stem was alive in all six AN1 trees.

Inoculation results from this project should be considered preliminary, since young elm trees are not as susceptible to DED as are mature trees (Townsend *et al.*, 1995; Gartland *et al.*, 2000). Furthermore, susceptibility can vary according to season and environmental conditions (Sutherland *et al.*, 1997; Hubbes, 1999), and the greenhouse conditions used in this study may not represent true field conditions. Expanded field inoculation tests are currently being planned.

2.6 Specific Regulatory Measures Adopted

The ESF39A AMP was designed specifically to be harmless to humans and animals, and since it is only expressed in the vascular system, the authors do not believe it poses any special risks. At the time of writing, the SUNY College of Environmental Science and Forestry has a 5-year USDA permit for temporary controlled field-testing of transgenic elm trees (see Section 3.2). Long-term permissions and other permits are pending.

3. FUTURE ROAD MAP

3.1 Expected Products

Most genotypes of American elm are relatively easy to propagate via softwood cuttings (Doran and McKenzie, 1949; Hartmann and Kester, 1983). This will facilitate the large-scale production of planting stocks of resistant clones that could be released as patented named varieties. The transformation events described in this chapter will be evaluated in field tests along with “Liberty”, “New Harmony”, “Valley Forge”, and possibly other reportedly DED-resistant American elm varieties. If the ESF39A AMP does confer DED resistance at least as strong as the other resistant American elm varieties, the authors plan to, at a minimum, patent, clonally propagate, and release the most resistant transformation event as a new variety. The authors would prefer to transform, test, and release several additional clonal varieties, each derived from a different genetic background and transformation event. However, all these goals depend on the availability of funding, the level of resistance provided by the ESF39A peptide, and the cost and effort required to successfully obtain a

“Determination of Nonregulated Status” from the United States Department of Agriculture Animal and Plant Health Inspection Services Biotechnology Regulatory Service (USDA-APHIS BRS).

3.2 Addressing Risks and Concerns

The transgene products described in this study were designed specifically to be harmless to plants and animals, and since they are not expressed in pollen, and humans do not consume elm products, potential allergic reactions should not be a concern. The transgenic trees will be field-tested under permits from the USDA-APHIS BRS. Once transgenic lines are identified that demonstrate substantial pathogen resistance and are substantially equivalent to nontransgenic clones, applications for deregulation will be submitted to the two US agencies that have regulatory oversight over the release of transgenic plants: USDA-APHIS BRS and the Environmental Protection Agency (EPA). Since the elms do not produce a food crop, they do not need to be reviewed by the Food and Drug Administration (FDA). To ensure transparency of the research and raise public awareness, educational outreach plots mirroring the larger field trials have been planted (Figure 7).

Ideally, genes for American elm disease resistance could be isolated from DED-resistant species within *Ulmus*, and employed as transgenes controlled by wound-induced or vascular-specific promoters from other closely related species (Hollick and Gordon, 1993; Connors, 2001). It could also be beneficial to study relative gene expression levels in American elm trees that show unusually high levels of tolerance to DED, such as the Jefferson Elm that was originally thought to be an American–Asian hybrid. A gene encoding a chitinase-like protein has been isolated from this elm clone, and was found to be effective in reducing brown patch disease when expressed in creeping bentgrass (Chai *et al.*, 2002). It is possible that simply changing the expression pattern of an existing elm gene through the use of a different promoter could provide increased levels of resistance not found in most American elms. These existing genes could include chitinases, as well as those that control tylose formation, vessel diameter, or mansonone production. Other potential sources for transgene products could



Figure 7 Public educational outreach plot featuring transgenic American elm

be based on existing DED control mechanisms, including a gene product based on d-factors (the naturally occurring viruslike particles in some *O. novo-ulmi* populations). These would make use of DED devices that have already evolved specifically with this pathogen, and would likely reduce public concern associated with donor genes from distantly related organisms.

The transgenic American elms resulting from transformations with naturally occurring, DED-related genes or promoters would be resistant to *O. novo-ulmi* specifically, and effects on nontarget organisms would theoretically be almost nonexistent. However, microarray or other gene expression analysis and isolating disease resistance genes can be complicated and time-consuming processes. Therefore, the most efficient alternative for the time being is to use known genes with the highest fungal resistance and fewest unintended effects possible. AMPs, as described in this publication, clearly have great potential for control of DED.

One important consideration for any DED research program is susceptibility to elm yellows. The causative organism of this disease, variably called a phytoplasma (Lingua *et al.*, 2002) or a mycoplasma-like organism (Zhao *et al.*, 2004), is part of a weakly defined group of microorganisms that cause yellows diseases by infecting phloem tissue in a variety of plant groups. They are usually spread by hoppers or other sucking insects, and cannot be cultured *in vitro*, so inoculation experiments rely on propagation of infected insect vectors or grafting of infected plant tissue. Symptoms of elm yellows, as the name suggests, start with yellowing of leaves and usually result in death within a few seasons of infection (Sinclair, 2000). Identification of infected trees before symptoms are visible (or if symptoms may be confused with those of DED) can be accomplished by PCR with phytoplasma-specific primers (Lee *et al.*, 1993). One potential way to address elm yellows is to transform elms with

phloem-specific promoters (Zhao *et al.*, 2004), which could be used to control expression of AMPs or other gene products. The vascular-specific promoter (*ACS2*) used in the current project should be tested to determine whether it is active in both phloem and xylem vessels, which has been reported for homologous gene promoters in other plant systems (Zhang *et al.*, 2000).

It is of course possible that pathogens could develop resistance to almost any transgene product. However, assuring the continued presence of wild-type susceptible individuals, avoiding large plantings of a single clonal transformant, and transforming plants with multiple genes (Dixon, 2001) are all effective strategies for reducing pathogen-acquired resistance. *Agrobacterium*-mediated transformation with multiple constructs (Li *et al.*, 2003) is one method of “pyramiding” resistance genes, or a single construct can be modified to express multiple protein products in different tissues (Francois *et al.*, 2002).

3.3 Expected Technologies

The current research suggests that higher pSE39 expression levels could be more effective against *O. novo-ulmi*. This may necessitate the use of a different promoter, or it could be as simple as testing or creating more transformed lines, since expression can vary greatly between multiple lines transformed with the same construct (Peach and Velten, 1991). In addition to the AMPs described above, transgenes that hold particular promise for phytopathogen resistance in trees include chitinases (Pappinen *et al.*, 2002; Pasonen *et al.*, 2004), oxalate oxidases (Liang *et al.*, 2001; Polin *et al.*, 2006), and RNA interference (Escobar *et al.*, 2002) (see Powell *et al.*, 2006 for a more thorough review of tree transgenesis research progress to date). Oxalate oxidase (OxO) may be especially appropriate for transgenic tree restoration work (Grover and Gowthaman, 2003; Powell *et al.*, 2006). This enzyme converts oxalic acid to H₂O₂, which can have direct antifungal properties and reduce fungal pathogenicity, as well as serving as a defense response signal to stimulate natural plant defenses (Stuiver and Custers, 2001). Oxalate oxidase can also be used as a quantifiable reporter gene, since a spectrophotometric enzyme assay shows where and how much it is being

expressed (Simmonds *et al.*, 2004). Transgenic plants expressing OxO have been shown to be resistant to fungal pathogens *in vivo* (Liang *et al.*, 2001; Livingstone *et al.*, 2005). OxO may be particularly well suited for use against DED, as De Rafael *et al.* (2001) showed that H₂O₂ significantly slowed growth of *O. novo-ulmi*, and it is also part of the natural defense response in the DED-resistant *U. pumila*. *O. novo-ulmi* has not been tested for production of oxalate that could then be converted to H₂O₂, but many other ascomycetous phytopathogens are known to produce oxalate (Foster, 1951; Dutton and Evans, 1996), and the potential benefits for use of OxO as a transgene should warrant further investigation. OxO has also shown promise for *in vivo* use against insect pests, so simultaneous control of the fungus and vector may be possible. Specifically, European corn borer feeding and larval growth were both reduced when OxO was expressed in corn leaves (Ramputh *et al.*, 2002).

3.4 Public Perceptions and Economic Consequences

Informal surveys have indicated that public perception of transgenic heritage trees for restoration purposes may be higher than that of transgenics used to increase crop yields (W. Powell, personal observations; Fenning *et al.*, 2005). The vascular-specific promoter used in this research was cloned from the American chestnut tree, and if appropriate disease-resistant genes and regulatory sequences were found in other trees, public acceptance may be even greater.

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Black Walnut

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Black walnut (*Juglans nigra* L.), also known as eastern black walnut or American walnut, is a premier, commercial, hardwood timber (and nut) species. The genus *Juglans* is derived from the Roman name *Jovis glans* meaning “nut of Jove (Jupiter)” (Goodell, 1984), and *nigra* meaning “black” in reference to the dark wood, bark, and nut husks. Ancient fossil records date walnuts to the middle to upper Cretaceous period (Elias, 1980). Native to the deciduous forests of the eastern United States from Massachusetts to Florida and west to Minnesota and Texas, and occurring naturally in southern Ontario, Canada, black walnut is seldom found in pure stands, but rather in association with five mixed mesophytic forest cover types: (1) sugar maple (*Acer saccharum* Marsh.); (2) yellow poplar (*Liriodendron tulipifera* L.); (3) yellow poplar-white oak (*Quercus alba* L.)—northern red oak (*Quercus rubra* L.); (4) beech (*Fagus grandifolia* L.)—sugar maple; and (5) silver maple (*Acer saccharinum* L.)—American elm (*Ulmus americana* L.) (Williams, 1990). Historically, the bark of black walnut was used by several Native American tribes, including the Cherokee, Delaware, Iroquois, and Meskwaki, in tea as a cathartic, emetic, or disease remedy agent, and chewed or applied for toothaches, snake bites, and headaches (Moerman, 1998, 2003).

Caution: the bark should be used cautiously in medicine because it is poisonous. The Cherokee, Chippewa, and Meskwaki also used the bark to make a dark brown or black dye (Moerman, 1998, 2003). The Comanche pulverized the leaves of black walnut for treatment of ringworm, the Cherokee used leaves to make a green dye, and the Delaware used the leaves as an insecticide to dispel fleas (Moerman, 1998, 2003). The nut meats were also a food source for Native Americans, and the nuts are still consumed today by people and are an important food source for wildlife, especially squirrels (Goheen and Swihart, 2003). A toxic chemical “juglone” (5-hydroxy-1, 4-naphthoquinone), naturally occurring in the leaves, buds, bark, nut husks, and roots of black walnut, is a highly selective, cell permeable, irreversible inhibitor of the parvulin family of peptidyl-prolyl *cis/trans* isomerases (PPIases) and functions by covalently modifying sulfhydryl groups in the target enzymes (Henning *et al.*, 1998; Chao *et al.*, 2001). Certain plants, especially tomato, apple, and several conifer species, are adversely affected (allelopathy; foliar yellowing, wilting, and even death) by being grown near the roots of black walnut trees (Goodell, 1984; Dana and Lerner, 1994). Horses can contract acute laminitis, an inflammation of the foot, when black walnut wood chips or sawdust is used for stall bedding or stables, and paddocks are located too close to walnut trees (Galey *et al.*, 1991).

1.2 Botanical Description

Black walnut is a member of the family Juglandaceae, section *Rhysocaryon* (Manning, 1978). In general, *J. nigra* will not cross with species in the sections *Cardiocaryon* or *Trachycaryon*, but *J. nigra* will cross with *J. ailantifolia* (*Cardiocaryon*) (Williams, 1990). *J. nigra* will also hybridize to some extent with other *Juglans* species (*Dioscaryon* and *Rhysocaryon*) and one hybrid is recognized: *J. nigra* \times *J. regia* = *J. \times intermedia* Carr. (USDA-NRCS, 2004). *J. nigra* is the largest and the most valuable timber tree of all the *Juglans* species, and it is hardy to USDA (United States Department of Agriculture) hardiness zone range of four to nine (Dirr, 1998). Black walnut is a large tree and on good sites may attain a height of 30–38 m, diameter of 76–120 cm, and can exceed 100 years of age (Williams, 1990; Dirr, 1998; USDA-NRCS, 2004). Black walnut is shade intolerant, and control of competing vegetation is especially important in new plantations for the first 3–4 years. Black walnut grows best on moist, deep, fertile, well-drained, loamy soils; although it also grows quite well in silty clay loam soils or in good agricultural soils without a fragipan (Williams, 1990; Cogliastro *et al.*, 1997). These sites include coves, bottomlands, abandoned agricultural fields, and rich woodlands. Black walnut forms a deep tap root, wide-spreading lateral roots, and has been cultivated since 1686. Black walnut is monoecious with male and female flowers maturing at different times (McDaniel, 1956). Staminate catkins (5–10 cm) develop from axillary buds on the previous year's wood and appear as small, scaly, conelike buds, and the female flowers occur in two to eight flowered spikes borne on the current year's shoots (Brinkman, 1974; Williams, 1990; Flora of North America Editorial Committee, 1993; Dirr, 1998). The female flowers more commonly appear first (protogyny) and flowering occurs with or shortly after the leaves. Because flowering is dichogamous, self-pollination is unlikely, thereby promoting outcrossing. The fruit is a drupelike, furrowed nut enclosed in a thick, indehiscent yellowish-green husk that develops from a floral involucre (Brinkman, 1974). Fruits are subglobose to globose, rarely ellipsoid, 3.5–8 cm, warty, with scales and capitate-glandular hairs (Flora of North America Editorial Committee, 1993). The fruit

occur singly or in clusters of two to three and are edible, sweet, oily, and high in protein (Reid, 1990). The nut is subglobose to globose, rarely ellipsoid, 3–4 cm, very deeply longitudinally grooved, and the surface between the grooves is coarsely warty (Flora of North America Editorial Committee, 1993). Leaves are alternate, pinnately compound, 30–60 cm long, with 9–23 leaflets, nearly glabrous and somewhat lustrous dark green, pubescent and glandular beneath, with petioles 6.5–14 cm long covered with glandular hairs (Flora of North America Editorial Committee, 1993; Dirr, 1998). Black walnut stems are stout, densely gray-downy, smooth and reddish buff; have a chambered light brown pith (paler than that of butternut, *Juglans cinerea*), and a distinctly notched leaf scar. Terminal buds are ovoid or subglobose, 8–10 mm long, and weakly flattened (Flora of North America Editorial Committee, 1993). Lateral buds are smaller, often superposed, and grayish in color. *J. nigra* has a dark gray or brownish bark, deeply split into narrow furrows and thin ridges, the ridges are chocolate in color when cut, forming a roughly diamond-shaped pattern. The wood of black walnut is semi-ring porous (Miller, 1976), with the sapwood nearly white and the heartwood varying from light to dark brown. Vessel element lengths average 517–666 μm , fiber-tracheid lengths 1204–1634 μm , ray height 229–355 μm , and pore diameter averages 170–202 μm (Miller, 1976). The wood is heavy, hard, strong, normally straight grained, and has good resistance to shock (Forest Products Laboratory, 1999). The chromosome number of black walnut is $2n = 32$ (Woodworth, 1930). Black walnut trees produce seed at about 12 years of age, with good seed crops occurring every 2–3 years (Brinkman, 1974). Seeds of black walnut, like most *Juglans* spp., have a dormant embryo, but dormancy can be broken by fall sowing or by moist prechilling of seeds at 1–5°C for 3–4 months (Brinkman, 1974; Williams, 1990). Commercial propagation is by seed or grafting of selected cultivars (Reid, 2001).

1.3 Economic Importance

Black walnut is one of the largest hardwood trees found in the United States, and is valued economically and ecologically for its wood and

edible nuts. Quality black walnut wood demands high market prices for many uses including furniture, veneer, cabinets, interior architectural woodwork, flooring, and gunstocks. Black walnut wood with figured grain demands even higher market prices. Curly and wavy figure can produce interesting characteristics in veneers, and these can arise from walnut butts, crotches, burls or in rare cases, occur throughout the wood of the whole tree. The nut is an important food source for wildlife and is also consumed by humans. The majority of black walnut trees occur in natural stands, with walnut plantations (approximately 13 800 acres) accounting for 1% of all the black walnut volume (ft³) in the United States (Shifley, 2004). There are 11 states that currently have the greatest volume of black walnut growing stock on timberland, and these include Missouri, Ohio, Iowa, Indiana, Illinois, Tennessee, West Virginia, Kansas, Pennsylvania, Virginia, and Michigan (Shifley, 2004). Since the last (1997) comprehensive inventory and summary of the black walnut resource in the eastern United States, the number and volume of black walnut trees has increased, except in Michigan, Virginia, and Pennsylvania where walnut volume is level or decreasing (Shifley, 2004). In addition to the multimillion dollar US market consumption of walnut wood, for the period 2001–2005, the United States exported walnut lumber to 68 area/countries (80 069.9 m³; \$60 110 242; averages for this period) and walnut logs to 51 area/countries (88 814.2 m³; \$57 693 282; averages for this period) (USDA-FAS, 2006). Black walnut yields edible nuts that are used in baking (cookies, cakes, etc.) and ice cream products. The Hammons Products Company (Stockton, MO; <http://www.black-walnuts.com>) is the world's premier processor and supplier of American black walnuts for both food and industrial uses. The Lodi Nut Company (Lodi, CA; <http://www.lodinut.com>) is a large retail supplier of bulk black walnut nuts and gift baskets. Selection of black walnut trees for nut quality and production has developed slowly over the years, but over 700 cultivars have been named and the percent of edible kernel has improved to over 34% (Reid, 1990; Reid *et al.*, 2004). Black walnuts are low in saturated fats (3.4 g per 100 g edible nut), have zero cholesterol, and are high in polyunsaturated (35.1 g per 100 g edible nut) and monounsaturated fats (15 g per 100 g

edible nut) (USDA-ARS, 2004). Black walnuts are also a good source of protein (24.1 g per 100 g edible nut) and fiber (6.8 g per 100 g edible nut) containing low levels of sugar (1.1 g per 100 g edible nut) (USDA-ARS, 2004). Processed black walnut shell is a soft grit, durable, nontoxic, biodegradable abrasive product used for many industrial purposes. It is used for metal cleaning and polishing, in oil well drilling, for plaster effect in paints, as filler in explosives, and as a grit agent in soaps, cosmetics, and dental cleansers (Thomas and Schumann, 1993). Because of the various economic values mentioned above, breeders have been interested in employing conventional breeding to improve some of the economic traits.

1.4 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

The majority of black walnut conventional breeding has focused on improvement of the species for timber traits and these activities have mostly occurred in the Midwest. Although monoculture plantations of black walnut for timber production date from the 1600s, modern efforts at genetic research were initiated in the early 1950s (Minckler, 1952, 1953; Wright, 1954). In the last 50 years, genetic research has included studies of breeding methods, development of breeding resources, genetic variance, heritability and selection age, adaptation, and use of hybrids.

Height and diameter growth are the most studied traits in black walnut and these traits can help predict adaptation to a site. In early generations of selection, heritability estimates for these growth traits are very high. It was reported by Rink and Clausen (1989) that results of three progeny tests at age 13 showed heritability (h^2) for height was about 0.41, and there were significant family \times site interactions. Kung *et al.* (1974) found h^2 for height growth was approximately 0.4, although Rink (1984) and Beineke (1974) indicated a value slightly higher ($h^2 = 0.55$). Heritability of diameter growth is in the same range as height ($h^2 = 0.35$ – 0.65) (Beineke and Stelzer, 1991; Rink and Kung, 1995). However, Woeste (2002) found a lower value ($h^2 = 0.28$)

based on a 35-year-old progeny test. Hammitt (1996) and Rink (1997) found that heritability of this trait was near zero at outplanting, increased over the first 10 years, and stabilized around age 15.

Estimates of the heritability of tree form range from 0.4 to 0.5 (Beineke, 1989; Beineke and Stelzer, 1991). Estimates for the other heritability factors include foliation date (0.92), defoliation date (0.73), sweep (0.32), number of crooks (0.24), branch angle (0.20), and branch number (0.41) (Beineke, 1974). Heritability estimates for multiple stems (0.18), leaf drop date (0.13), insect damage (0.27), and leaf angle (0.32) were reported by Bey (1970).

Walnut anthracnose causes foliage to senesce and trees to defoliate earlier than more resistant genotypes. Anthracnose resistance may be highly heritable (Funk *et al.*, 1981; Woeste, 2002), and this could be important in extending the growing season and thus the growth of black walnut. Attempts to associate anthracnose resistance with growth were not conclusive in earlier published studies (Todhunter and Beineke, 1984), but recently, control with fungicides has shown to increase late season growth in industrial plantations (Pardillo, personal communications).

The heritability of important wood quality traits such as heartwood formation and color and wound recovery require destructive sampling of mature trees. Nelson (1976), Rink (1987), and Woeste (2002) all report that heartwood area has a moderate to high heritability ($h^2 > 0.4$), possibly because this trait is strongly associated with tree vigor (Woeste, 2002). Rink (1987) was unable to find any genetic component to heartwood color.

The rotation age for black walnut is more than 60 years, thus selection of juvenile trees for their anticipated rotation-age value is essential. Juvenile-mature correlations and rank correlations for height and diameter growth based on progeny tests were reported by various authors (McKeand *et al.*, 1979; Rink, 1984; Beineke, 1989; Rink and Kung, 1995). In general, these studies found family selection for high heritability traits of height and diameter growth can begin by about age 8, but that within family selection should be delayed until after age 12. The optimal age for selection depends on thinning schedules and site quality, as these factors influence selection intensity,

intertree competition, and trait heritability (Kung, 1973).

Provenance trials to measure the effects of long-distance seed movement have been a fundamental part of black walnut improvement. These provenance trials were established to characterize regional genetic variability and to determine the relationship between the latitude of seed sources and tree growth. The underlying rationale was that trees from southern sources would leaf out earlier and lose their leaves later than trees from more northern latitudes. Longer growing seasons, in turn, would translate into faster growth. Frost injury, which can cause poor form, and dieback from winter injury, were considered potential drawbacks to the use of southern seed sources. Reports by Bey and Williams (1975) indicated that trees from provenances south of the planting site would perform well compared to local sources. Bey (1979, 1980), and Bresnan *et al.* (1992, 1994) refined this analysis. Results from a large number of provenance studies provide a general guideline that recommends planting of seeds from sources 200 miles (322 km) to the south for optimal growth. The use of grafted trees to evaluate site effects and adaptability is only now underway (Woeste and McKenna, 2004).

Rink *et al.* (1989) were able to determine the mating system parameters for black walnut using allozyme systems. The goal of the research was to determine the level of inbreeding and outcrossing of black walnut in native stands and the allocation of genetic variance at marker loci among and within populations. Rink found that black walnut had a high outcrossing rate (about 90%) and was highly heterozygous based on eight loci. The 26 maternal trees in the study were significantly more heterozygous than their progeny, and the authors suggested this might have been caused by selection against inbred progeny. These findings were substantiated by later research (Rink *et al.*, 1994; Busov *et al.*, 2002). These later studies also showed that the mating parameters of black walnut seed orchards were similar to those found in wild stands, and that as much as 94% of the variance in the isozyme marker loci was distributed within populations, i.e., that very little differentiation among populations could be detected at the level of allozymes. Fjellstrom (1993) found high levels of heterozygosity in black walnut using restriction fragment length polymorphisms, and Woeste has

indicated that black walnut is highly heterozygous at microsatellite loci (Woeste *et al.*, 2002) as well.

Interspecific *Juglans* hybrids have unusual vigor. The “Royal” hybrid (*J. hindsii* × *J. nigra*) has been impractical in the Midwest because of poor cold tolerance, but in milder climates such as California and Pacific Northwest, it appears to have excellent potential as a timber tree (Forde and McGranahan, 1996). Hybrids between the *J. nigra* × *J. regia* known as *J. × intermedia* Carr. have attracted the most interest in the United States (Wright, 1966; Funk, 1970) and Europe (Hussendorfer, 1999; Germain, 2004). Funk (1970) summarized the hybridization research of McKay (1965) and others who found that while *J. × intermedia* had variable vigor, it was nearly sterile, and all attempts to restore fertility through the production of amphidiploids failed. The clonal deployment of *J. × intermedia* Carr. has occurred in France, and the possible uses of seedling hybrid rootstock for timber production in the Midwestern United States was discussed by Woeste and McKenna (2004). In addition, they cite the potential of *J. nigra* × *J. major* hybrids as a rootstock for drier sites. The identity and parentage of *Juglans* hybrids can be verified using intersimple sequence repeats (Potter *et al.*, 2002).

1.5 Limitations of Conventional Breeding and Rationale for Transgenics

In some cases, genes for traits of interest do not exist within the native population of black walnut or addition of the trait requires introgression of several or more genes at the same time in order to achieve expression of the trait. Some examples for black walnut are resistance to glyphosate and sulfometuron methyl herbicides, flowering control or sterility, early heartwood formation, and curly grain. Genes for some of these traits have been described and methods have or will be developed in order to insert them into the black walnut genome. In other cases, genes for some traits have yet to be identified and characterized. In theory, using transgenic technologies, foreign genes can be inserted, plants selected, and the transgenes expressed that will result in elite clones with the new traits that could not be accomplished with breeding.

2. DEVELOPMENT OF TRANSGENIC WALNUTS

2.1 Review of Prior Literature

Fang and Wang (2000) achieved somatic embryogenesis from black walnut tissues and developed an *Agrobacterium*-mediated transformation system to insert genes into the somatic embryos. In this research, immature embryos and leaf explants from black walnut grown in China were acquired 1 month after pollination. Woody plant medium (WPM) developed by Lloyd and McCown (1981) with 2.0–6.0 mg l⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.1–0.5 mg l⁻¹ KT (kinetin) was used to induce callus formation, which occurred on the explants after 1 month of culture. Then WPM with 0.1–0.2 mg l⁻¹ 2,4-D, 0.1–0.5 mg l⁻¹ KT, and 1 mg l⁻¹ hydrolyzed casein was used to induce the formation of somatic embryos from callus, and different stages of somatic embryos developed on the medium. Microshoot and root development were observed from the somatic embryos when cultured on WPM basal medium. The induction ratio of callus and somatic embryos from immature embryo explants (67.7% and 25.36%, respectively) were higher than that from immature leaf explants (15.4% and 2.4%, respectively), thus immature embryo explants were used to develop the genetic transformation system. *Agrobacterium*-mediated transformation was carried out following the method by McGranahan *et al.* (1988), but somatic embryo pieces around 5 mm and an *Agrobacterium* strain containing binary vector with *uidA* and *nptII* (neomycin phosphotransferase II) genes were used for infection. Co-cultivation was carried out on somatic embryo induction medium for 2–3 days, following by selection on the same medium with 100 mg l⁻¹ kanamycin and 500 mg l⁻¹ cefotaxime. β -glucuronidase (GUS) staining was used to assist selection of transformed embryos. Completely transformed somatic embryos were acquired after five generations of secondary embryo production, but the selection efficiency reached 90% by the third to fourth generation. Plantlets with shoot and root were developed after 20 days cultured on WPM with 0.5 mg l⁻¹ BA (benzyladenine), 0.2 mg l⁻¹ IBA (indole-3-butyric acid), 1.0 mg l⁻¹ GA (gibberellic acid), and 100 mg l⁻¹ kanamycin. Transgenes were verified by

polymerase chain reaction (PCR) and southern hybridization of transgenic embryos and plantlets. Comparison experiments with different stages of somatic embryos indicated that globular and torpedo embryos had higher transformation and secondary embryo production efficiency than cotyledonary embryos and callus, and it was very difficult to induce secondary embryos from mature cotyledonary embryos.

Kanamycin resistant black walnut transgenic lines were developed by Bosela *et al.* (2004). In this project, somatic embryos were induced from immature zygotic embryo cotyledon pieces of open-pollinated elite black walnut trees. More than 40 lines of somatic embryos of different genotypes with different induction media were established, some of which are still constantly maintained *in vitro* on a 3–5-week subculture cycle on MS (Murashige and Skoog) or DKW (Driver and Kuniyuki) media. Globular and cotyledonary embryos were induced on media without or with 2,4-D. Globular and cotyledonary embryos were proliferated by repetitive secondary embryo production, where the secondary embryos were directly attached to the parent somatic embryo or lacked obvious connection. Different *Agrobacterium* strains containing different binary vectors harboring *uidA* (with or without intron) and *nptII* genes were used to infect immature cotyledonary somatic embryos 1.0–1.25 cm in width for 20–30 min. Co-cultivation was carried out on basal medium with 100 μ M acetosyringone for 60 h in the dark, following by several washes with and culture on antibiotic containing media. Selection were carried out on medium with 250 mg l⁻¹ kanamycin for one experiment, and T-DNA transfer ratio of 75–100% was obtained for 10 genotypes as indicated by GUS staining, but less than 10% of the primary embryos produced secondary embryos on the selection media. In another experiment (Smagh, 2005), five different genotypes were infected with *Agrobacterium*, with T-DNA transfer ratio from 54% to 100% among genotypes. Secondary embryo production on 100 mg l⁻¹ kanamycin was 55% for infected embryos. After three generations of secondary embryo production, completely transformed embryos were obtained and verified by PCR.

In a current study to develop herbicide resistant black walnut, the selection dose of the active

ingredient of ArsenalTM has been determined for black walnut somatic cotyledonary embryos (Liang, personal communication) and construction of binary vector containing an ArsenalTM resistance gene for black walnut transformation is underway. No transformation experiments have been performed to date.

One of the critical steps in production of transgenic black walnut is to induce the transformed tissue to regenerate roots. The presence of flavonoids in walnut tissues is speculated to inhibit the adventitious rooting process, which is often crucial for propagation of clonal hardwood trees, so an antisense gene strategy was used to target the chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis, and investigate how the flavonoid content could influence rooting capacity (El Euch *et al.*, 1998). In this study, cotyledons of an immature hybrid walnut (*J. nigra* \times *J. regia*) were used as explants to induce somatic embryos, which were maintained by repetitive embryogenesis on basal DKW medium. For transformation, embryos 2–5 mm in width were used for infection with an *Agrobacterium* strain containing the antisense vector that harbored a *uidA* gene with intron, a *nptII* gene, and a antisense chalcone synthase (*chs*) gene, or control vector that harbors only *uidA* and *nptII* genes. Co-cultivation occurred on basal DKW (Driver and Kuniyuki, 1984) medium for 48 h at 24 °C. DKW medium with 500 mg l⁻¹ kanamycin, 250 mg l⁻¹ cefotaxime, and 500 mg l⁻¹ tacarpen was used as selection medium. Of the first generation of secondary embryos produced after transformation, 20 out of 80 embryos stained positive in histochemical GUS tests. Embryos produced in following generations were maintained as homogeneous lines, and 13 lines transformed with the antisense vector were obtained. A control transformed line and an untransformed line were also maintained. Embryo epicotyls were placed on DKW medium with 1 mg l⁻¹ BAP (6-benzylaminopurine) and 1 μ g l⁻¹ IBA for 3 weeks to produce microshoots, which were sub-cultured four times on a 3-week cycle before been tested by various means. Northern blotting and RT-PCR were used to characterize the *chs* genes present in the hybrid walnut. CHS activity and flavonoid content were determined and six antisense lines expressed flavonoid deficiency at the mRNA (messenger-RNA) level and very low or undetectable levels

of myricitrin and quercitrin, and undetectable amounts of flavanols were found in the stems. Microshoots were tested in two ways to test rooting capability of the whole microshoot or only the stem segments. Morphological changes were noted. The rooting percentage was higher in most of the antisense lines where the flavonoid content was decreased, than in the control lines with normal flavonoid content. Auxin content was determined, but no variation was detected between antisense and control lines.

2.2 Methods Developed: *Agrobacterium* Strains and Genetic Construct Development

For *Agrobacterium* transformation, several different strains have successfully been used along with various constructs. In the first case, El Euch *et al.* (1998) used the disarmed C58/pMP90 *Agrobacterium* strain, pKY/GIN/AS.B as the antisense *chs* vector, pKY/GIN as the control vector, and pKY/GIN/AS.B was placed between nopaline synthase (*nos*) promoter and terminator; *uidA* with a plant intron placed between cauliflower mosaic virus (CaMV) 35S promoter and NOS terminator. A 400 bp *chs* gene fragment (CHS.B) was placed in reverse direction with duplicated CaMV 35S promoter and followed by *rbcs* terminator. Fang and Wang (2000) used the EHA 105 *Agrobacterium* strain that contained pMOG410 with *nptII* and *uidA* selection and marker genes (with intron) in the T-DNA region. Bosela *et al.* (2004) used the GV3101/pMP90 *Agrobacterium* strain with two different binary vectors, pBI121 and pBISN1, in different experiments. Using the pBI121 vector, *nptII* was driven by *nos* promoter and *uidA* (without intron) was driven by CaMV 35S promoter. With the pBISN1 vector, *nptII* was driven by the *nos* promoter; but *uidA* (with intron) was driven by a “super promoter” provided by S. Gelvin at Purdue University.

2.3 Selection of Transformed Tissue Using Antibiotic Resistance

In order to select transformed tissue in association with untransformed plant tissue for generation of transgenic black walnut, it is necessary to have a

selectable marker gene. The selective marker gene is inserted during the transformation process along with the donor gene into the donor genome. With black walnut, the kanamycin (*nptII*) resistance gene has been used most often as the selective marker.

Transgenic tissue is selected using the *nptII* gene and the *uidA* gene is inserted so that the transformation event can be later verified (Bosela *et al.*, 2004; Smagh, 2005). In the black walnut system, somatic embryos used as the target for transformation are induced to generate secondary somatic embryos on the cotyledon surface. Since it has been found that secondary embryogenesis originates from one single cell with walnut species (Polito *et al.*, 1989; Aly *et al.*, 1992), then transgenic cells have the potential to produce a completely transgenic secondary embryo. A sublethal kanamycin dose, the rate at which untransformed embryos produce no or only a few secondary embryos, has been used to select putative transgenic embryos (Smagh, 2005). At this particular dose, after transformation, secondary embryos are produced from transgenic tissue. Transformed walnut cells will have obtained kanamycin resistance while maintaining the ability to produce secondary embryos.

Two different strategies can be followed when choosing doses to use for selection pressure. A high selection pressure can be applied, if the repetitive secondary embryogenesis system is highly productive and selection of only transformed tissue is the objective. The disadvantage is that some incompletely transformed or chimeric embryos might not be able to survive at a high antibiotic dose. In the second approach using low selection pressure, even partially transformed embryos will produce secondary embryos from transformed cells or patches of tissue. This will ensure that partially and completely transformed secondary embryos will survive and produce putatively transformed secondary embryos. In this latter case, there will be some escapes, which can be killed by antibiotic selection during later culture on medium containing kanamycin. Nontransformed tissue can also be detected by histochemical GUS assays (Smagh, 2005), but this is a destructive procedure.

If the transformation event occurs in only one cell and the transformed cell differentiates and multiples to form a secondary embryo,

the result will be a completely transformed embryo. It can be cultured to produce secondary embryos to start a new transgenic line of walnut embryos. If transformation event occurs after cell differentiation process, then secondary embryos will not be completely transformed, because some cells will have originated from untransformed cells, resulting in chimeric embryos. Using chimeric embryos, transformed cells within the chimeric somatic embryo will eventually generate completely transformed secondary somatic embryos when selection is continual on kanamycin-containing medium. Only at this point can these embryos be used to start a new transformed embryo line.

2.4 Regeneration of Whole Plants

Maturation and germination of black walnut somatic embryos was documented initially by Deng and Cornu (1992). The authors were able to germinate 45% of the embryos into complete plantlets by maturing them with cold treatment for 2 months or desiccation for 3–5 days. Addition of GA to germination medium increased germination. Although acclimatization in the greenhouse was not tried, survival of plantlets was observed after transplanting into peat and vermiculite. Routine production of complete plants has been reported in other *Juglans* species, but not much success has been achieved in black walnut. Smagh (2005) was able to germinate transformed black walnut somatic embryos, although few of them had elongation of the shoot apex. Somatic embryos (1.5–2.0 cm in length) were first desiccated in 95% relative humidity at 25°C for 3 weeks and then placed on germination medium (half basal DKW with 1% sucrose and 100 mg l⁻¹ kanamycin) in petri plates in light for 3 weeks. Sixty-nine percent of the embryos germinated with roots only and with 2% of the embryos, shoot initiation was observed. After 3 weeks, germinated embryos with root elongation were transferred to shoot development medium (basal DKW + 2% sucrose, 20 mg l⁻¹ adenine sulfate and 2.5 µM zeatin) in 200 ml jars. After 4 weeks in culture, 22% had shoot elongation. The remaining explants developed callus on the roots and died. Embryos with both root and shoot elongation did not show any further elongation and were converted

into shoot cultures by discarding the roots and transferring the remainder of the explant to DKW medium containing higher concentrations of zeatin (6.25 µM). In studies by other authors (Deng and Cornu, 1992; Tang *et al.*, 2000) they used embryo explants at earlier stages of development, which might lead to better germination. New experiments are being designed by X. Liang (personal communication) to look at effect of cold treatment before germination and use of somatic embryos that have not been exposed to extended time to the *in vitro* environment.

2.5 Testing: Transgene Insertion, Transgene Function, and Whole Plant Testing

Transformed trees need to be tested at the molecular level, in the greenhouse and in field tests to confirm gene insertion and gene function before propagating the trees for commercial production. Expression of inserted genes is necessary to determine gene function because T-DNA insertions into the nuclear genome occur randomly. Random insertion can cause the transgene to be nonfunctional or it can disrupt the expression of another gene through insertion in the functional area of that gene in the genome. In addition, if the transgene is inserted in the wrong orientation, this improper insertion may cause the transgene to become nonfunctional.

The most accepted method for detection of transgenes in the target cells at molecular level is through PCR. A primer or unique DNA sequence of approximately 15–20 nucleotides, which is only present in the transgene, will amplify a particular region of the transferred gene and this amplified product can be detected using agarose gel electrophoresis. This method is very simple and easy, but it cannot detect the number of copies of the transferred gene. Southern hybridization can detect the number of copies of transgenes inserted into genomic DNA. In this method, a labeled DNA sequence fragment is hybridized to genomic DNA fixed on a membrane. Each inserted copy of the transgene in genomic DNA will be detected on membrane in an appropriate background. Both PCR and southern hybridization methods should be performed both with somatic embryos and plant leaves to insure there are no escapes during selection procedure.

Further tests of gene function are required to insure that no essential part of the transgene has been truncated. This can be achieved by testing the expression of a foreign gene inserted along with the desired gene. A selectable marker such as *uidA* can be detected *in vitro* by culturing putatively transformed embryos on kanamycin medium and germinating these embryos in kanamycin containing medium. All embryo cells lacking this gene will die. A destructive method for testing transgenics and gene insertion into plant cells is the histochemical GUS assay. If the complete *uidA* gene is integrated into plant genomic DNA, indigo coloration will appear in embryos treated with X-Gluc solution containing substrate for β -glucuronidase enzyme made by *uidA* gene. Chemical reaction taking place will cause indigo coloration, which can be detected visually or under a light microscope, which will be a positive indicator of gene expression and proper insertion of T-DNA region into plant genomic DNA.

Field testing of transgenics is necessary to determine if transgene expression is stable over the long term, if the level of gene expression is at a sufficient level to warrant commercial production of the bioengineered tree, and if the gene insertion has caused any unexpected phenotypic changes or changed the species ecological fitness.

2.6 Regulation of Transgenic Walnuts

USDA's Animal and Plant Health Inspection Service (APHIS) monitors release and regulation of transgenic plants. APHIS has considerable experience in assessing annual row crop plants for agricultural and environmental safety, but there are no rules and regulations for transgenic trees for commercial deployment. Recent advances in tree genetics and biotechnology has accelerated the pace of transgenic tree production and the issue of regulating open-pollinated transgenic trees is currently being addressed. Regulations for open-pollinated transgenic tree species such as black walnut are currently being formulated. APHIS has asked for public input on this issue and the agency is likely to publish directions in the near future.

An important issue that will be considered for deployment of transgenic black walnut is the escape of transgenes to sexually compatible species outside a managed site. In case of herbicide-

resistant trees, spread of herbicide resistance genes into compatible species of trees could make these trees weedy. Also, before receiving approval for deployment, controlled field trials would be required and guidelines could be different from annual row crops due to large size, perennial nature, and outcrossing capacity of trees.

In addition to APHIS that regulates plants that could become agricultural pests, black walnut transgenics could either be regulated the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA). FDA would regulate genetically engineered black walnut used for foods, drugs, cosmetics, and medical devices and EPA would regulate bioengineered trees expressing pesticidal properties (Anonymous, 2003). There is speculation that APHIS might publish regulations that would require sterility in trees and the deployment of transgenic trees would not be allowed to contain any foreign genes such as *nptII* or *uidA* that had been used as selectable markers.

3. FUTURE ROAD MAP

3.1 Expected Products

Applied research efforts are being focused on those traits that are most needed by growers, either for management purposes or to maintain global competitiveness. Those traits in greatest demand are likely to be made available first. Because our ability to commercialize any transgenic tree is predicated on the development of a durable confinement strategy (see below), flowering control is of the highest priority. Recent work has shown that reducing competing vegetation is one of the most important factors influencing seedling survival (Jacobs *et al.*, 2004), so herbicide tolerance is another area of emphasis. The success of forest-tree plantations is often severely hampered by animal browse (Trumbull *et al.*, 1989; Martin and Baltzinger, 2002; Jacobs *et al.*, 2004). In some areas, up to 85% of regeneration failures are the result of damage from deer (Marquis, 1981). In addition to affecting seedling development, herbivory can also have long-term effects on performance (Ward *et al.*, 2000). Although this concern is not currently getting much attention, it will become increasingly important over time. Finally, being able to manipulate the formation

of figured wood and heartwood will add value to the timber produced by walnut, so genes affecting these traits are also being investigated.

3.2 Risks and Concerns

Public acceptance will be needed before transgenic trees can be deployed commercially. To enhance our chances of achieving this outcome, it is incumbent upon plant scientists who are developing these biotechnologies to cooperate with their colleagues in the behavioral and social sciences so they can conduct studies concerning the societal, economic, and political aspects of their use. These studies should determine the level of public knowledge of tree biotechnology, and evaluate how this awareness will impact citizens and policymakers in the context of resource availability and global competitiveness. Without this type of interdisciplinary activity, it is unlikely that we will be able to take advantage of advancements resulting from the genomics revolution that is currently underway in plant biology.

A key aspect of public and regulator acceptance is transgene confinement. Before genetically engineered trees can be commercialized, APHIS, which has overarching regulatory authority over the deployment of transgenic trees, is likely to require a strategy to mitigate the risk of transgene spread and persistence in the environment. With this in mind, researchers worldwide are attempting to genetically engineer flowering control in trees (Meilan *et al.*, 2001; Lemmetyinen and Sopanen, 2004). By manipulating the genes involved in the control of floral development, it should be possible to engineer reproductive sterility. This approach may help alleviate public and regulatory concerns over the commercialization of transgenic trees containing other introduced, desirable traits. Blocking the production of sexual propagules (e.g., pollen and seeds) or flowers entirely is also likely to promote vegetative growth (Eis *et al.*, 1965; Tappeiner, 1969; Teich, 1975), leading to shorter harvest rotations. Moreover, flowering-control research could lead to ways for either delaying the onset of flowering, which could itself serve as a confinement strategy, or shortening their juvenile periods, which could lead to shorter breeding cycles.

3.3 Expected Technologies

In order to genetically engineer a plant, it is necessary to stably introduce a gene into the genome of an individual plant cell. The transformed cell must then be coaxed into differentiating into a whole plant, i.e., regeneration. Either process can limit our ability to recover transgenic plants. Although efficient transformation and regeneration systems have been developed for poplars (species within the genus *Populus*) (DeBlock, 1990; Confalonieri *et al.*, 1995; Han *et al.*, 2000; Meilan and Ma, 2006), most other tree species, including black walnut, are refractory with respect to one or both processes. Protocols originally developed for Persian walnut (*J. regia* L.) (McGranahan *et al.*, 1988, 1990; Dandekar *et al.*, 1998; Escobar *et al.*, 2000; Breton *et al.*, 2004) were later applied to black walnut (Bosela *et al.*, 2004), but the efficiency with which transgenic plants were recovered from the latter was low.

One limitation of *Agrobacterium* transformation is the restricted host range of this bacterium; many plants, especially various tree species, remain highly recalcitrant to transformation. Recent breakthroughs in transformation technology have permitted *Agrobacterium*-mediated transformation of some varieties of rice and maize, both of which had been notoriously difficult to transform. It is likely that knowledge gained from transforming monocotyledonous plants can be utilized to develop more efficient systems for trees such as black walnut.

Over the past 10 years, the S. Gelvin (2000, 2003) laboratory at Purdue University has investigated the role of plant-host genes involved in *Agrobacterium*-mediated plant transformation. They have identified more than 125 of these genes in the model plant *Arabidopsis thaliana* (Zhu *et al.*, 2003). In some cases, overexpression of these genes in *Arabidopsis* increases the frequency of transformation (Mysore *et al.*, 2000; Yi *et al.*, 2002, 2006; Hwang and Gelvin, 2004). Using what has been learned with this model plant system, it should now be possible to make rapid progress with other plants, including trees.

Currently, immature zygotic embryos are used as starting materials for establishing black walnut tissue cultures. Embryogenic calli derived from these explants are, in turn, co-cultivated with *Agrobacterium*. Newly transformed cells are then

hormonally induced to differentiate into somatic embryos. There are two complications associated with this approach. First, there is no way of knowing *a priori* how an individual line will perform *ex vitro*. Thus, lines must be propagated, acclimated, and grown in soil prior to transformation, in order to ensure that the best lines are being used as starting material for transformation. Second, embryogenic masses often begin to differentiate into somatic embryos prior to being transformed. This leads to the production of chimeric plantlets, which are only partially transformed. To overcome this obstacle, successive generations of somatic embryos must be induced in order to recover fully transformed plants. This is a very costly procedure. To avoid both of these difficulties, research efforts should be focused on developing a direct organogenesis system.

Prior to transformation, a selectable marker gene is physically linked to a gene that imparts a trait of interest (e.g., herbicide tolerance, insect resistance, altered wood properties, etc.). Because this marker gene often confers resistance to an antibiotic or herbicide, the cells that are actually transformed can be isolated on a medium containing the appropriate selection agent. While this method is convenient, it is also problematic. First, various selection agents can have dramatic and negative effects on regeneration. Second, the presence of a selectable marker gene is usually an impediment to gaining public acceptance of and regulatory approval for genetically engineered plants. Finally, performing subsequent rounds of transformation may not be possible because only a limited number of selectable marker genes are available.

Assuming the need for a selectable marker and for flowering control, a minimum of three transgenes will need to be inserted if only one gene of interest is introduced. In many cases, however, more than one desirable gene is sought. Because each transgene should be tested individually before being stacked, but there are physical limits to the size of T-DNA that *Agrobacterium* can insert, one must conduct successive rounds to introduce multiple traits. Given all of this, it is essential that we have a way to remove the selectable marker gene afterward.

The following three gene-excision systems have been the focus of recent research: *Cre/lox* (Russell

et al., 1992), FLP/FRT (Lyznik *et al.*, 1996), and R/RS (Onouchi *et al.*, 1995). Their vectors typically include: (1) a recombinase gene, usually under the control of an inducible promoter, and (2) recognition sites that flank the DNA targeted for removal. However, these systems are differentially effective, if at all, in various plants. Thus, it is necessary to determine which is the most appropriate for use with black walnut. For each system, one must ascertain the efficacy of the recombinase and how cleanly it excises the target. In addition, it is important to have an inducible promoter that functions reliably in the chosen host.

Recently, alternative selection systems have been developed for plants. These are based on a growth medium that lacks a substance needed for proper metabolic activity or physiological development. A particularly attractive option exploits the inability of a cell to regenerate a whole plant without the addition of a phytohormone, or its derivative, to the culture medium at a precise step in the regeneration process. For example, most plant transformation protocols rely on the addition of cytokinin to a medium that induces the differentiation of adventitious shoots or embryos from transgenic calli. The β -glucuronidase gene (GUS), a common reporter, encodes an enzyme that cleaves glucuronide residues. The glucuronide derivative of benzyladenine is biologically inactive; if it is the sole cytokinin incorporated in the induction medium, regeneration cannot occur. However, upon hydrolysis by GUS, a biologically active cytokinin is then liberated to induce regeneration. This supplement must necessarily be transitory because cytokinin can inhibit subsequent root development.

Another positive selection strategy involves inserting a gene whose product imparts a metabolic advantage to the transformed cell. For example, mannose is a sugar that plants are unable to metabolize; cells starve when grown on a medium containing mannose as the sole carbon source. When taken up by the cells, this sugar is phosphorylated by a native hexokinase. However, plants lack a native phosphomannose isomerase gene, which encodes an enzyme that catalyzes the conversion of mannose to a useable six-carbon sugar (Joersbo *et al.*, 1998). Similarly, xylose isomerase, another enzyme that plants normally lack, is able to convert xylose to a sugar that can be utilized (Haldrup *et al.*, 1998). Regeneration

protocols that exploit positive selection strategies such as these are up to 10-fold more efficient than those that rely on more traditional, negative selection strategies (i.e., antibiotic or herbicide resistance).

3.4 Political and Economic Consequences

The current costs and burdens associated with regulatory agencies in the United States (Meilan, 2006) are having a strong negative effect on the development of the forest biotechnologies by academic and industrial researchers. Revisions in the current system are needed to encourage investment in transgene-based innovations, thus facilitating the production of improved varieties (Bradford *et al.*, 2005). Suggested changes include (1) developing a regulatory system that is based on phenotype rather than the method used to produce it, following long-standing National Academy of Science recommendations (National Research Council, 1989, 2000, 2002); (2) eliminating personal liability for academic researchers conducting approved research; and (3) establishing a means for early evaluation of the benefits and risks of gene dispersal that allows certain types of long-term field trials, similar to what is currently being done for conventional breeding programs.

Because of recent advances, the technology for creating transgenic trees is much more advanced than the research that evaluates their ecological impacts. A comprehensive, interdisciplinary scientific approach that combines experimental results with model projections is badly needed. The knowledge we acquire from this work will inform the public debate, and must be completed before a multitude of social and regulatory issues can be clarified and resolved. Failure by the forest products sector to engage in this cooperative research could lead either to the establishment of transgenic trees with unintended consequences, or to an inability to realize the numerous advantages that this technology has to offer (Farnum *et al.*, 2006).

Although there has been considerable resistance in Europe to the use of genetically modified trees, this has not been the case in Asia. For example, there are recent reports that insect-resistant transgenic poplars have already been

commercially deployed on 300–500 ha in China (Boerjan, 2005; Sedjo, 2006). Undoubtedly, this was done first with poplar because of the ease with which it can be transformed and regenerated. Given their willingness to do this with trees that have so many wild relatives with which they are interfertile, there is no reason to believe that they would not continue with other tree species soon, without regard for environmental consequences. Although we in the United States should proceed more cautiously than that, if we do not embrace this technology, our competitiveness will continue to erode.

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Casuarinaceae

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1. INTRODUCTION

1.1 History, Origin, and Distribution

The family Casuarinaceae is a group of 96 species of multipurpose trees and shrubs that grow naturally in Southeast Asia, Malaysia, Australia, and the Melanesian and Polynesian regions of the Pacific (Wilson and Johnson, 1989). Casuarinas are characterized by a coniferlike appearance due to morphologically distinctive foliage with the leaves reduced to tiny teeth on green, jointed, needlelike branchlets (Figures 1–3). The name Casuarina is derived from the Papuan word “kasuari”, which refers to the similarity between the drooping foliage of the tree and the feathers of the Cassowary bird. The most widely used common names for Casuarinaceae species are sheoak or she-oak. Other common names include ironwood, Australian pine, bull-oak, and beefwood.

Macrofossils from the Eocene, Miocene, and Plio-Pleistocene indicate that in the past, Casuarinaceae had a much wider distribution including New Zealand and southern South America. Fossil pollen has also been identified in southern Africa, in the suboceanic Ninetyeast Ridge, and possibly in India (Wilson and Johnson, 1989).

Casuarinaceae members are tolerant to adverse edaphic and climatic conditions and most species tolerate extreme heat. They grow in a wide range

of different environments, from tropical forests to arid woodlands and coastal dunes (Barlow, 1983). They frequently occur as pioneer vegetation at early stages of plant succession following disturbances, such as fire, landslides, volcanic eruption, and flooding. In Australia, Casuarina forests occur in all the territories and cover a total area of over two million hectares.

Three main species, *Casuarina equisetifolia*, *Casuarina glauca*, and *Casuarina cunninghamiana* have been planted outside their native habitat (National Research Council, 1984). Vast plantings of *C. equisetifolia* have been established in China along the coast fronting the South China Sea. They form a green belt that stretches for 3000 km and varies from 0.5 to 5 km in width (Zhong and Zhang, 2003). *C. cunninghamiana* has been extensively planted in Argentina and neighboring countries and in Egypt. *C. glauca* has been successfully introduced in the saline soils of Israel, Cyprus, India, Kenya, Malawi, South Africa, Egypt, and Florida (National Research Council, 1984).

1.2 Botanical Description

The family Casuarinaceae is unique among angiosperms and, having no close relatives, is assigned to an order of its own, Casuarinales (Beadle, 1981). Formerly, all species were included



Figure 1 *Casuarina glauca* shelterbelt in Egypt

in a single genus, *Casuarina*, but accumulated evidence on morphology, anatomy, cytology, biogeography, and chromosome size and number resulted in the recognition of four genera: *Allocasuarina*, *Casuarina*, *Ceuthostoma*, and *Gymnostoma*. The *Allocasuarina* and *Casuarina* genera include, respectively, 59 and 17 species; *Gymnostoma* includes 18, and only 2 species have been described for *Ceuthostoma* (Dommergues *et al.*, 1999). Morphological and chromosomal characters suggest that the least specialized genus, and therefore likely to be the most primitive



Figure 2 Plantation of *Allocasuarina verticillata* in Tunisia



Figure 3 Photosynthetic branchlet of *C. glauca*

in the family, is *Gymnostoma*, while the most specialized and diverse genus is *Allocasuarina* (Wilson and Johnson, 1989). The growth habit of Casuarinaceae ranges from woody shrubs to large trees; for example, *C. cunninghamiana*, which grows to heights of 12–30 m (National Research Council, 1984).

The chromosome number in *Allocasuarina* ranges between $n = 10$ and $n = 14$ and polyploidy is frequent. In *Casuarina* and *Gymnostoma*, the chromosome number is, respectively, $n = 9$ and $n = 8$, and there is no polyploidy (Barlow, 1983). The chromosome number has not been determined for *Ceuthostoma*.

The genome size and base composition of *C. glauca* and *Allocasuarina verticillata* were determined by flow cytometry (Schwencke *et al.*, 1998). *C. glauca* has a small genome size of $2C = 0.7$ pg (picogram), 59% AT (adenine-thymine), 40.5% guanine-cytosine (GC). *A. verticillata* has a larger genome of $2C = 1.9$ pg, 59.3% AT, 41.1% GC. These small genomes are a valuable trait for genomic and molecular studies.

1.3 Economic Importance

In the field, Casuarinaceae bears nitrogen-fixing root nodules (so-called actinorhizal nodules) resulting from association with the soil actinomycete *Frankia* (Figures 4–5) (Baker and Mullin, 1992). Due to this symbiotic association, Casuarina trees have an outstanding ability to grow vigorously on



Figure 4 Nitrogen-fixing nodules of *C. equisetifolia* observed in the field

soils that are otherwise too deficient in nitrogen to sustain plant growth. The amount of nitrogen fixed by Casuarina is comparable to the amounts fixed by legumes and their *Rhizobium* symbionts (60 kg ha^{-1} per year) and these trees contribute significantly to the N-economy of ecosystems (Diem and Dommergues, 1990). Casuarina roots also form mycorrhizal associations with arbuscular mycorrhizal and ectomycorrhizal fungi that facilitate the uptake of minerals, such as phosphorus (Diem, 1996; Duponnois *et al.*, 2003). Some species can differentiate proteoid roots; these are unique features made of tightly packed rows of rootlets, which may increase the ability of the host plant to absorb nutrients and thus tolerate



Figure 5 Two-month-old nodules of *A. verticillata* obtained *in vitro*

nutrient-deficient soils (Lamon, 2003). With the combination of the different symbionts, Casuarina is one of the best species for the reforestation of low-fertility lands. In some tropical lowland agroforestry systems, it is associated with crops such as coffee, cashew nut, coconut, groundnut, sesame, and various grain legumes to enhance their quality and growth (National Research Council, 1984).

Taken collectively, Casuarina trees have many uses (National Research Council, 1984; Diem and Dommergues, 1990). They are capable of stabilizing shifting sand dunes, stabilizing eroding hillslopes, and reclaiming marshy soils that are periodically inundated. Because of their resistance to salt-laden winds, many species such as *C. glauca* and *C. equisetifolia* are widely used to stabilize coastal sand dunes (Zhong and Zhang, 2003). They are also planted as windbreaks to protect crops. Casuarina trees have proved to be one of the most effective shelter trees during typhoons and Tsunami in Asia. Some species such as *C. equisetifolia* and its hybrids grow rapidly (a growth rate of 3 m per year has been reported in India) and have an attractive dense crown; consequently, these species are often planted as ornamental plants for urban beautification, parks and seaside resorts, and along roadsides.

Another important use of Casuarina in the tropics is the production of firewood. In comparison to other fuelwood crop species, Casuarina ranks well for calorific value in relation to wood volume (about $5000 \text{ kcal kg}^{-1}$). People in China use the stumps and even litter for fuel. Because Casuarina wood splits on drying, it is difficult to use the wood for lumber and furniture making. However, it can be used in rural construction as poles for house construction, electric poles, and the masts of boats. The wood is very hard, with a density of 1000 kg m^{-3} , and is resistant to decomposition in soil and saltwater. Thus, Casuarina poles are used in Vietnam for anchoring fishing nets in the mouths of the rivers. In India, the wood of *C. equisetifolia* is also pulped for paper, and in Madagascar, the bark is extensively used for tanning leather.

1.4 Traditional Breeding

Improving the yield and adaptability of Casuarinaceae to biotic and abiotic stresses is a

big challenge in tropical regions. Two types of approaches have been used: the first is to improve the microsymbionts (*Frankia* and mycorrhiza); the second is to develop host plants with superior provenance. However, when selection of optimal *Frankia* partners was based on the nitrogen-fixing potential of different Casuarina clones, the data obtained established that the clone effect was more important than the symbiont effect. It was suggested that this observation was linked to the large intraspecific variability of Casuarina, which results from the allogamous, monoecious (though sometimes dioecious), and anemophilous character of this tree (Sougoufara *et al.*, 1992).

Strategies for plant improvement include the selection of species and provenances, conventional plant breeding using seedlings, and techniques based on vegetative propagation and tissue culture (Duhoux *et al.*, 1996; Zhong *et al.*, 2003). Casuarina trees produce good seed crops almost every year, and regenerate easily from seed. These trees are also easy to propagate by cutting, which has the advantage of being a rapid method of perpetuating the traits of superior trees. Other methods for propagating *in vitro* plants include the use of immature female inflorescences collected from mature trees (Duhoux *et al.*, 1986).

Breeding objectives include selection of trees with improved performance under saline, waterlogged, nutrient-impovertished, drought-prone, and coastal conditions. Differences in tree susceptibility to diseases and pests (Hassan, 1990) have also triggered studies on selection of Casuarina provenances and clones (Sougoufara *et al.*, 1992). Amongst the pathogens that cause damage to the wood, bacterial wilt induced by *Pseudomonas solanacearum* has been reported in *C. equisetifolia* in India and China. Vegetative propagation and selection of Casuarina for resistance to bacterial wilt succeeded in China (Liang and Chen, 1982). Several clones with satisfactory levels of resistance to bacterial wilt and possessing fast-growing characters were obtained and propagated in large coastal areas in South China. Other pests that attack the tree include insects such as crickets and grasshoppers, defoliators (*Lymantria xyli*), stem borers (*Apate monachus*), and sap feeders (*Icerya* spp.). Major root diseases are caused by *Trichosporium vesiculorum* and *Rhizoctonia* spp. (National Research Council, 1984).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Casuarina plantations are faced with a number of problems including diseases and pests. In the tropics, there is also a need for trees that can tolerate salty or polluted soils, as well as drought. Long breeding cycles, large size, high levels of heterozygosity, and the economics of producing and evaluating large segregating populations of trees are some of the difficulties encountered in breeding forest trees.

Genetic engineering offers prospects for generating novel forest tree genotypes at an accelerated rate. One major advantage of this approach over conventional breeding is that only the characteristics of interest are inserted into the recipient plant while the original genetic framework remains unchanged. Although genetic engineering in trees is still in its infancy, several studies have clearly established its potential for introducing novel genetic characters, such as herbicide tolerance, insect resistance, or modifying lignin content (Tzfira *et al.*, 1998; Ahuja, 2000; van Frankenhuyzen and Beardmore, 2004).

2. GENETIC TRANSFORMATION

2.1 Organogenesis in Casuarina

The ability to regenerate a plant species following *in vitro* cultivation of a transformed cell remains a critical factor for the success of a genetic transformation procedure. Shoot regeneration from calli has been described for both *C. glauca* and *A. verticillata* (Duhoux *et al.*, 1996).

Epicotyls from 4- to 5-week-old seedlings were cut into 2-cm pieces and cultured on a MSC (Murashige and Skoog medium modified for Casuarina) medium containing half-strength MS salts, Nitsch and Nitsch vitamins, 20 g l⁻¹ sucrose at pH 5.6 and supplemented with 0.5 µM naphthalene acetic acid (NAA), and 2.5 µM benzylaminopurine (BA). After 2 months, callogenesis was observed on 40% of *C. glauca* explants and on 90% of *A. verticillata* epicotyl fragments. Buds elongated into shoots within 6 months on 41% of *C. glauca* calli. Shoots proliferation was observed within 4 months on 85% of *A. verticillata* calli.

When *C. glauca* shoots were about 3 cm in height, they were excised and root formation induced by a 3-day treatment with 10 μ M indole-3-butyric acid (IBA) (Franché *et al.*, 1999). The same procedure was followed for *A. verticillata* using 25 μ M NAA (Franché *et al.*, 1997).

2.2 Antibiotics for the Selection of Transformed Tissue in Casuarinaceae

The sensitivity of Casuarinaceae epicotyls to the antibiotic kanamycin was established before the first gene transfer experiment (Le *et al.*, 1996). Experimental results established that *C. glauca* is more sensitive to antibiotics than *A. verticillata*. Complete inhibition of callus growth on untransformed explants cultivated on a callus-induction medium can be obtained at a kanamycin concentration of 50 mg l⁻¹ for *C. glauca* whereas 100 mg l⁻¹ is necessary for *A. verticillata*. Unpublished data concerning *C. equisetifolia* indicate that selection for transformed cells requires 50 mg l⁻¹ of kanamycin.

Cefotaxime is the antibiotic used to eliminate *Agrobacteria* in plant tissues. In Casuarinaceae, it had no adverse effect on shoot differentiation when used at a concentration ranging from 250 to 500 mg l⁻¹ (Le *et al.*, 1996).

2.3 Gene Transfer Methods

2.3.1 Direct gene transfer based on particle bombardment

Particle bombardment is a valuable technology for transient expression of new gene constructs in target plant cells and can be used to generate transgenic plants in species that are recalcitrant to *Agrobacterium* (Sanford, 2000; Kikkert *et al.*, 2005). This method was tested on cotyledons, epicotyls, and hypocotyls excised from 2-month-old *C. glauca* seedlings (Franché *et al.*, 1999).

A helium particle gun was used to accelerate 1 μ M tungsten beads coated with a plasmid containing the β -glucuronidase gene (*gus*) under the control of the constitutive cauliflower mosaic virus (CaMV) promoter 35S. Bombardment of the cotyledons led to the most efficient and reproducible data for transient expression. An average of three *gus*-expressing units was observed

on the cotyledons 2 days after bombardment. The low efficiency of gene transfer is mainly linked to the small size (about 2 mm) of the cotyledons from *C. glauca*. Stable genetic transformation has never been reported in Casuarinaceae using this approach, and the low frequency of stable transgene integration is a major limitation.

2.3.2 Gene transfer based on *Agrobacterium rhizogenes*

Susceptibility of Casuarinaceae to *Agrobacterium rhizogenes* was demonstrated in 1991 (Phelep *et al.*, 1991). The agropine-type strain A4 (Petit *et al.*, 1983) and the cucumopine-type strain 2659 (Davioud *et al.*, 1988) were found to induce hairy roots on seedlings of *A. verticillata*. This property can be used to produce partially transformed plants (composite plants) of *C. glauca* (Diouf *et al.*, 1995) and fully transformed plants of *A. verticillata* (Phelep *et al.*, 1991).

The rapid procedure for composite *C. glauca* relies on the induction of a hairy root system by *A. rhizogenes* while the aerial part of the plant remains untransformed (Figures 6–7). Young seedlings of *C. glauca* were wounded on the hypocotyl and inoculated with *A. rhizogenes* A4RS containing the binary vector pBIN35S-*gus-int* that carries a derivative of the β -glucuronidase reporter gene *gus-int* expressed only upon transfer to the plant cells and not in *Agrobacterium* (Vancanneyt *et al.*, 1990). After 2 weeks, highly branched roots exhibiting rapid growth were observed at the inoculation site. The normal root system was removed at the stem base, and the composite plant decontaminated with cefotaxime at 300 mg l⁻¹. GUS reporter gene activity was observed in about 50% of *C. glauca* hairy roots, indicating a cotransfer of the genes carried on the T-DNA (transfer-DNA) of the pBIN vector and those of the root inducing (Ri)-plasmid. After inoculation with *Frankia*, nodulation was observed on 40% of the transformed roots. Using this “composite plant” approach, the expression and the function of a chimeric gene can be studied in both roots and nodules of *C. glauca* within 4 months. The same approach can be used on *A. verticillata* (Franché, unpublished data).

Fully transformed plants of *A. verticillata* can be obtained following genetic transformation by



Figure 6 Hairy roots developing on the hypocotyl of *C. glauca* after inoculation by *A. rhizogenes* 2659

the A4 or 2659 strains of *A. rhizogenes* (Phelep *et al.*, 1991). After inoculation with A4, 20% of the epicotyls from 2-month-old seedlings developed hairy roots. After excision, the transformed roots were grown on a MSC medium supplemented with $1.25\ \mu\text{M}$ NAA and $0.5\ \mu\text{M}$ BA. Two months later, shoot regeneration was observed on a few of A4-induced roots. In contrast, shoot regeneration occurs spontaneously on 90% of the *A. rhizogenes* 2659 transformed roots after 3 months of culture on a hormone-free medium. All the shoots developed roots when placed in a nutrient medium with $0.5\ \mu\text{M}$ of IBA. However, the phenotype of the transgenic plants differed from that of the control plants (Figure 8). The transgenic regenerants were characterized by a better developed root system, plagiotropic, and more branched than the normal nontransformed

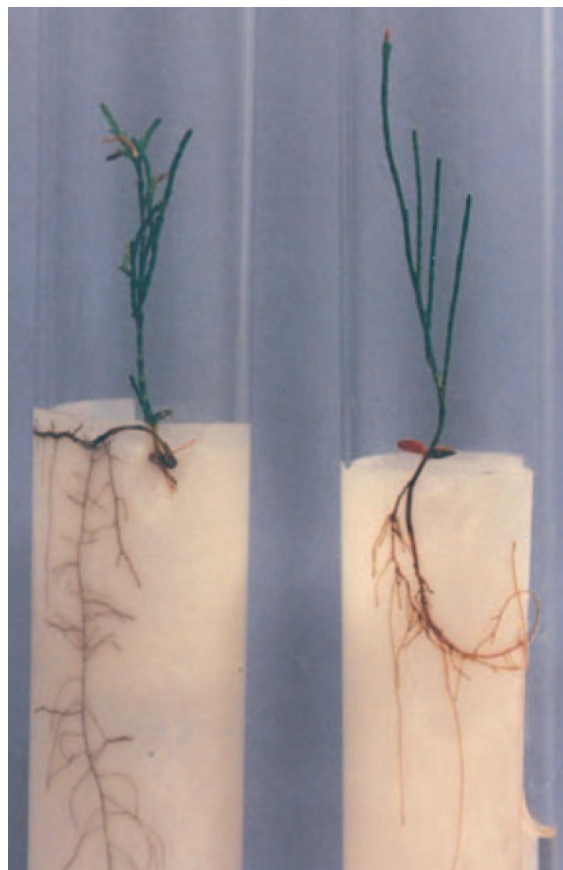


Figure 7 Composite plant (left) obtained after inoculation by *A. rhizogenes* A4 and control plant (right) of *C. glauca*

roots. The root dry weight of the transformed plant was five times higher than that of the control plants. The aerial system showed reduced apical dominance with highly branched shoots and the dry weight of the transgenic was two times higher than that of the normal regenerant.

2.3.3 Gene transfer based on *Agrobacterium tumefaciens*

The natural susceptibility of members of the Caesariaceae family to *Agrobacterium tumefaciens* was used to develop a gene transfer procedure for both *A. verticillata* (Franchet *et al.*, 1997) and *C. glauca* (Smouni *et al.*, 2002). A number of physiological and environmental factors, such as the presence of plant phenolic compounds (acetosyringone), sugars, pH, temperature, and



Figure 8 Control plant (right) and transgenic plants (middle and left) fully transformed by *A. rhizogenes* A4

osmoprotectant compounds, influence the induction of virulence genes and, consequently, the efficiency of T-DNA transfer from *A. tumefaciens* to the wounded plant cells (Gelvin, 2000). Preliminary experiments performed with *C. glauca* showed that optimal transformation rates are obtained when epicotyls from 45-day-old seedlings are co-cultivated for 3 days at pH 5.6 with the *A. tumefaciens* strain C58C1 (pGV2260) (Le *et al.*, 1996). The efficiency of genetic transformation by the strain LBA4404 (pLBA4404) (Hoekema *et al.*, 1983) is 1.5–2.5 times lower than that obtained with C58C1 (pGV2260) (Vancanneyt *et al.*, 1990).

In the optimal conditions previously described, respectively 26% and 39% of the epicotyls from *C. glauca* and *A. verticillata* that were genetically transformed with the strain C58C1 (pGV2260; pBIN19-35S-*gus-int*) developed one to three calli growing in the presence of kanamycin



Figure 9 Bud differentiation on a transgenic callus of *C. glauca* obtained after genetic transformation by *A. tumefaciens* C58C1 (pGV2260)

(50 mg l⁻¹ for *C. glauca* and 100 mg l⁻¹ for *A. verticillata*) (Figure 9). About 95% of these calli expressed β -glucuronidase reporter gene activity and the integration of the transgenes was further confirmed by polymerase chain reaction (PCR) and Southern blot analyses. Transgenic plants were regenerated in approximately 9–10 months for *C. glauca* and in 4–6 months for *A. verticillata*. The phenotype and the nodulation efficiency conferred by *Frankia* was found to be similar in transgenic Casuarinaceae and in nontransformed control plants (Figure 10), and the transgenic nodules fixed nitrogen at the same rate as those of the nontransformed control nodules.

The transformation procedure developed on *C. glauca* and *A. verticillata* has several major advantages: kanamycin selection is efficient and there are very few escapes; only one medium is required for both bud differentiation and shoot elongation; and it is possible to regenerate numerous trees from a single callus in a reasonable period of time.

2.4 Promoters for the Expression of Valuable Traits in Casuarinaceae

2.4.1 Promoters for constitutive expression

The CaMV 35S promoter (Odell *et al.*, 1988) is the most widely used sequence for constitutive



Figure 10 One-month-old nontransformed plant (left) and transgenic rooted plant (right) obtained after genetic transformation by *A. tumefaciens* C58C1 (pGV2260)

expression of foreign genes in plants. The expression pattern conferred by this sequence has been reported in transgenic 35S-*gus* *C. glauca* (Smouni *et al.*, 2000, 2002) and *A. verticillata* plants grown either *in vitro* for 2 months or for 1 year in a glasshouse (Obertello *et al.*, 2005).

In 2-month-old transgenic *C. glauca* plants, 35S was expressed constitutively in the shoots, and reporter gene activity generally increased with distance from the shoot apex (Smouni *et al.*, 2002). In the primary root, activity was detected throughout the main root except in the elongation zone. Observations on main root transverse sections showed intense blue staining in the vascular system, including the xylem, phloem tissues, and pericycle. The large, thin-walled cells of the cortex and rhizoderm were lightly stained. As noted in the aerial parts of the plant, the intensity of staining increased with root diameter and distance from the root tip. In emerging lateral roots, promoter activity was restricted to the root cap. In older lateral roots,

blue staining was also visible in the proximal region of the vascular tissue (Smouni *et al.*, 2002). In actinorhizal nodules, intense GUS activity was seen in the vascular bundle, the phellogen, and in strands of uninfected cells filled with polyphenols. 35S-*gus* expression was undetectable in *Frankia*-infected cells. Analysis of 35S-*gus* *C. glauca* grown for 1 year in a glasshouse revealed a nearly constitutive pattern of expression (Smouni *et al.*, 2002). The *CaMV* promoter was highly active in both roots and shoots, except in peripheral suberized tissues. These data were confirmed by detailed fluorometric analysis of a transgenic 35S-*gus* *C. glauca* tree (Smouni *et al.*, 2000).

In the search for promoters that drive strong constitutive expression in *A. verticillata*, the specificity of *gus* expression conferred by four different promoters has been compared (Obertello *et al.*, 2005). The sequences studied include the 35S promoter, the enhanced double 35S (*e35S*) (Kay *et al.*, 1987), the *e35S-5ocs* that contains four copies of the *ocs* (octopine synthase) element (Ellis *et al.*, 1987), and the ubiquitin 1 (*UBQ1*) promoter from *Arabidopsis thaliana* (Callis *et al.*, 1990). The 35S promoter was constitutively expressed except in the shoot apex and in lateral roots. The *e35S* and *e35S-4ocs* drove high constitutive expression in non-nodulated *A. verticillata*. In contrast, the *UBQ1* promoter that is nearly constitutive in *Arabidopsis* was poorly expressed in *Casuarina*. None of the four promoters drove expression in the *Frankia*-infected cells of actinorhizal nodules.

2.4.2 Promoters for root- or nodule-specific expression

Promoters tested for root- and nodule-specific expression include *cgMT1*, a sequence from a metallothionein (MT) gene from *C. glauca* (Laplaze *et al.*, 2002), and two heterologous promoters from hemoglobin genes (Franche *et al.*, 1998a).

PcgMT1 is a 1.2-kb fragment corresponding to the promoter region of a type 1-MT gene isolated from a nodule complementary DNA (library of *C. glauca* (Laplaze *et al.*, 2002). In transgenic *A. verticillata* plants, *PcgMT1* drives a constitutive *gus* expression in lateral roots whereas the aerial part did not express the reporter gene except in the oldest shoot

region. In transgenic nodules of *A. verticillata*, β -glucuronidase expression was restricted to large *Frankia*-infected cells. No activity was observed in noninfected cells of the cortex, in the periderm, vascular bundle, or meristematic zone. Induction experiments performed on whole plants revealed that *PcgMTI* responds to wounding, oxidative stress, and pathogen infection. Thus, this promoter appears to be a valuable sequence to drive expression in roots, in *Frankia*-infected cells, and in plants under oxidative stress.

Symbiotic hemoglobin is a protein present at high concentrations in the nitrogen-fixing nodules of both legumes and nonlegumes, where it facilitates oxygen diffusion to nitrogen-fixing endosymbiotic bacteria (Appleby, 1992). Two different hemoglobin promoters were studied in transgenic Casuarinaceae: the promoter region of the hemoglobin gene from soybean (*lbc3*) (Lauridsen *et al.*, 1993) and from *Parasponia andersonii* (Bogusz *et al.*, 1988). *lbc3* is a symbiotic gene expressed at high levels in soybean nodules (Lauridsen *et al.*, 1993). *P. andersonii*, a nonlegume belonging to the family Ulmaceae, lives in symbiotic association with *Rhizobium* (Trinick, 1979); the *Parasponia* hemoglobin sequence is expressed both in the nitrogen-fixing nodules and at low levels in the root tissue (Bogusz *et al.*, 1988). In transgenic *C. glauca* and *A. verticillata*, the soybean and *P. andersonii* hemoglobin promoters direct expression of the *gus* gene in *Frankia*-infected cells; some blue staining was also observed in the root tip of the *Parasponia* construct (Franché *et al.*, 1998a). No expression was observed in the aerial parts of the transgenic plants. So far, these two hemoglobin promoters are considered to be the best sequences to drive a high level of transgene expression in *Frankia*-infected cells.

2.5 *gus* and *gfp* as Reporter Genes in Transgenic Casuarinaceae

Reporter genes have been used as convenient markers to visualize gene expression and protein localization *in vivo* in a wide spectrum of procaryotes and eucaryotes. A comparison of the *gus* and *gfp* (green fluorescent protein) reporter genes was undertaken to determine which marker is the most suitable for gene expression studies in transgenic Casuarinaceae (Santi *et al.*, 2003).

The β -glucuronidase assay invented by Jefferson is very sensitive, and it is possible to obtain both qualitative (histochemical) and quantitative (fluorometric) data (Jefferson *et al.*, 1987). As previously mentioned in this chapter, the first genetic transformation experiments of Casuarinaceae proceeded through the use of the *gus* reporter gene (Franché *et al.*, 1997). Histochemical assays established that the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) can easily penetrate calli, shoots, and roots, but does not penetrate directly in nodules. Reporter gene activity can only be observed in 45- μ m-thick nodule sections obtained on a vibratome. This treatment is absolutely necessary to compensate for the poor penetration of the GUS substrate in nodules of transgenic Casuarinaceae.

One major problem that is sometimes encountered in fluorometric β -glucuronidase analyses performed on woody plants is linked to the presence of phenolic compounds, which can interfere with GUS activity (Jefferson *et al.*, 1987). This inhibition has been observed in protein extracts of Casuarinaceae, mainly in nodules and to a lesser extent in roots. Adding polyvinylpyrrolidone (PVPP) during preparation of protein extracts can overcome the problem. With the addition of insoluble PVPP (0.5 g g⁻¹ fresh weight tissue) to the protein extraction buffer, GUS activities measured in actinorhizal nodules and roots increased by a factor of 70 and 9.8, respectively. It has been suggested that PVPP can specifically absorb phenolic compounds that act as inhibitors of GUS enzymatic activity (Serres *et al.*, 1997).

Because the GUS assay is toxic and destructive, it cannot be used to monitor gene expression *in vivo*. This is a major obstacle for kinetic analyses of symbiotic gene expression during differentiation of actinorhizal nodules. The GFP from the jellyfish *Aequorea victoria*, which emits bright fluorescence upon excitation with ultraviolet or blue light GFP, thus appears to provide a useful alternative to GUS (Chalfie *et al.*, 1994). Analysis of transgenic *35S-mgfp5-ER* (Haseloff *et al.*, 1997) *A. verticillata* plants revealed clearly visible fluorescence in the transformed root system (Santi *et al.*, 2003). In shoots, fluorescence was only detected in the young scales of the apex but it was masked by chlorophyll in other parts of the shoots. Under UV light, prenodules and nodules exhibited strong green GFP fluorescence making them easy to

identify in the lignified root system. However, in control nodules obtained after inoculation of nontransformed *A. verticillata* plants, a low level of green autofluorescence was observed. This level of autofluorescence of the nodule makes analysis of weak promoters difficult for gene expression studies in transgenic Casuarinaceae. Their study thus requires the use of more sophisticated methods such as confocal microscopy.

3. FUTURE ROAD MAP

3.1 Transgenic Casuarinaceae as a Tool for Functional Genomics in Actinorhizal Plants

In the past decade, some progress has been made in the knowledge of the plant genes that are expressed at different stages of Casuarina nodule differentiation, and genomic approaches have been initiated (Franché *et al.*, 1998b; Obertello *et al.*, 2003; Hoche *et al.*, 2006). The genetic transformation procedures developed in the Casuarinaceae family now make it possible to perform functional analysis of plant symbiotic genes. These approaches include promoter studies, over- and underexpression of plant genes using sense and antisense constructs, and RNA interference. Promoter-reporter gene fusions have already been shown to be particularly appropriate in Casuarina when the expression of the symbiotic genes is limited to discrete subpopulations of cells within a given organ such as a root or a nodule (Laplaze *et al.*, 2002; Santi *et al.*, 2003; Svistoonoff *et al.*, 2003).

3.2 Transgenic Casuarinaceae as a Tool for Evolutionary Studies of N₂ Fixing Symbioses

The symbiotic association between *Frankia* and Casuarina exhibits striking differences to the *Rhizobium*-legume symbiosis (Pawlowski and Bisseling, 1996; Wall, 2000; Vessey *et al.*, 2005). *Frankia* is a filamentous, branching, gram-positive actinomycete, whereas *Rhizobia* are gram-negative unicellular bacteria. Legume root nodules are stemlike structures with peripheral vascular bundles and infected cells in the central

tissue, whereas actinorhizal nodules conserve the structure of a lateral root with a central vascular bundle and peripheral infected cortical tissue. Nevertheless, recent phylogenetic data showed that all nodule-forming plants belong to a single clade suggesting a single origin of the predisposition for nitrogen fixation in the angiosperms (Soltis *et al.*, 1995).

Transgenic Casuarinaceae trees provide valuable tools to investigate the conservation of the mechanisms for nodule-specific expression between legumes and actinorhizal plants. Using transgenic Casuarina expressing *gus* driven by promoters from nodulin genes of legumes, transgene expression during ontogenesis of the actinorhizal nodules can be investigated and the data compared to those reported in legumes. This approach has already been used for a number of symbiotic promoters from both early nodulin genes such as *enod40* (Santi *et al.*, 2002) and *enod 12* (Sy *et al.*, 2006), and from late nodulin hemoglobin genes (Franché *et al.*, 1998a). A deeper understanding of the different types of nitrogen-fixing nodules will facilitate the development of future strategies to modify lateral root development on nonsymbiotic plants to enable some to associate with nitrogen-fixing bacteria.

3.3 Gene Transfer for the Improvement of Casuarinaceae

Gene transfer procedures based on *A. tumefaciens* and knowledge of gene expression conferred by heterologous promoters such as the 35S in Casuarinaceae pave the way for genetic engineering of these tropical trees. Furthermore, the isolation and characterization of promoters from Casuarina will contribute to providing sequences that are well suited for the expression of valuable traits in these tropical trees.

Strategies can be developed to engineer Casuarina to resist major pathogens, such as *Rhizoctonia solani*, and insect pests, such as *L. xyli*. Transgenic trees that are more tolerant to adverse edaphic conditions, such as salt and drought, would also be very valuable in tropical regions. Other aspects may include modification of lignin content and/or composition to obtain trees that are more suitable for industrial uses.

Paper production has more than tripled in the last 35 years and the paper industry suffers from the high cost of removing lignin from cellulose, which also has a negative environmental impact (Jouanin and Goujon, 2004). Another goal linked to lignin modification could be to prevent *Casuarina* wood from splitting when it dries. This is currently a major drawback for the use of *Casuarina* wood for the manufacture of furniture.

At the time of writing, no transgenic *Casuarina* trees have yet been planted in the field. Additional information on the stability of transgene expression in field-grown Casuarinaceae exposed to changing environments is thus needed to determine the real potential of genetic engineering for the introduction of valuable new traits in this tropical tree family. Furthermore, to prevent an uncontrolled escape into the environment, efforts should be made to obtain sterile transgenic Casuarinaceae trees that do not form fertile pollen or seeds.

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Black Locust

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1. INTRODUCTION

1.1 History, Origin, and Distribution

Robinia pseudoacacia L. (black locust, false acacia, robinia, honey locust, pea flower, post locust, yellow locust, green locust, and white locust) is a nitrogen-fixing deciduous tree native to eastern North America from the Ozark–Ouachita region to the southern Appalachians. It is widely cultivated as an ornamental tree in many parts of the world (Figures 1a and b). The genus *Robinia* is dedicated to Jean Robin (1550–1629) and his son Vespasian Robin (1579–1662), herbalists to the kings of France and the first to cultivate black locust in Europe at the beginning of the 17th century. The specific name of *pseudoacacia* comes from its obvious similarity to the subtropical acacias of Africa. Since its discovery, the tree has become widely distributed not only in Europe but also throughout the temperate and Mediterranean zones of the world. Because it is a nitrogen fixer and has rapid juvenile growth, it is widely planted as an ornamental, for shelterbelts, and for land reclamation. It is suitable for fuel wood and pulp, and provides cover for wildlife, browse for deer, and nesting cavities for birds. *Robinia* is the second most widely planted hardwood genus in the world (Keresztesi, 1980). The species has escaped cultivation and become naturalized throughout the United States, southern Canada, and parts of Europe and Asia.

1.2 Botanical Description

Black locust belongs to Division Magnoliophyta, Class Magnoliopsida, Order Fabales, Family Fabaceae, Genus *Robinia*, and species *pseudoacacia*. The species is described as a medium-sized tree, generally 12–18 m in height and 30–76 cm in diameter. On better sites it may reach 30 m in height and 122 cm or more in diameter. The leaves are alternate and compound, 15–30 cm long with 7–19 elliptical leaflets, folding at night. The flowers, numerous in drooping racemes 10–20 cm long at the base of leaves, are fragrant and white, about 2 cm long. Blooming in late spring, they are pollinated by honeybees and other insects. The fruit of black locust is a legume 5–10 cm long and 2–3 cm wide, containing four to eight seeds. The fruit ripen during September and October, opening on the tree, and seeds are dispersed from September to April (Olson, 1974). Dry seeds can be stored and they retain their viability for as long as 10 years, if placed in closed containers at 0–5 °C. Young trees grow very fast on good sites, but the species matures early and the growth rate decreases rapidly after 30 years, especially on poor sites. Black locust also produces stump sprouts and root suckers prolifically. In some areas, natural reproduction by root suckers exceeds that by seeds (Huntley, 1990).

The native range of black locust is classified as humid, with two local areas of superhumid climate (Thornwaite, 1931), but black locust seems to do



Figure 1 (a) Adult black locust (*R. pseudoacacia* L.) tree [Reproduced from <http://www.forestryimages.org> with permission of Chris Evans, University of Georgia]; (b) diagram of black locust flowers, leaves, and fruits [Reproduced from <http://www.forestryimages.org> with permission of Zelimir Borzan, retired from the University of Zagreb]

well in many different habitats. The range includes the cool temperate moist forest, warm temperate montane moist forest, warm temperate montane wet forest, and warm temperate moist forest life zones (Sawyer and Lindsey, 1964).

Black locust grows below 1000 m altitude over a wide range of soils with the exception of extremely dry or clayey ones (De Gómez and Wagner, 2001). The most common orders of soil within its native range are Inceptisols, Ultisols, and Alfisols, and the most common soil great groups are Hapludults, Paleudults, Dystrochrepts, and Eutrochrepts (Soil Conservation Service, 1970). The species is intolerant to shade and unless it is a dominant tree, will not grow in dense woods (Trimble, 1975).

In its native area, black locust is severely damaged by coleopteran insects such as *Megacyllene robiniae* Foster (the locust borer, Figure 2) and *Odontota dorsalis* Thunberg (the locust leaf miner), preventing growth of the tree for timber production (Hoffard, 1992). In Hungary, where these pests are not found, it is utilized

as a multipurpose tree (Keresztesi, 1988). In other countries, except for reclamation, most forest managers consider the tree to be a weed species and a strong competitor against more desirable species (Hanover, 1993). Recent



Figure 2 Wood damage caused by locust borer (*M. robiniae* Forster) [Reproduced from <http://www.forestryimages.org> with permission of James Solomon, USDA Forest Service]

studies identified several allelopathic substances including robinetin, myricetin, and quercetin from leaf tissues, suggesting a potential role of these flavonoids in *R. pseudoacacia* invasion in introduced habitats (Nasir *et al.*, 2005).

Black locust has a small genome size ($1C = 0.65$ pg, picogram) (Bennett and Leitch, 2005) and is primarily diploid ($2n = 20$), although it can occur as a polyploid. In fact, a tetraploid clone has been described in Korea (Kim and Lee, 1973). Several cultural varieties have been recognized in the United States (Bongarten, 1992; Hanover, 1993) and Europe (Keresztesi, 1988). Shipmast locust (*R. pseudoacacia* var. *rectissima*), a clone of unknown origin, is listed by Little (1979) as a natural variety. Surles *et al.* (1989) reported a high degree of polymorphism (71%) for 18 enzyme systems in the species. Four hybrids resulting from crosses with Kelsey locust, *Robinia kelseyi* Hutch. (*R. × slavinii* Rehd.); New Mexico locust, *Robinia neomexicana* Gray (*R. × holtii* Beissn.); clammy locust, *Robinia viscosa* Vent. (*R. × ambigua* Poir.); and bristly locust, *Robinia hispida* L. (*R. × margaretta* Ashe) are recognized (Little, 1979).

Black locust contributes high levels of nitrogen to the soil from nitrogen-fixing bacterial symbiosis (De Gómez and Wagner, 2001). Studies on rhizobial strains nodulating black locust demonstrated that the species is nodulated with a high diversity of rhizobial strains, most of which belong to the genus *Mesorhizobium*. This rhizobial diversity may contribute to the success of black locust as a pioneer tree species in the temperate zone (Ulrich and Zaspel, 2000).

1.3 Economic Importance

Black locust is widely planted in the United States, Europe, and Asia for erosion control, reclamation of drastically disturbed sites, windbreaks, nurse crops, amelioration of sites, honey production, and ornamental use. It is also suitable for use in fuel plantations (Eigel *et al.*, 1980; Geyer and Naughton, 1980).

Black locust wood is very heavy and hard, exceedingly strong and stiff, and has very high shock resistance and high nail-holding qualities. The heartwood has high decay resistance due to flavonoids and can endure for over 100 years in the

soil (Barrett *et al.*, 1990; Hizioglu and Kamdem, 1995). Because of its durability, the primary use of black locust wood has been for fence posts, mine timbers, poles, railroad ties, insulator pins, ship timber, tree nails for wooden ship construction, boxes, crates, pegs, stakes, and novelties. However, in the United States, severe damage by the locust borer mentioned earlier, as well as the fungi (*Phellinus rimosus* and *Polyporus robiniophilus*) that attack the stem, causing deformation or breakage, has virtually removed the species from lumber production (Hanover, 1993). In Hungary, where the insect is not a problem, black locust is a major timber tree (Keresztesi, 1988). The wood is also suitable for chemical pulping systems in the paper industry (Miller *et al.*, 1987; Hanover and Mebrahtu, 1991).

Black locust's virtues, including nitrogen-fixing ability, inexpensive propagation by seed, rapid vegetative propagation, adaptability to a wide range of site conditions, rapid juvenile growth, high heat content of the wood, and prolific regrowth after cutting, make the species widely used for erosion control, reforestation, and woody biomass plantings. Commercial energy production may eventually become one of its primary uses in the United States (Miller *et al.*, 1987).

Black locust is valued for its beneficial effect on neighboring trees in the form of nitrogen-fixing activity around the roots and in elements released from decomposing litter (Landgraf *et al.*, 2005). It is an outstanding tree for reclamation (Kim and Lee, 2005) and phytostabilization of unsanitary landfills (Mertens *et al.*, 2004). Planting the species on mine sites provides the advantages of watershed protection, site amelioration, aesthetic enhancement, screening, and food and habitat for wildlife. Leaves of the species have also been used to monitor heavy metal contamination in soils (Celik *et al.*, 2005).

In Hungary, black locust plantings are vital to commercial honey production (Keresztesi, 1988). The leaves are used for livestock feed (forage) in the Republic of Korea and in Bulgaria (Keresztesi, 1983, 1988). For this purpose, in the highlands of Nepal and northern India, where black locust is naturalized, a tetraploid black locust clone, *R. pseudoacacia* "Gigas", with leaves three times larger than other genotypes and with higher protein content, is used (Baertsche *et al.*, 1986).

1.4 Traditional Breeding: Breeding Objectives, Tools and Strategies, and Achievements

Because black locust has been widely planted, the proper seed treatment and nursery practices are well described. Black locust produces abundant seeds, but the seeds remain dormant. Seed coats are impermeable and germination must be induced by scarification. Soaking in concentrated sulfuric acid, soaking in boiling or near-boiling water, and mechanical scarification have proven successful to induce germination (Hartmann *et al.*, 1997). The species can also be propagated by root cuttings and by grafting using a whip, side veneer, cleft, or wedge graft (Parr, 1983; Ward, 1993), but success rates vary among clones. Grafted or rooted shoot sections produce lateral growth, while sprouts from roots grow upright (Prentice, 1987). Sprouts grow more rapidly than seedlings.

The first breeding work began in Hungary in 1930, and many improved cultivars for specific uses such as wood and nectar production have been registered (Keresztesi, 1983). Clonal selection, early pruning, and close spacing have been effective means to produce straight-stemmed black locust plantations. Breeding programs have been developed in the United States for biomass production (Miller *et al.*, 1987; Barrett *et al.*, 1990; Bongarten *et al.*, 1992) and for other traits such as growth rate, borer resistance, or stem form (Hanover, 1993). These programs were carried out at the Michigan State University and the University of Georgia (Kennedy, 1983; Miller *et al.*, 1987). All traits showed family differences, but no variation by region of origin was found (Mebrahtu and Hanover, 1989). Superior individuals have been reproduced from stem and root cuttings, and established in plots of single clones to be used for seed production and forage/biomass studies. Selection and propagation of trees with superior vigor, form, and resistance to borers have been attempted.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Traditional breeding efforts have been so far inefficient, mainly due to the difficulty in obtaining controlled crosses (Han *et al.*, 1999), making it

difficult to fix desirable alleles in a particular highly heterozygous background (Williams and Savolainen, 1996). Thus, conventional breeding is rather slow and unproductive and cannot be used efficiently for the genetic improvement of trees. In black locust, selection and propagation of trees with superior vigor, form, and resistance to borers have been attempted using traditional vegetative propagation methods. Early results indicate significant differences in borer attack among clones and sites; however, differences were small and to date have had no practical application.

Plant tissue culture and genetic transformation methods offer an important option for the effective multiplication and improvement of trees within a limited time frame (Giri *et al.*, 2004). Genetic engineering assumes additional significance because of the possibility of introducing a desired gene in a single step for precision breeding of forest trees. Genetic transformation requires the development of methods for *in vitro* regeneration of the species. Three black locust *in vitro* regeneration pathways have been reported: axillary bud elongation and multiplication from nodal or apical buds (Chalupa, 1983, 1987; Barghchi, 1987; Davis and Keathley, 1987; Han *et al.*, 1997); differentiation of adventitious buds from various plant organs (Brown and Sommer, 1982; Davis and Keathley, 1985; Han and Keathley, 1989; Arrillaga and Merkle, 1993; Han *et al.*, 1997); and somatic embryogenesis from immature embryos (Merkle and Wiecko, 1989; Arrillaga *et al.*, 1994) (Figure 3). These techniques will allow mass propagation and/or conservation of the selected genotypes

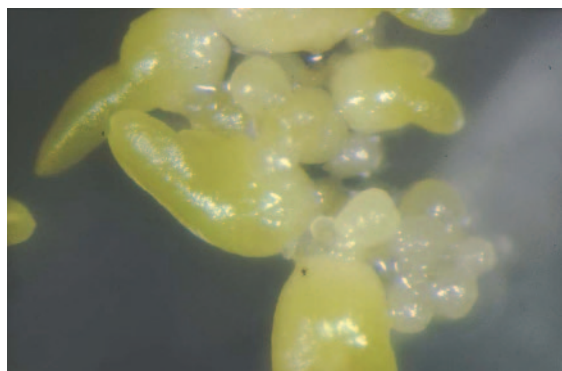


Figure 3 Somatic embryos from immature black locust seeds

(Verleysen *et al.*, 2005) and are the basis for the application of transgenic technology that will produce trees with superior qualities such as disease and pest resistance and high wood quality.

2. REVIEW ON TRANSGENICS DEVELOPED

In spite of its feasibility to be propagated by *in vitro* techniques, there are a few reports on successful protocols to regenerate normal transgenic black locust plants. The latest review on this subject (Han *et al.*, 1999) reported the susceptibility of black locust to *Agrobacterium* infection (Davis and Keathley, 1989) and the production of transgenic plants after co-culture with either *Agrobacterium rhizogenes* (Han *et al.*, 1993) or *Agrobacterium tumefaciens* (Ahn, 1995).

Briefly, Davis and Keathley (1989) inoculated cotyledons and hypocotyls with five *A. tumefaciens* strains and one *A. rhizogenes* strain. Infection with wildtype A6, A348, A247, and A208 *A. tumefaciens* strains incited unorganized tumors, whereas wild A4 *A. rhizogenes* strain induced hairy roots. A modified A281 *A. tumefaciens* strain carrying the binary vector pGA472 with the neomycin phosphotransferase (*nptII*) gene produced kanamycin-resistant callus (Davis and Keathley, 1989). In all cases, Southern hybridiza-

tion indicated that transfer-DNA (T-DNA) was integrated into the DNA of tumors or calli. Han *et al.* (1993) obtained hairy roots after inoculating hypocotyl explants with *A. rhizogenes* R1601 strain carrying the *nptII* gene. Subsequently, hairy roots were isolated and cultured in the presence of kanamycin to induce callus and further shoot regeneration. The integration of the transgene in the regenerated plants was confirmed by Southern hybridization. Nevertheless, only abnormal plants with dark green and wrinkled leaves and hairy root phenotypes were obtained (Han *et al.*, 1993). Finally, the review reported on the production of transgenic black locust shoots following inoculation of hypocotyls with *A. tumefaciens* LBA4404 strain carrying the binary vector pBSW1. Integration of transgenes was confirmed by Southern hybridization (Ahn, 1995).

Recent results describing procedures to obtain transgenic black locust plants are summarized in Table 1 and discussed in the following sections.

2.1 Plant Material and Culture Media

To the best of our knowledge, the explant sources used for genetic transformation of black locust are as follows: (1) an embryogenic culture line (BL2-1C) initiated from immature embryos as described by Merkle and Wiecko (1989) and

Table 1 Black locust transformation protocols based on *Agrobacterium tumefaciens* infection

Bacteria strain	Plasmid	Transgenes	Selectable marker	Protocol	Explant	Observations	References
LBA4404	pSMAH704	<i>gus/hpt</i>	Hygromycin	Vacuum infiltration	Leaf Stem	No plants Plants No Southern data	Igasaki <i>et al.</i> , 2000
EHA101	pSMAH704	<i>gus/hpt</i>	Hygromycin	Vacuum infiltration	Leaf Stem	Plants Plants No Southern data	Igasaki <i>et al.</i> , 2000
GV3101	pSMAH704	<i>gus/hpt</i>	Hygromycin	Vacuum infiltration	Leaf Stem	Plants Plants MUG assay Southern data	Igasaki <i>et al.</i> , 2000
LBA4404	PBI121	<i>gus/nptII</i>	Kanamycin	Immersion	Hypocotyl	Plants No Southern data	Kanwar <i>et al.</i> , 2003
AGL1	PTAB16	<i>gus/bar</i>	Phosphinothricin	Sonication	Cotyledon	Plants PCR Southern data CR assay Herbicide application	Zaragoza <i>et al.</i> , 2004

Table 2 Media composition used in black locust transformation experiments

Component	MS	RPR	MSBIB	FM
Macronutrients	MS	MS	MS	Finer
Micronutrients	MS	MS	MS	MS
Vitamins	MS	B5	B5	B5
Sucrose (g l ⁻¹)	30	30	30	60
Zeatin (μM)	—	11.4	—	—
BA (μM)	5	11.1	4.4	—
2,4-D (μM)	—	0.2	—	8.8
IBA (μM)	—	—	26.4	—
NAA (μM)	5	—	—	—
Glutamine (g l ⁻¹)	—	—	—	2.2
Gelling agent (%)	?	Gellan gun (3)	Difco-bacto (8)	Phytagar (8)
Antibiotic/herbicide (mg l ⁻¹)				
Kanamycin	50	—	—	—
Geneticin	—	—	—	10
Hygromycin	—	20	—	—
Phosphinothricin	—	—	4	—
Carbenicillin	500	500	—	—
Cefotaxime	—	500	—	—
Timentin	—	—	150	—
References	Kanwar <i>et al.</i> , 2003	Igasaki <i>et al.</i> , 2000	Zaragoza <i>et al.</i> , 2004	Arrillaga and Merkle, 1994

maintained as a suspension of proembryogenic masses (PEMs); (2) hypocotyls from 15-day-old seedlings germinated on sterilized sand (Kanwar *et al.*, 2003); (3) cotyledons from freshly isolated embryos (Zaragoza *et al.*, 2004); and (4) leaf or stem segments from *in vitro* grown plants (Igasaki *et al.*, 2000).

Culture media consisted of Murashige and Skoog (MS) (Murashige and Skoog, 1962) macronutrients and micronutrients salts, MS or B5 vitamins (Gamborg *et al.*, 1968), and different combinations of plant growth regulators (auxins and cytokinins). In the case of the embryogenic culture used for transformation, PEMs were maintained on a modified 10A40N medium (Finer and Nagasawa, 1988) supplemented with 8.8 μM 2,4-dichlorophenoxyacetic acid (2,4-D)

(here designated FM, Finer's medium). Media composition, antibiotics, and selective agents are described in Table 2.

2.2 Selection of Transformed Tissue

Before attempting transformation, sensitivity of the target material to several selective agents (antibiotics such as kanamycin, geneticin, and hygromycin, and herbicides such as phosphinothricin) was tested (see Table 3). Explant performance was recorded after 3–4 weeks. Hypocotyl and leaf explants were cultured on a kanamycin-containing medium. For hypocotyls, 0, 10, 20, 30, and 40 mg l⁻¹ kanamycin were used (Kanwar *et al.*, 2003), whereas 25, 50, 100, and

Table 3 Selective agents and concentrations tested for genetic transformation in black locust

Tissue	Genes	Selective agent	Range tested (mg l ⁻¹)	Optimal concentration	References
PEMs	<i>nptII</i>	Kanamycin	0–200	None	Arrillaga and Merkle, 1994
		Geneticin	0–50	10 mg l ⁻¹	
Hypocotyl	<i>nptII</i>	Kanamycin	0–40	50 mg l ⁻¹	Kanwar <i>et al.</i> , 2003
Leaf	<i>nptII</i>	Kanamycin	0–200	None	Igasaki <i>et al.</i> , 2000
	<i>hpt</i>	Hygromycin	0–40	20 mg l ⁻¹	Igasaki <i>et al.</i> , 2000
Cotyledon	<i>bar</i>	Phosphinothricin	0–15	4 mg l ⁻¹	Zaragoza <i>et al.</i> , 2004

200 mg l⁻¹ were tested for leaves (Igasaki *et al.*, 2000). The kanamycin sensitivity test performed by Kanwar *et al.* (2003) revealed that callus from hypocotyl explants was inhibited in the presence of 10 mg l⁻¹ kanamycin, but these authors used a higher kanamycin concentration (50 mg l⁻¹) for transformation experiments (Kanwar *et al.*, 2003). In contrast, Igasaki *et al.* (2000) found that this antibiotic did not inhibit callus growth in nontransgenic tissue, even when used at concentrations of 200 mg l⁻¹. Therefore, they tested the effect of hygromycin B at 0, 5, 10, 20, and 40 mg l⁻¹ on leaf explants. The results suggested that the *hpt* (hygromycin phosphotransferase) gene was most suitable as a selectable marker gene, since 20 mg l⁻¹ hygromycin B inhibited growth. This level was used in subsequent transformation experiments. Tolerance of cotyledon explants to phosphinothricin (PPT) at 0, 5, 10, and 15 mg l⁻¹ was tested. The presence of 5 mg l⁻¹ PPT inhibited organogenesis. Therefore, for selection of putative transgenic black locust shoots, 4 mg l⁻¹ PPT were added to the culture medium (Zaragoza *et al.*, 2004). In PEMs, the capability of kanamycin (25, 50, 75, 100, 150, and 200 mg l⁻¹) and geneticin (0, 5, 10, 15, 20, and 25 mg l⁻¹) to inhibit growth of nontransformed black locust PEMs demonstrated that kanamycin was not as effective a selection agent as geneticin. Even at 200 mg l⁻¹, kanamycin failed to inhibit growth, whereas a severe growth inhibition was observed at concentrations of geneticin higher than 10 mg l⁻¹ (Arrillaga and Merkle, 1994).

2.3 Direct DNA Transfer

Arrillaga and Merkle (1994) used the biolistic approach in an attempt to transform black locust PEMs. First, factors affecting DNA delivery, such as gas pressure propelling the macrocarrier and gap distance between the rupture disk and the macrocarrier, were tested. The best results in terms of transient β -glucuronidase (GUS) expression were obtained with a rupture disc that broke at 1100 psi (7.5 MPa) and a gap distance of 6.5 mm. In these experiments, plasmid pBI121, containing the selectable marker gene *nptII* driven by the nopaline synthase promoter (NOS), and the reporter gene *gusA* fused to the

cauliflower mosaic virus (CaMV) 35S promoter was used. Subsequently, PEMs were bombarded with plasmid (pBI2S21) where the *gusA* gene was fused to the promoter from a constitutively expressed *Arabidopsis* actin gene, *Ac2*. GUS transient expression assayed 2 days following bombardment and measured as the number of blue spots per plate, increased up to sixfold (900 spots/plate) when the *Ac2* promoter was used (Figure 4). Although some PEMs grew on a selection medium containing 10 mg l⁻¹ geneticin (Figure 5), no stable integration of either transgene was detected by polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay. Therefore, attempts to regenerate somatic embryos from the geneticin-resistant material were discontinued.

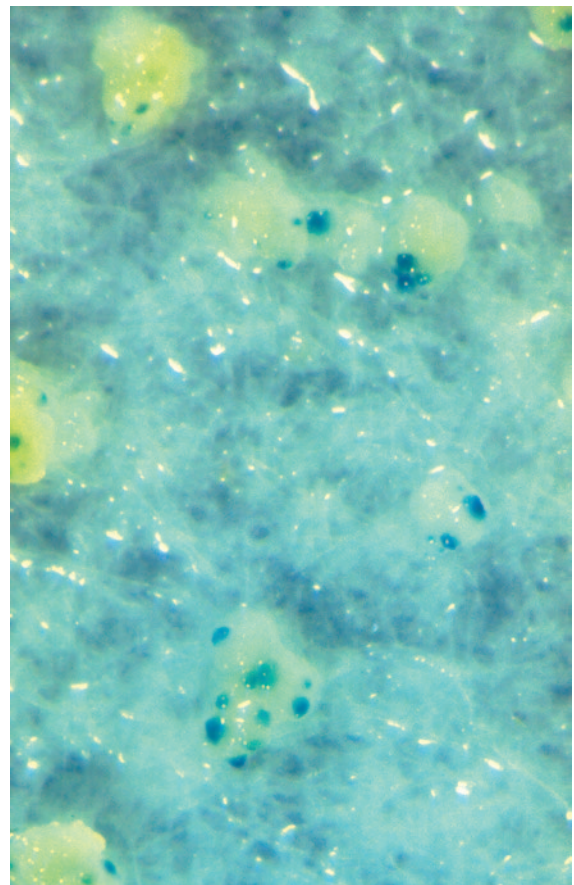


Figure 4 Transient GUS expression in black locust proembryonic masses after particle bombardment

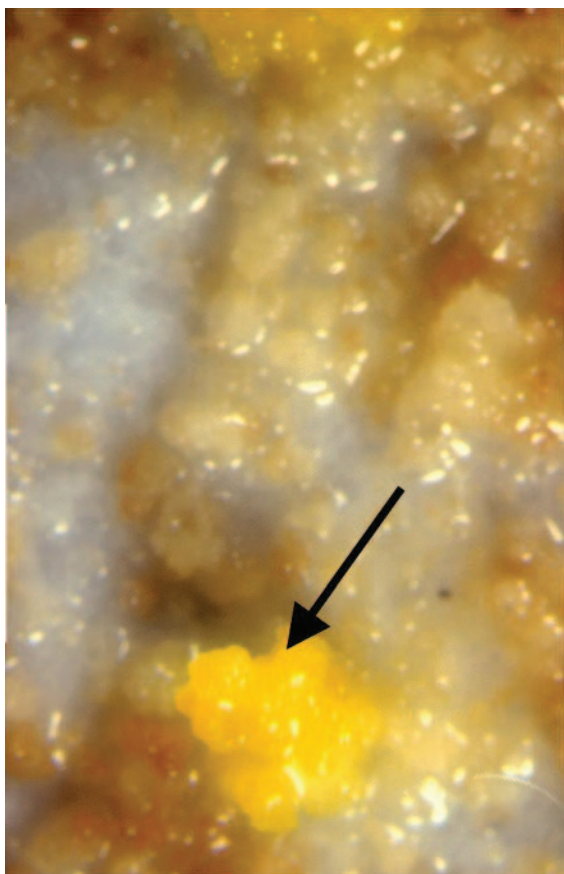


Figure 5 Putative black locust transgenic PEMs growing on geneticin containing medium (see arrow)

2.4 *Agrobacterium*-mediated Transformation

2.4.1 Bacterial strains and plasmids

Several disarmed *A. tumefaciens* strains have been used to transfer T-DNA to black locust: LBA4404 (Hoekema *et al.*, 1983), EHA101 (Hood *et al.*, 1986), GV3101 (Koncz and Shell, 1986), and AGL1 (Lazo *et al.*, 1991). These strains harbored the following binary vectors (see Table 3): pBI121 (containing the *gusA* and *nptII* genes both under the control of CaMV 35S promoter); pSMAH704 contained genes for GUS and HPT (Igasaki *et al.*, 2000), and pTAB16 that includes the *bar* (bialaphos resistance) gene, which confers tolerance to the herbicide PPT and the *gusA* gene, both driven by CaMV 35S promoter (Schröder

et al., 1993). The *gusA* gene in pTAB16 plasmid is not expressed in bacteria due to the insertion of a plant intron in the coding region.

2.4.2 Transformation procedures

The published transformation protocols employ different explants, bacterial strains, and plasmids. Because of this variation, procedures are detailed separately. In all cases, *Agrobacterium* was grown overnight and diluted to the appropriate concentration ($1\text{--}5 \times 10^8$ cells/ml) before being used for infection.

Leaf and stem explants (Igasaki *et al.*, 2000). Leaf and stem explants were vacuum infiltrated for 20 min with the bacteria suspension containing 20 μM acetosyringone, then blotted with sterile filter paper and incubated for 2 days. After removing bacteria with several washes on the medium containing carbenicillin and cefotaxime, explants were transferred to the selection medium (Table 2). Regenerated shoots were subcultured to the same medium without growth regulators.

Hypocotyl explants (Kanwar *et al.*, 2003). Prior to infection, explants were preconditioned for 0, 24, 48, or 72 h on a regeneration medium without antibiotics. After infection by immersion 8–10 s in the bacterial suspension, hypocotyls were blotted dry and transferred to the selection medium (Table 2). Calli growing on the selection medium were transferred to the selective MS shoot-induction medium with 0.5 μM NAA (α -naphthaleneacetic acid), 1 μM BA (benzyladenine) and 50 mg l^{-1} kanamycin.

Cotyledon explants (Zaragoza *et al.*, 2004). Freshly isolated cotyledons were preconditioned for 72 h on a regeneration medium with 100 μM acetosyringone. Explants were either incubated or sonicated for 20 min in different bacteria concentrations (optical density 0.3, 0.6, 0.8). Transient GUS expression (see below) was used to determine the best bacterial delivery treatment 3 days after infection. Further experiments included preconditioning of the explants (0, 72, or 96 h) and the period of time before applying herbicide to the culture medium (0 or 3 days). After 3 days of co-culture, explants were transferred to the selection

medium (Table 2). Regenerating explants were transferred to the same medium with 4.4 μM BA and 0.5 μM IAA (indole-3-acetic acid), PPT, and timentin (see Table 2).

2.4.3 Testing transgene activity

Histochemical GUS assay. Histochemical GUS assays (X-gluc) were carried out according to Jefferson *et al.* (1987) and were used for transient expression experiments (Zaragozá *et al.*, 2004) and to detect stable integration in hypocotyl-derived callus, cotyledons, and leaves. Cells and tissues were fixed in 0.3% formaldehyde and 0.3 M mannitol prior to the GUS assay. For fluorimetric assay of GUS activity, 4 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) was used as substrate (Igasaki *et al.*, 2000).

Molecular analysis of the regenerated plants. Genomic DNA was extracted using the Doyle and Doyle (1990) or Murray and Thompson (1980) protocols. Molecular analyses were performed on putative transgenic and control plants by PCR and Southern hybridization. DNA was digested with *Eco*R1, *Bam*H1, and *Hind*III to excise the *bar*, *hpt*, and *gusA* genes, respectively. Filters were hybridized with PCR-labeled *bar*, *hpt*, or *gusA* probes. The presence of introduced DNA was determined by nonradioactive digoxigenin (Zaragozá *et al.*, 2004) or by radioactive (Igasaki *et al.*, 2000) methods.

Chlorophenol red assay (Zaragozá *et al.*, 2004). To test the ability of transgenic plants to detoxify PPT and avoid ammonium accumulation, the chlorophenol red (CR) assay as described by Kramer *et al.* (1993) was used. Putatively transgenic shoots (4 cm length) and controls, previously micropropagated through axillary bud proliferation on the Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) medium, were cultured in test tubes containing SH with 2 mg l^{-1} PPT and 50 mg l^{-1} CR, gelled with 0.7% agar. The pH of the medium was adjusted to 6.0, a pH at which the medium is a deep red color. The change of color to yellow, indicating that ammonium was not accumulated, was evaluated after 3 days.

Herbicide application. Regenerated plants were acclimatized and tested for their response to herbicide by a leaf spray assay. Transgenic and control plants were sprayed with an aqueous solution containing 160 mg l^{-1} of glufosinate ammonium and 0.1% Tween 20. Results were scored after 4 days.

2.4.4 Regeneration of whole transgenic plants

The three protocols reviewed here are all suitable to produce black locust transgenic plants. Nevertheless the results obtained indicated that the following factors affect transformation efficiency.

Bacterial strain and explant. In a pioneering work, Davis and Keathley (1989) demonstrated that black locust can be infected by a wide range of *Agrobacterium* wild strains. More recently, transgenic plants were obtained after infection with LBA4404, EHA101, GV3101, and AGL1 strains. A general conclusion regarding the best strain for black locust transformation is not feasible since the bacteria carried different plasmids and were used to infect a variety of explants. However, when three different *A. tumefaciens* strains carrying the same plasmid were tested to infect leaf and stem explants, the GV3101 strain led to a transformation frequency higher than that obtained with EHA101 and LBA4404 (Igasaki *et al.*, 2000). The authors also identified stems as the best tissue for such transformation. Arrillaga and Zaragozá (1999) also found that LBA4404 was less effective than EHA105 and AGL1 for infection of black locust cotyledons.

Bacterial delivery. Sonication and vacuum infiltration have been successfully used for bacteria delivery to cotyledons and leaf or stem explants, respectively. Assays of transient GUS expression in control and sonicated cotyledons demonstrated that 1 min of sonication significantly enhanced the percentage of cotyledons with GUS activity (96.7 vs. 75.3 %) as well as the number of blue spots ≥ 1 mm (Figure 6).

Preconditioning of explants. Preculture of explants on a regeneration medium 2–4 days prior to

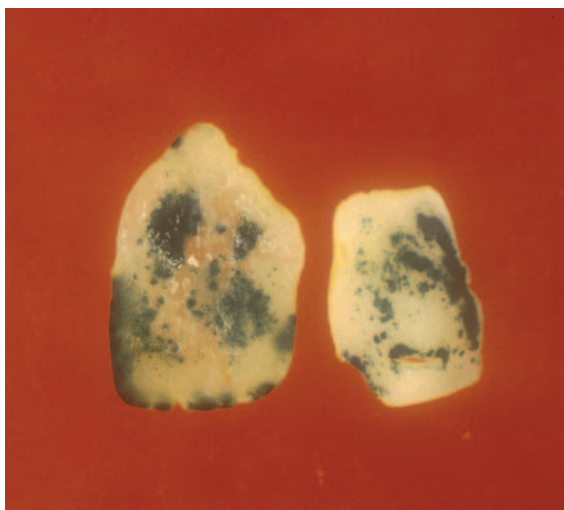


Figure 6 Transient GUS expression on cotyledon explants 3 days after sonication in a bacterial suspension

transformation was required to obtain transgenic plants from black locust cotyledons (Figure 7) and hypocotyls (Zaragoza *et al.*, 2004; Kanwar *et al.*, 2003, respectively). This procedure seemed to be unnecessary for leaf and stem explants.

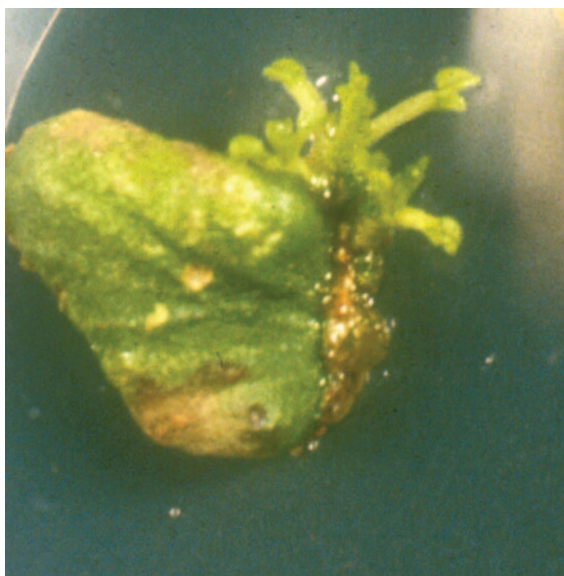


Figure 7 Regeneration of black locust shoots on PPT-containing medium

2.4.5 Transgene integration

Gus activity was detected by X-gluc (5-bromo-4-chloro-3-indolyl β -D-glucuronide) assays in callus growing on a kanamycin-containing medium (Kanwar *et al.*, 2003) and on leaves isolated from transgenic plants regenerated in the presence of hygromycin or PPT (Igasaki *et al.*, 2000; Zaragoza *et al.*, 2004). GUS activity was also determined by the MUG assay, showing that enzyme activity was higher in transgenic lines than in nontransformed plants, with values ranging from 6.5 to 12.8 nM 4-methylumbelliferone (4-MU)/mg protein/min in transgenic plants versus 0.17 nM in controls (Igasaki *et al.*, 2000).

The integration of the *hpt* and *bar* transgenes was confirmed by PCR and Southern hybridization. No variation in the number of copies of the *hpt* gene was observed since all the three transgenic plants assayed integrated a single copy of the transgene. On the contrary, three out of the six plants analyzed for the *bar* gene showed a single insertion of the gene and the other three had two insertions of the gene. The stability of the *bar* transgene in the micropropagated transgenic plants was confirmed periodically by PCR analysis (Zaragoza *et al.*, 2004). Kanwar *et al.* (2003) did not report any molecular characterization on the integration of the *nptII* gene.

CR assays showed that all *bar* PCR-positive plants induced changes from red to yellow indicating that phosphinothricin acetyl transferase (PAT), the enzyme encoded by the *bar* gene, was able to detoxify PPT and ammonium was not accumulated (Figure 8).

Herbicide tolerance assays performed on excised leaves and whole transgenic plants demonstrated that the level of PAT expression in these plants was high enough to confer resistance to ammonium glufosinate. Control plants died 4 days following herbicide application, while transgenic plants survived and leaves remained green (Figure 9).

From the literature reviewed, it can be concluded that it is possible to regenerate phenotypically normal transgenic *R. pseudoacacia* plants using *Agrobacterium* strains and that several genes can be used as selectable markers (*nptII*, *hpt*, and *bar*). Results, however, differ among authors. Igasaki *et al.* (2000) suggested that neither kanamycin nor geneticin were suitable selection



Figure 8 CR assay: control plant (right) and two *bar*-positive transgenic plants (left)



Figure 9 Herbicide assay: two controls (left) and three *bar*-positive (right) black locust plants 4 days after herbicide application

agents for black locust since nontransgenic calli grew up in the presence of these aminoglycosides. Similar results were obtained when cotyledons were infected with LBA4404 carrying the vector pBI121 (Arrillaga, personal communication) or following particle bombardment of PEMs with the same plasmid (Arrillaga and Merkle, 1994). These findings, however, are inconsistent with the reports of Han *et al.* (1993), Ahn (1995) and Kanwar *et al.* (2003) all of whom used kanamycin for selection following *A. tumefaciens*- or *A. rhizogenes*-mediated transformation.

3. FUTURE ROAD MAP

Black locust is among the most widely planted tree species in the world because it is ornamentally attractive, drought tolerant, fast growing, fixes nitrogen, has very hard, durable wood, and is adaptable to many sites and climates. In spite of the biological and economic importance of the species, and its amenability to both tissue culture and genetic transformation, the actual impact of biotechnological tools on the genetic improvement of black locust to date has been minimal. In fact, with the exception of a paper reporting herbicide (glufosinate ammonium)-resistant transgenic black locust (Zaragoza *et al.*, 2004), there is no work reporting engineering of black locust with genes of potential economic importance. Thus, the transformation challenge for the near future in black locust must lie in extending the established protocols to genes of interest. The recently completed sequencing of the *Populus trichocarpa* genome (Tuskan *et al.*, 2006) would facilitate, by sequence comparisons, the identification of genes that are likely to be involved in producing valuable traits in black locust. Among potentially valuable genes are those related to pest and disease resistance, improved wood characteristics, and faster growth. Taking into account the well-known uses of black locust, the biotechnological approaches that may have a high impact for the genetic improvement of the species are as follows:

- (1) Pathogen and pest resistance. Black locust is severely damaged by insects and disease, probably more so than any other eastern US

- hardwood species. Ubiquitous attacks by the locust stem borer (*M. robiniae*), locust leaf miner (*O. dorsalis*), and by the heart rot fungi *P. rimosus* and *P. robinophilus* make growing black locust for timber production impractical. Thus, one of the first goals may be to engineer the tree with genes conferring insect or disease resistance, as has been demonstrated in other hardwood species (Giri *et al.*, 2004).
- (2) Manipulation of wood formation. Advances are being made toward describing developmental mechanisms regulating secondary growth in trees (Groover and Robischon, 2006). In black locust, various genomic tools, including expressed sequence tags and complementary DNA microarrays, have been employed for the study of wood formation (Yang *et al.*, 2003, 2004; <http://biodata.ccgb.umn.edu>). All these advances, in conjunction with the genome sequence of poplar, should provide the identification of black locust genes that control wood formation, a prerequisite for any transgenic strategy aimed at altering the rate of wood biogenesis and properties of wood.
 - (3) Yield improvement. The increasing economical pressure for wood and wood products may require use of intensive management of plantations with genetically engineered trees to produce biomass efficiently (Boerjan, 2005). This is of particular interest for black locust, since the species has been a focus of attention for biomass production for fuel. Since black locust is a leguminous tree, efforts in this field should be primarily focused on the improvement of the nitrogen fixation process and subsequent ammonia assimilation. Another important approach that could contribute to increased yield is the modulation of cell wall extensibility by up- or downregulation of genes implicated in cell wall metabolism. Tree size, morphology, and performance can be also accomplished through modifications in plant hormone levels (Giri *et al.*, 2004). Finally, the prevention of flowering could be another strategy to increase vegetative growth; one way of achieving this might be to express cytotoxic genes from floral-specific promoters (Skinner *et al.*, 2000).
 - (4) Phytoremediation. Because of their large biomass and longevity, trees are an ideal tool for phytoremediation, the use of plants to stabilize, reduce, or detoxify pollutants in soil or water. Given the successful deployment of black locust on mine spoils (Merkle and Nairn, 2005), the tree may be a suitable target for engineering with phytoremediation genes.
 - (5) Black locust metabolomics. In addition to the genome sequence and methods to analyze the transcriptome, a comprehensive analysis of phenotypes and biological processes will require metabolomics, a tool that is generally much less developed (Boerjan, 2005). This attains a special significance in black locust, which has been cited as having a number of interesting traditional medicinal uses, as well as being poisonous to man and animals. However, as Tian *et al.* (2001) state, few chemical investigations have been conducted to identify the active principles in this plant. In fact, these authors identified an unusual compound (robinlin), a novel C-11 homo-monoterpene, with strong bioactivity in the brine shrimp lethality test. Thus, the identification of all the metabolites of the species (their metabolome) and measurements of their dynamics under many different challenges will be of paramount importance. Integrated approaches combining metabolomics with transcriptomics and proteomics may contribute to the metabolic profiling of black locust in the near future. This also will facilitate the identification of genes associated with pest and disease resistance as well as novel metabolites of biomedical interest.
 - (6) Marker-assisted breeding programs. The use of genetic maps based on molecular markers linked to genes of interest facilitates the selection of simple and complex traits, thus accelerating their incorporation into breeding materials. Also, molecular markers are utilized for the characterization of the population structure (the distribution of variability within and between populations) and in paternity testing. Although markers have been utilized in many hardwood tree species, there have been no studies of this type in black locust.

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Sandalwood

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1. INTRODUCTION

1.1 Taxonomy, Distribution, and Ecology

Sandalwood is the general name given to a small group of parasitic shrubs or trees in the genus *Santalum*, family Santalaceae. The taxonomic and molecular phylogeny of these species has recently been reviewed, with a comprehensive summary on 16 species and their variants (Harbaugh and Baldwin, 2007). Members of the genus *Santalum* are widespread, growing throughout India, Indonesia, Australia, New Guinea, Hawaii, and many South Pacific islands. The term sandalwood refers to those species that bear fragrant heartwood and have been utilized commercially for this purpose. *Santalum album*, often referred to as East Indian or tropical sandalwood has long been harvested for its valuable, odorous wood (Rai and Sarma, 1990). The genetic origins of *S. album* appear to be East and West Timor, as well as a small population in northern Australia (Harbaugh and Baldwin, 2007). A more detailed study of the Indo-Papuan species *Santalum macgregorii* and *S. album* in particular has found few mutations in the chloroplast genome, suggesting recent speciation (C. Jones *et al.*, in preparation). In India, it occurs throughout the Indian subcontinent, particularly in the southern states of Karnataka, Kerala, Tamil Nadu, and Andhra Pradesh (Rai, 1990). *Santalum spicatum* is one of Western Australia's oldest export industries and it is still harvested commercially. To a lesser extent, *Santalum lanceolatum*, commonly known

as “plumbush” or northern sandalwood, has been harvested in Queensland and South Australia (Applegate *et al.*, 1990b; Statham, 1990; Applegate and McKinnell, 1993). In the Pacific, *Santalum yasi*, *Santalum austrocaledonicum*, and *Santalum insulare* were extensively utilized (Jiko, 1993; Ehrhart, 1998).

Sandalwoods are slow growing root hemiparasites, meaning they derive some water and nutrients from the roots of neighboring trees and shrubs (Loneragan, 1990; Radomiljac and McComb, 1998; Radomiljac *et al.*, 1999; Tennakoon and Cameron, 2006). Host trees vary, depending on the *Santalum* species and their native ecosystem. In India, *S. album* utilizes a wide range of hosts, and occasionally self-parasitizes (Rai, 1990). *S. album* occurring in Timor similarly parasitizes numerous species, including *Eugenia guajava*, *Casurina junghuhniana*, *Cassia siamea*, *Schleichera oleosa*, and *Pterocarpus indicus* (Suriamihardja and Suriamihardja, 1993). In Australia, *S. spicatum* associates with *Acacia accuminata* and *Eremophila* spp. while *S. lanceolatum* is often found in association with *Acacia cambagei* and *Melaleuca* spp. (Applegate and McKinnell, 1993). Growth rates for each species will vary according to rainfall, soil type, and climate. Tropical *S. album* may take 40–60 years to reach maturity (Rai, 1990).

In contrast, *S. spicatum* is the slowest growing species taking up to 100 years to reach a commercial size (Applegate *et al.*, 1990a). Inflorescences of *Santalum* spp. are usually auxiliary or terminal cymose panicles, petals are 4- or 5-merous,

fused to the perianth, and purple-brown in color (Hewson and George, 1984). The flowers have inferior ovaries yielding one single-seeded drupe. Flesh of the fruit is typically purple; however, *S. spicatum* fruits are dry and fibrous, enclosing a hard nut (Hewson and George, 1984). Although only limited work has been done on the cytological features of sandalwood, root-tip squashes have indicated that *S. album* is diploid and has 20 chromosomes (Kulkarni *et al.*, 1998). Other members of the genus are believed to be tetraploid, particularly the non-oil-yielding *Santalum acuminatum* (Byrne *et al.*, 2003b) and Papua New Guinean *S. macgregorii* (C. Jones *et al.*, in preparation). Several other species within the genus may also be polyploid, and this is currently being investigated (D.T. Harbaugh, personal communication). *S. album* and *S. spicatum* are outcrossing insect-pollinated trees; however, self-pollination is possible (Rugkhla *et al.*, 1997).

1.2 History and Economic Importance

All of the commercially utilized sandalwoods are pulled from the ground at harvest, the upper limbs are cut, and bark removed. Logs are then cut into billets, which are either processed or exported directly (Loneragan, 1990). Usual downstream processing involves grinding the wood into a fine powder and processed into incense or joss sticks. These are used in many Asian ceremonies and religious events (Loneragan, 1990; Rai and Sarma, 1990). The ground wood is also distilled by either hydro- or steam-distillation to produce the neat oil, often used as a fixative for many perfumes. Supercritical CO₂ has been utilized as an alternative to distillation with similar results (Piggot *et al.*, 1997; Moretta *et al.*, 1998). *S. album* yields more heartwood oil than other members of the genus, with typical values around 6.2% (w/w) (Shankaranarayana and Parthasarathi, 1987; Verghese *et al.*, 1990; Shankaranarayana *et al.*, 1998). Extractable oil yield can vary from zero to nearly 9% (w/w) from within one tree (Jones *et al.*, 2006). *S. yasi* from Fiji is reported to yield about 5% oil, *S. austrocaledonicum* around 3–5%, and *S. spicatum* around 2% (Applegate *et al.*, 1990a).

Sandalwood oil production from India is variable. However, the Food and Agriculture

Organization of the United Nations reports an estimated 40 tons per annum over the period of 1987 to 1993 (Anonymous, 1995). Indonesia, the world's second largest producer of *S. album* products, produced an average of 15 tons of oil per annum for the same period. In the 10 years from 1995 to 2005, Indonesia provided the bulk of *S. album* products to the world markets, while India's production levels fell significantly due to scarcity and legislated resource protection. In March 2006, the highest auction price in India for cleaned *S. album* logs was Aus\$105 400 per ton (Source: Tropical Forestry Services Pty. Ltd, Australia).

Sandalwood, despite being a renewable plant resource, is suffering from significant decline in India, Indonesia, the South Pacific, and Australia. This is primarily due to overharvesting and illegal poaching of native stands, or by biological hindrance from grazing animals or sandal spike disease (Loneragan, 1990; Rai and Sarma, 1990; Statham, 1990). Harvesting of *S. spicatum* from native stands is likely to continue for some time although considerable effort is going into developing the species into an extensive plantation crop (Radomiljac, 1998; Brand, 2002; Woodall and Robinson, 2002a, b). In Western Australia, *S. spicatum* and *S. album* are being planted in the southwest and far north of the state, respectively. Plantations of *S. album*, as well as small plots of *S. austrocaledonicum* and *S. macgregorii*, are being trialed at the Frank Wise Agricultural Research Station, Kununurra, Western Australia. The oldest *S. album* trees from these trials are approaching 20 years, and preliminary experiments have shown yields of valuable heartwood oil after 10 years. *S. lanceolatum* is being trialed in Queensland (Bristow *et al.*, 2000), while a concerted effort to re-establish *S. austrocaledonicum* as a tree crop is also underway in Vanuatu (Tate *et al.*, 2006). *S. insulare* from eastern Polynesia is critically endangered due to overharvesting, and as such a conservation program is being implemented (Butaud *et al.*, 2005).

1.3 Traditional Breeding and Improvement Programs

Despite being utilized for centuries, sandalwood has received little attention from a tree improvement perspective. Efforts to understand the complex ecology of the species have largely

hampered field trials, and as such most tree improvement work has been devoted to selection of superior phenotypes from naturally occurring populations. Heritability studies for desirable traits such as tree form, health, early heartwood onset, high heartwood content, and essential oil yield have not been conducted extensively. Although once complete, these investigations would certainly provide a solid rationale for further tree improvement.

Probably the most significant steps toward genetic improvement of sandalwood species have been through genetic diversity studies: identifying restriction fragment length polymorphisms (RFLPs), microsatellite or simple sequence repeat polymorphisms, and random amplified polymorphic DNAs (RAPDs). By gaining some measure of the level of variation within and between sandalwood populations, researchers have been able to assess potential for improvement. *S. spicatum*, *S. austrocaledonicum*, *S. album*, and *S. insulare* have been studied intensively in this regard. *S. spicatum* was assessed for genetic diversity using both nuclear and chloroplast RFLP probes (Byrne *et al.*, 2003a, b) revealing modest levels of genetic variation but few rare alleles and considerable inbreeding coefficients compared to other widespread forest species such as *Eucalyptus* spp. (Hines and Byrne, 2001). Although this particular study used anonymous alleles, these polymorphisms may represent genotypic variation in gene structure and function, which could in turn affect important phenotypic traits such as form, growth rate, and heartwood oil production. *S. austrocaledonicum*, a sandalwood tree confined to the South Pacific islands of Vanuatu and New Caledonia, was recently examined for population structure and genetic diversity using microsatellite markers (Bottin *et al.*, 2005). In contrast to *S. spicatum*, *S. austrocaledonicum* has a very limited distribution, bound by ocean in all directions, significantly restricting gene flow and germplasm dispersal. This probably explains the high mean fixation index, sometimes referred to as the inbreeding coefficient F_{is} , compared to *S. spicatum*. On the basis of this comparison, one may predict that there is considerable scope for improvement in *S. spicatum* compared to *S. austrocaledonicum*; however, caution must be exercised when comparing different techniques and unknown alleles. Whilst microsatellites and

RFLPs represent co-dominant genetic markers, meaning data can be pooled for most forms of analyses, the analytical methods are fundamentally different. Often, the detected polymorphisms may not be directly related to functional genes.

S. insulare, a sandalwood growing throughout the French Polynesia was studied intensively by chloroplast microsatellite polymorphisms (Butaud *et al.*, 2005). As populations of *S. insulare* are restricted to islands, gene flow between populations was particularly low. About one-third (36%) of the total genetic diversity was between archipelagos, 31% between islands within a given archipelago, and 33% within islands. Further studies on the same populations found that clonality was common, and that up to 58% of the trees examined were likely to be clones of another individual (Lhuiller *et al.*, 2006). Clonal reproduction in nature is common for many species of sandalwood. *S. lanceolatum* in Victoria, southeastern Australia, for example, is represented by only one small population of clones (Warburton *et al.*, 2000). The level of genetic variation in *S. lanceolatum* in its northern distributions (Queensland, Northern Territory and northern Western Australia) is not well understood. Clonal reproduction of *S. lanceolatum* has been used to advantage, as regrowth from pulled trees appears to result in healthier root suckers (Bristow *et al.*, 2000).

Genetic diversity in *S. album* has been studied using isozyme variation (Suma and Balasundaran, 2003) and RAPDs (Shashidhara *et al.*, 2003; Suma and Balasundaran, 2004). Both these techniques have helped researchers to understand the extent of diversity remaining within the natural distribution of sandalwood in India. Unfortunately, no comprehensive study has included Indonesian material, although a study by RFLPs of *S. album* growing in northern Western Australia has included some Timorese material for comparison (C. Jones *et al.*, in preparation).

Of all of the work compiled on the genetic diversity of *S. album*, the consensus seems to be that while little diversity exists within populations, there appears to be significant diversity between natural populations. Suma and Balasundaran (2003) found that 78% of all variation was described by differences between populations, while the remainder was within individual populations. The populations studied

were geographically isolated (several hundred kilometers apart) with different climatic zones. Gene flow is hence quite low between populations. Shashidhara and co-workers (2003) found that RAPDs were effective in distinguishing genotypes and estimating relatedness. Levels of genetic dissimilarity were low (15%) between nearby populations, and high (91%) when compared to *S. spicatum* (the designated out group). This would be expected as *S. spicatum* is unique to arid southwestern Australia and resides some 6000 km away. However, when the out group was excluded from the same analysis, 75% was the highest level of genetic dissimilarity. From these findings one may conclude that the level of genetic diversity for *S. album* in India is sufficient for a tree improvement program. Traditional breeding methods for woody perennials, most notably open-pollinated seed orchards, are well established for many forest species, particularly *Eucalyptus* (Chaix *et al.*, 2003). Seed orchards have been established for *S. album* in India and Australia using this principle. The phenotypes of the progeny seed collected from such orchards are difficult to assess due to natural variability in the plantation environment. While RAPD is an accepted means for estimating relatedness, the technique has been shown to overestimate within-population variability in the absence of a suitable out group (Powell *et al.*, 1996). In the Forest Products Commission collection of *S. album* in the Ord River Irrigation Area, Kununurra, Western Australia, genetic variation estimated by RFLPs was low, but phenotypic variation in oil yield was quite substantial. This may be due to subtle nucleotide sequences undetected by this technique (C. Jones *et al.*, in preparation).

In addition to genetic improvement, improved plantation management has taken the sandalwoods to a new level of productivity. For many years, the seemingly simple process of growing seedlings, planting them in the field, and maintaining trees until harvest has been very difficult and often inefficient. It was not until the early 1990s that research into suitable silvicultural practices was conducted, and eventually a fairly reliable production system for *S. album* was published (Radomiljac, 1998). Likewise, *S. spicatum* recently underwent a similar research (Brand, 2002; Woodall and Robinson, 2002a, b). In both instances, it was essential for

a short-term pot host to be grown alongside *Santalum* sp. seedlings. At the time of planting into the field, a medium-term host must be well established nearby and a suitable long-term (longer than 5–7 years) host must eventually be present. Sufficient haustorial connections must be made in the early stages of development. Since all of the sandalwoods are root hemiparasites, early host–parasite relationship is key to their survival. In a recent comprehensive study of the histology and anatomy of *S. album* haustoria, Tennakoon and Cameron (2006) found that proto-haustoria will develop in young seedlings, which remain unattached to host roots. But the formation of a penetration peg, which taps into a host's root xylem is not present in these immature organs. Once attached to the host, a peg is formed. Furthermore, the presence of parenchymous tissue in the host–parasite border supports the hypothesis that cross-membrane solute transport is occurring, and hence some solute selectivity could be involved. Most significantly for the plantation industry, if this process is selective in terms of the nutrients and chemical signals that are moved from host to sandalwood tree, the influence of host species and root connectivity is bound significantly to affect tree growth, vigor, and essential oil productivity.

Not surprisingly, the complex ecology of the sandalwoods makes phenotypic selection difficult. Plant nutrient and water demands are complicated further by host contributions, as well as planting density, pruning regime, and light penetration/requirements. All of these factors contribute to the overall health and vigor of the sandalwood tree, making genetic selection fraught with error. Simple oil yield and heartwood content analysis of standing trees have been used as a selection tool (Jones *et al.*, 2007), but the genetic contribution to any variation identified is substantially masked by agronomic practices and variations in environment niches across the plantation.

In the last 20 years, tissue culture has provided some useful avenues into large-scale clonal propagation of sandalwood. Somatic embryogenesis (Rao and Bapat, 1995; Rugkhla and Jones, 1998; Rai and McComb, 2002) along with molecular studies on intra- and interspecific hybrids (Rugkhla *et al.*, 1997; McComb and Jones, 1998) have been major advances, and trees cloned

in this fashion have shown growth characteristics comparable to naturally regenerated trees. The ability to clonally reproduce large numbers of specific genotypes is essential for any future transgenic work. Currently, tissue culture technique is proving useful for propagation but the process is expensive and time consuming, and application to genetic transformation is lacking.

Unlike the usual applications of tissue culture for plantlet regeneration, Valluri *et al.* (1991) devised a process whereby *S. album* cell cultures were grown in a bioreactor where phenolic compounds were measured as an indicator of secondary metabolic activity. This rationale ensured that the optimum growing abiotic conditions were discovered before further exploitation of the system. Bioreactors offer the benefit of having all necessary genetic machinery operating in a hygienic, controlled environment.

1.4 Scope for Transgenics in Sandalwood

Clearly, traditional tree improvement methods can, and should be, used to deliver more desirable trees. However, the application of transgenic technology to sandalwood may offer a faster route to the same end. One cannot disregard the value of knockout or knock-down sandalwood genotypes to improving our understanding of gene structure, transcription, and regulation. Further to experimental applications, highly active promoters may be introduced to economically important genes such as terpenoid synthases, prenyltransferases, and transcription factors.

Unfortunately, almost none of the *Santalum* genome or transcriptome has been sequenced, let alone functionally characterized. A thorough evaluation of all relevant genes is essential before embarking upon the introduction of foreign genes to sandalwood. The long rotation time for sandalwood trees is probably the main driving force to develop transgenic lines. Traditional crossing and selection trials would take at least 40 to 50 years to evaluate; provided a suitable phenotype screen is established (i.e., one that accounts for environmental variability). Given the long period between harvests for sandalwood, this may not be of concern. However, if genetically superior seedlings could be identified at the nursery stage, or even propagated *in vitro*, growers could

plant sandalwood with certainty of better long-term returns.

2. TRANSGENICS DEPLOYED SO FAR AND POTENTIAL OPTIONS FOR THE FUTURE

2.1 Current Status

Transgenic sandalwood has only ever been produced in two experiments, both a decade apart. In 1998, a proof of principle experiment by Veena and Rao (1998) inserted the bacterial derived marker genes β -glucuronidase and the selective gene neomycin phosphotransferase II (*NPTII*) bound by a binary vector were inserted into the *S. album* genome using *Agrobacterium tumefaciens*. A suite of binary vectors containing the marker genes were trialed: pKIWI105, pBI121, and pIG121-Hm. Of these pKIWI105 proved to be the most efficient. Standard assays for *GUS* (β -glucuronidase) and *NPTII* were used to select transformed *S. album* embryos, and polymerase chain reaction (PCR) with insert-specific primers confirmed that the new genes were present in the regenerated seedlings.

The vectors pKIWI 105 and 110 are binary vectors, which have been used previously in other plant gene transformations (Janssen and Gardner, 1989; Yao *et al.*, 1995). The transfer-DNA (T-DNA) component of the vector inserted into *S. album* used a nopaline synthase promoter (*NOS*) to drive expression of *NPTII* gene, enabling kanamycin-based antibiotic selection. The construct also contained a P35S cauliflower mosaic virus promoter to drive expression of the *uidA* gene (encoding the β -glucuronidase enzyme). *GUS* expression was restricted only to true positives by utilizing defections in both pKIWI105 and pIG121-Hm. The *uidA* gene in pKIWI105 lacked a ribosomal binding site while the vector pIG121-Hm contained an intron in the same gene construct. This ensured that *GUS* expression would only occur under plant transcriptional machinery and not bacterial.

Veena and Rao (1998) used PCR to confirm the presence of *GUS* genes and hence cells were shown to be transgenic; however, the shoots emerging from infected embryos were chimeric. Chimeras are a common problem, although

repeated subcloning may have eventually rectified this. Until recently, this was the only example in the literature of the introduction of foreign genes into a sandalwood species. Just this year, Shekhawat *et al.* (2008) repeated the experiment, only instead of using cotyledon stage plantlets, embryogenic cell suspension cultures of *S. album* were infected with *A. tumefaciens* containing the pCAMBIA 1301 vector. Stable, high level GUS protein expression was noted. Both experimental procedures relied heavily on tissue culture technique, indicating the application of transgenics to sandalwood will be slow for some time.

Although not technically a transgenic experiment, there is a growing body of evidence that gene transfer between parasitic plants and their hosts has occurred in the past, resulting in polyphyletic distributions (Nickrent *et al.*, 2004; Davis *et al.*, 2005). While still poorly understood, there may be some merit to the possibility of genetic information being transferred between morphologically and genetically distant angiosperms. If this is the case, then there is considerable potential for transfer of specific genes via the parasitic connections sandalwoods have with their hosts. Further work is clearly needed to investigate this phenomenon.

2.2 Potential Application of Resistance Gene Transfer

There are many potential applications for the insertion of foreign genes into sandalwood, not least resistance to sandal spike disease. Spike disease is a major concern for growers in India, where the majority of natural stands remain. Symptoms include yellowing of leaves and in severe cases defoliation, poor form, and low fruit set. Efforts to combat the phytoplasma responsible for the disease have been made, but follow-up research is lacking (Lakshmi Sita, 1996; Khan *et al.*, 2004). If resistance genes were to prove useful against spike disease, there is certainly potential to insert them into the genome of sandalwood. To this end, Lakshmi Sita and Bhattacharya (1998) isolated and cloned a proline-rich protein (PRP) complementary DNA from *S. album* leaves. PRPs serve as biopolymers involved in the maintenance of cell wall integrity, and may be involved in systemic resistance to pathogens. At present, this appears to be the only functional genetic

analysis of potential resistance mechanisms in sandalwood. Further work in this area is certainly needed.

2.3 Potential Improvement by Metabolic Engineering

Sandalwood is highly prized for essential oils contained within the heartwood. The oils consist of the sesquiterpene alcohols α -santalol, β -santalol, α -trans-bergamotol, and *epi*- β -santalol. These compounds make up nearly 90% of the distilled oil (Verghese *et al.*, 1990). These appear to be the 12-hydroxy products of the olefin precursors, α - and β -santalene, α -bergamotene, and *epi*- β -santalene. Other minor compounds make up the remainder of the extracted oil. The biosynthesis of these oils appears to occur in the transition zone between sapwood and heartwood (Jones *et al.*, 2006).

Terpene biosynthesis begins with the 5 carbon unit of isopentyl pyrophosphate (IPP). This compound is synthesized from either the mevalonic acid pathway, localized in the cytoplasm of cells (Gershenzon and Croteau, 1990; Dewick, 2002), or it can be derived from plastids via the deoxy-D-xylulose 5-phosphate pathway (Arigoni *et al.*, 1997; Rohmer, 1999). Condensation with the isomer of IPP, dimethylallyl pyrophosphate (DMAPP) leads to the universal 10 carbon monoterpene precursor geranyl pyrophosphate (GPP). Further condensation with DMAPP leads to the 15 carbon farnesyl pyrophosphate (FPP) while the diterpene precursor geranyl geranyl pyrophosphate (GGPP) is derived from a third condensation with DMAPP (Gershenzon and Croteau, 1990).

Terpene synthases (TPS) are responsible for converting the linear precursors GPP, FPP and GGPP into cyclic compounds. Scores of TPS have been isolated from a vast array of plant species and most have been functionally characterized by either heterologous expression in *Escherichia coli* or from cell-free extractions. The TPS gene family is large, with high homology among taxonomic groups (Bohlmann *et al.*, 1998). Many of these genes encode proteins that catalyze the formation of multiple products (Munck and Croteau, 1990). Site-directed mutagenesis studies by Hyatt and Croteau (2005) found that only very subtle amino

acid changes can have a large impact on the terpenoids produced.

Sandalwood oil biosynthesis has only recently received detailed attention. Our research group has found strong correlations between the santalenes and bergamotene, suggesting multiple product formation from a single TPS in *S. album* (Jones *et al.*, 2006). This work has been followed up with gene isolation, functional characterization, and expression studies (C. Jones *et al.*, in preparation). The gene structure and expression properties of TPS in sandalwood may be exploited through modification or selection of specific genotypes conducive to high essential oil production.

However, before this kind of work can proceed, one must understand the normal pattern of oil formation in wild-type sandalwood. Normally, in the case of *S. album*, fragrant heartwood does not form until around 10 years (Rai, 1990) and it continues to expand outward as the tree matures. Ideally, a tree worthy of selection would yield heartwood earlier, and the ongoing expansion of fragrant heartwood would continue throughout the tree's life at a faster rate. At some point in the first few years of tree development, a signal induces oil biosynthesis. Interestingly, almost all members of the *Santalum* genus yield sesquiterpenes; and even the related African sandalwood *Osyris tenuifolia* (Santalaceae) produces sesquiterpenes in its heartwood. All of these species are root parasites, so it is curious to note the link between root parasitism and secondary metabolite formation in vascular tissues.

As detailed by Tennakoon and Cameron (2006) the haustorial connection involves exposure to soil-borne pathogens, which may in turn elicit oil production. Oil biosynthesis appears to be localized to ray parenchyma (Jones, unpublished results), which seems logical as only ray parenchyma cells are metabolically active. Studies linking host species and haustorial connection to oil yield have not been compiled. Such investigations would surely present a valuable insight into this phenomenon. With respect to transgenic sandalwood, expression of the sesquiterpene metabolic pathway is probably controlled very tightly until a certain age; suggesting that overexpression may cost the plant significantly. Terpenoids are highly reduced chemicals that bear a considerable metabolic cost (Gershenzon, 1994), so production of sesquiterpenes must be limited

until sufficient photosynthetic capacity is attained. A comprehensive knowledge of secondary metabolism pathways and their regulation in sandalwood is required before it can be exploited.

Another potential application of transgenics in sandalwood is the knockout of undesirable genes. For example, *S. spicatum* attracts a lower value than Indian sandalwood for various reasons, one being the complex mixture of sesquiterpenes in the extracted oil. One of the many sesquiterpene compounds present in distilled oil is farnesol (Piggot *et al.*, 1997; Howes *et al.*, 2004). Farnesol has been implicated as a potential allergen (Hostynek and Magee, 1997; Schnuch *et al.*, 2004). If the farnesol synthase gene could be silenced or excised from the genome, the allergenic properties of the distilled oil may be reduced. At present, the distilled oil may be rectified by fractional distillation and preparative chromatography, such that farnesol can be isolated from the oil. While the application of farnesol-free transgenic sandalwood may seem rather superfluous, it would at least provide an immediate commercial use of knockout sandalwood lines.

2.4 Potential for Transforming Sandalwood Genes into Other Organisms

Further to transforming sandalwood with foreign genes, sandalwood genes may be inserted into other species. There is growing interest in this technology as the santalene chemical structure is particularly difficult to synthesize (Christenson and Willis, 1980; Krotz and Helmchen, 1994; Zhong and Schlosser, 1994). Transformation of faster growing annual plants with synthase genes derived from *Santalum* may provide a quicker and easier route to the complex tricyclic santalenes. There is a growing body of research confirming multigene transformations, including the recent transformation of caffeine biosynthesis in tobacco (Uefuji *et al.*, 2005). *E. coli* or yeast can also be engineered to produce medicinally important plant metabolites, as has been proven with the antimalarial compound artemisin (Chang and Keasling, 2006). Sandalwood fragrances may also be produced in the same way, while careful regulation of the cytochrome P 450 responsible for hydroxylation of the santalenes completes the process. Oil yields may be low compared to that

of a mature tree; however, the process can be automated for a continuous supply.

New compounds are still being discovered in various sandalwood species (Brunke *et al.*, 1995; Kim *et al.*, 2005). Some chemicals found in sandalwood tissues have shown potential for medicinal benefit (Kim *et al.*, 2006). It may be possible to engineer these compounds into host plants, fungi, or bacteria as their natural abundance in sandalwood is too low to be economically feasible.

2.5 Testing of Transgenic Sandalwood

If sandalwood were to be transformed with genes for pathogen resistance or increased essential oil production, the product would require rigorous testing. Again to do this, uniform field trials must be established along with nontransformed controls. At present, this is particularly difficult given the aforementioned issues with growing parasitic trees. Glasshouse trials would be useful for initial pathogen resistance tests; however, the long-term expression patterns of the gene(s) would need to be monitored and confirmed in the field. Heartwood oil yields can be monitored over time using established nondestructive sampling techniques (Jones *et al.*, 2007). As no mapping projects have been undertaken on sandalwood, nor has the genome been extensively sequenced, this will be difficult and time consuming. Nonetheless, if transformation of new genes into sandalwood is successful and the desired outcomes are expressed in the phenotype, growers will want the ability to continue growing these trees from seed.

Studies on sandalwood mating systems are somewhat deficient in the literature. Rugkhla *et al.* (1997) found that both *S. spicatum* and *S. album* were preferentially outcrossing; however, both species were able to self-pollinate. Fruit development and seed set also depended heavily on genotype. In circumstances where clonal propagation has occurred and genotypes are appropriate, self-pollination can occur and produce viable seed. This process must be taken into consideration if transgenic sandalwood species are released. Seed collected from these plants will also require testing to confirm hereditary patterns. Further crosses must be done in order to obtain a relatively pure line; however, this will take considerable time given

the long rotation cycle of sandalwood plantations. Flowering and fruit production are observed after only a few years, but the transgenic traits may not be revealed for many more years. It may also be necessary to plant the transgenic sandalwood at some distance from natural populations as cross-pollination is likely to occur. This should apply to all genetically improved plantations whether they are transgenic or bred using more traditional methods.

2.6 Regulatory Measures Adopted

In Australia, all genetic engineering and biotechnology regulation is covered by the Commonwealth Government Office of Gene Technology Regulator. Legislation is covered extensively in the *Gene Technology Act 2001*, and all research, development, and extension is bound by this act. Release of products created through transgenic technology is covered by other offices; in the case of foodstuffs the Australian and New Zealand Food Safety Authority and for other products, including potential transgenic sandalwood products, the Therapeutic Goods Administrator.

Indian transgenic research is also bound by legislation covered by the *Environmental Protection Act 1986*, and the rules concerning genetic engineering were added in 1989. Several governing authorities are responsible for monitoring the research and release of genetically engineered goods, particularly the Ministry of Environment and Forests and the Department of Biotechnology. Any transgenic work on sandalwood that is done in either of these sandalwood producing countries is bound by the regulations set out by these authorities.

3. FUTURE ROAD MAP

3.1 Product Expectations

While there is certainly potential for transgenic sandalwood or other plants with sandalwood genes to be developed, there is often a large disparity between promised qualities and actual results. Production of transgenic woody plants is not a trivial task as it often relies heavily on tissue culture. Strong selective markers have improved

the odds of acquiring positive transformants, but the random location within the genome of an insert could make their regulation more difficult to follow.

Therefore, the following approach for transgenic research in all economically and noneconomically important sandalwoods should be adopted. A significant and sustained genetic mapping project should be undertaken, along with sequencing as much of the genome as possible. Genetic diversity studies should be followed up with gene flow and mating system studies, such that breeders will have known parameters in mind when performing crosses and developing new seed lines. Expression studies should be initiated concurrently, investigating transcriptional and metabolic responses to the many environmental factors that affect sandalwood growth and development. Functional characterization of as many secondary metabolic pathway genes should also be carried out and correlated with metabolite profiles *in vivo*. Extensive field trials must be established with all variables controlled, such that systematic processes can be fully explored. Promoter regions and transcription factors for many important pathways should also be considered; the sequences of these regions must be deduced.

Once these significant barriers to understanding normal gene expression in the sandalwoods are brought down, transgenic lines can be considered. Trials of different techniques of transformation should be considered. For example, gene-gun technology has not yet been trialed in sandalwood, and may prove more efficient than bacterial transformation. If young sandalwood flowers are inoculated with *Agrobacterium*, and the embryos transformed, the need for tissue culture could be avoided (Clough and Bent, 1998). Selectable markers like antibiotic resistance could be optimized, and the use of herbicide resistance constructs may also improve selection. Retention of herbicide resistance qualities in regenerated sandalwood plants may be useful, as weed management in plantations often risks killing sandalwood via weed-parasite haustorial connections.

3.2 Addressing Risks and Concerns

Transgenic sandalwood faces the same challenges and uncertainties as every other transgenic

organism produced to date. The public have grown wary of transgenic technology and the outrageous promises often claimed. Sandalwood is perceived as a natural product, far superior to any man-made chemical perfume. The same market that desires natural sandalwood will also question any transgenic lines of sandalwood, or even metabolically engineered bacteria as “unnatural”. Europe is a major producer of perfumes, and one of the largest customers of sandalwood oil. Current bans on genetically engineered products may hamper any efforts to test this technology. No doubt that organizations that intend to capitalize on transgenic sandalwood will have a strong marketing challenge ahead of them.

But in addition to marketing, the product must be safe and unlikely to harm consumers, or natural populations of sandalwood. Ultimately, the product must actually work and deliver the desired properties for which it was created. The sandalwoods have a very low weed risk, given the demise of their natural distribution. Hence, it is unlikely that transgenic lines will “escape”.

On the basis of current status of the technologies, market, and industry, transgenic sandalwood is unlikely to attract much commercial attention in the near future. Growing the plant under normal conditions and traditional breeding methods are already challenging enough. Furthermore, as the product is well established and has been shown to yield fragrant heartwood under plantation conditions, the need for transgenics is still low.

3.3 Expected Technologies

Transgenic sandalwood as a final product represents only part of the technology. As researchers seek to understand the genetic control of essential oil production, further applications in other species may become apparent or indeed using *ex planta* systems. Little work has been done since 1998, suggesting that modern techniques could be used to optimize transformation and selection. New transfer DNA (T-DNA) vectors and genotypes of *Agrobacterium* have since been developed, and are likely to work far more efficiently than those described by Veena and Rao (1998). Tissue culture free techniques of transformation should also be considered, particularly flower-dipping inoculation. *Santalum*

inflorescences vary widely, but generally have large numbers of individual flowers per stem. This would save considerable time but may in turn result in fewer transformants. Recognizing the natural promoters and terminators of the sandalwood genome will also help determine the make up of T-DNA constructs. Comparison of TPS and their regulatory elements among the *Santalum* genus will substantially aid our understanding of divergence in yield and composition.

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Teak

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1. INTRODUCTION

Teak (*Tectona grandis* Linn f.) is a deciduous forest tree species whose timber possesses several qualities. It is one of the world's premier hardwood timbers, rightly famous for its mellow color, fine grain, and durability. Other qualities include many properties such as attractiveness in lightness with strength, ease of working and carving, and resistance to termites, fungi, weathering, seasoning capacity without splitting, cracking, or materially altering shape. It has a good stability in fluctuating climates, strength, and decorative grains of wood. Teak is one of the most studied forest species due to its wide geographical distribution even in far places from its natural distribution. In fact, Tewari (1992) refers in his book "A Monograph of Teak" to more than 3000 papers on teak during more than 150 years including the most diverse aspects of the utilization of teak.

1.1 History, Origin, and Distribution

Teak is indigenous to continental Asia and is confined to the moist and dry mixed deciduous forest below 1000 m altitude in India, Myanmar, Thailand, and Laos. This species is also well established in Indonesia, but it is not clear whether this occurrence is natural or the results of an introduction by Hindu settlers in the 7th century. It is believed that teak was introduced to Java 400–600 years ago from India (Tewari, 1992). Due to its high timber qualities, high market demand, and ease of growing and domestication, teak plan-

tations have been widely established throughout the tropics since the 1850s. It has been widely planted between the latitudes of 28° N and 18° S in Asia and the Pacific region, as well as in Africa and Latin America. Under exotic conditions, plantations of the species has been successfully established in many countries including Sri Lanka, Bangladesh, and China in Asia; Ghana, Nigeria, Ivory Coast, Senegal, Togo, Benin, Sudan, and Tanzania in Africa; Trinidad and Tobago, Costa Rica, Puerto Rico, and Panama in Central America; and Brazil and Ecuador in South America. Teak planting in India began in the 1840s. Teak plantations were established in temporary association with agricultural crops, in Myanmar in 1856, and in Indonesia around 1880. The first introduction outside Asia was in Nigeria in 1902, with seed from India and Myanmar. Plantations in Ghana started around 1905 and in Côte d'Ivoire in 1929. The first teak plantation in tropical America was established in Trinidad and Tobago in 1913 with seed from Myanmar. Planting of teak in Honduras, Panama, and Costa Rica started at the end of the 1930s. There was a gradual increase in the area of teak plantations between the 1950s and 1960s. One of the major areas under teak plantation is in Java, Indonesia (Pandey and Brown, 2000).

1.2 Botanical Distribution and Taxonomy

Teak belongs to the genus *Tectona* and family Verbenaceae (Table 1). The name *Tectona* is derived from the Greek word "tekton", which

Table 1 Botanical classification of teak

Category	Name
Kingdom	<i>Plantae</i>
Division	<i>Magnoliophyta</i>
Class	<i>Magnoliopsida</i>
Order	<i>Lamiales</i>
Family	<i>Verbenaceae</i>
Genus	<i>Tectona</i>

means carpenter, while the name of a species “*grandis*” stands for large in Latin. The genus *Tectona* is represented by only three species: *T. grandis* Linn. f., *T. hamiltoniana* Wall., distributed in the dry zones of Myanmar, and *T. philippinensis* Benth and Hooker. f., confined to the Philippines islands (Tewari, 1992).

Because teak is a deciduous tree, generally teak trees remain leafless in winter and become dormant in their growth. Further, they also become leafless or exhibit dry nature of foliage during dry spells in summer and show apparent decrease in growth (Rajendrudu and Naidu, 1999). It can reach more than 30 m in height in favorable conditions. Crown opens with many small branches. Bark is brown, distinctly fibrous with shallow, longitudinal fissures. The rotation period is approximately 80 years. The root system is superficial, often no deeper than 50 cm, but roots may extend laterally up to 15 m from the trunk. Teak has somatic chromosome number of $2n = 36$ (Hedegart and Eigaard, 1965).

Teak is able to grow under a wide range of climatic and edaphic conditions. Optimal temperatures are from 25°C to 28°C, but teak can adapt to extreme temperatures. The annual rainfall should be between 1250 and 2500 mm, although teak has been found in some places with a 600 mm annual rainfall and in places with almost 4000 mm. The altitude range of growth is 0–1200 m in soils with a pH of 6.5–8 and good drainage. Teak grows very fast, particularly for a hardwood. It produces between 12 and 26 m³ of wood per year. Teak plants possess a great ability to overcome water stress (Rajendrudu and Naidu, 1997).

Teak has small flowers, found on the topmost branches in the unshaded part of the crown. Fruit is a drupe with four chambers. Each fruit may contain from zero to four seeds. One kilogram contains 1000–3500 seeds. Flowers normally have six petals in a ring. An inflorescence can contain

several thousand buds, but not all of them develop during flowering (Bryndum and Hedegart, 1969). Teak clones with a larger corolla tube diameter show higher fruit set (Vasudeva *et al.*, 2004). Pollen grain of teak has two shapes, prolate spheroidal (fertile) and perprolate (infertile) with a ratio of 3:2 between the forms (Choldumrongkul *et al.*, 2000).

According to a study on dry matter production in the reproductive components, from a massive flower production only about 0.5–0.7% flowers developed into fruits. Immature fruit abscission totaled 34% and 58% of the total number of fruits initiated in the 14- and 30-year-old trees, respectively (Karmacharya and Singh, 1992). Small fruits, with less than 9 mm diameter have low germination percentage leading to a less number of seedlings (Indira *et al.*, 2000).

Teak displays true annual rings, which are useful for determination of age and growth rate of the trees in wood production of managed stands. The occurrence of false rings often misleads the task of age and growth rate determination as continuous or discontinuous false rings are more frequent in juvenile teak (Priya and Bhat, 1998).

1.3 Economic Importance

In the first half of the last century, teak was one of the most important forest tree species in the tropical countries. However, with the development of pulp and paper industry in the last years, the main attention was turned toward plantations with fast growth like that of eucalypts and acacias.

1.3.1 Area coverage

The global planted teak area of the world as recorded in 1990 was about 1.6 Mha (million hectares), which is equivalent to about 75% of the high-grade tropical hardwood plantations. Based on the available information, it is estimated that there is a total of over 28 Mha of natural teak forest around the world. Details of teak resources in the Asia Pacific region are provided in Table 2. Some salient points on the Asia Pacific region are identified in the overview of the global teak resources as follow: from about 1.6 Mha of teak plantation in the world, the 99% occurs in Asiatic regions; from about 28 Mha of natural teak forest,

Table 2 Teak resources in the Asia Pacific region (area in hectare)^(a)

Country	Natural forest	Plantations	Total resources	Data up to
Bangladesh	–	62 700	62 700	1985
China	–	900	900	1992
India	8 900 000	276 640	9 276 250	1975
Indonesia	–	675 640	675 640	1990
Laos	16 000	5000	21 000	1992
Malaysia	–	3859	3859	1994
Myanmar	16 517 700	219 485	16 737 185	1994
Philippines	–	21 550	21 550	1990
Sri Lanka	–	70 813	70 813	1992
Thailand	2 500 000	170 240	2 670 240	1990
Vietnam	–	2037	2073	1994
Regional Total	27 933 700	1 516 674	29 450 374	

^(a) Source: Teaknet newsletter, Issue No. 1, December 1995 (Ko Ko Gyi and Kyaw Tint, 1995; Status of Management of Natural Teak Forest, presented at 2nd Regional Seminar on Teak, Myanmar)

the 60.5% occurs in Myanmar, indicating this country as dominant in the production of naturally grown teak; India possesses the second place for area with 31.2% of the total teak resources in Asia; Thailand is third with 9.2% of the total teak resources in Asia; Indonesia is the country with the largest teak plantation area in Asia, covering 47% of the total teak plantation area in the Asiatic region. Of the total area of forest plantations in 1995 in the entire world, 42.7% were softwoods and 57.3% were hardwood species, including the most valuable hardwoods: teak, mahogany, and rosewood (Krishnapillay, 2000; Pandey and Brown, 2000).

1.3.2 Production

Indicative estimates of teak standing volume (industrial wood) annually available place Asia as the dominant producer. According to five case studies, in Malaysia, the Melanesian Islands, Central America, Australia, and Ghana, governments have a significant role in initiating hardwood plantation development (Varmola and Carle, 2002). Besides governments' help, several other aspects have influenced teak forest production during the last decades: the worldwide reduction of the supply of tropical wood from natural forests; the extension of teak to zones very far from its natural distribution; the growth of forest products in the international market and the appearance of new centers of consumption; the advances in the processing technologies giving

other alternatives; the greater attention paid to the ecological aspects of the forests in its sustainable exploitation (Graudal *et al.*, 1999).

Size is more important influential feature in harvesting than age of maximum volume production because teak is planted for timber production. Its natural rotation age range can vary between 50 and 90 years, while the optimum commercial age is between 50 and 60 years. The average actual of mean annual volume increment at rotation age is $3 \text{ m}^3 \text{ ha}^{-1}$ per year. Some teak plantations have been established relatively recently in most countries outside its natural range. Thus, the most production of teak is largely restricted to the traditional large producers: Myanmar, India, and Indonesia. One of the most recent estimates provides an indicative total annual production of teak roundwood of $1\,795\,000 \text{ m}^3$ (Pandey and Brown, 2000).

1.3.3 Utilities

Establishment of forest plantations is not a popular activity among farmers because of the long time it takes for them to convert any income from the operations. Poor farmers generally cannot risk the long-term investments and credit. A combined system to produce food, livestock, and timber is necessary to allow them to take part in the potentially lucrative teak production (Roder *et al.*, 1995). Intercropping offers farmers the opportunity to obtain some benefits in the meantime, including those obtained for savings in

pest management due to increased diversity. Type of crop, spatial arrangements of plants, planting rates, and maturity dates must be considered when planning intercrops. Some studies to identify suitable crops for growing in interspaces of teak were carried out. *Trifolium alexandrinum* and *Vigna* beans could be grown with a minimum reduction in yield (Dagar *et al.*, 1995). Soybean mixed with teak not only makes the latter to grow better but also allows harvesting of the bean for food. Rice can be intercropped for the first 3 years.

1.3.4 Other economic attributes

Teak possesses optimal characteristics for the most variable uses. Its wood is a medium-weight timber that is rather soft. Grain is straight, wavy, or slightly interlocked, with rather coarse and uneven texture (Brennan and Radomiljac, 1998). Teak trees have a mean basic density of 610 kg m^{-3} and mean air-dry density of 700 kg m^{-3} . They present good basic wood properties (i.e., green density, basic density, air-dry density, and shrinkage), as well as recoveries, sawing and drying behavior, and working properties (i.e., sawing, sanding, color, planning, nailing, and staining), indicating this species to show potential for value addition (Brennan and Radomiljac, 1998). The wood grains are figured well, producing an attractive veneer, which is extensively used in the manufacture of furniture and interior fittings. The teak wood can be used for bridge building and constructions in contact with water since it possesses excellent characteristics of durability and resistance. In fact, it is used for ship decking and other construction works such as boat building. In house building, teakwood is often used for floors and for garden furniture. Among other attributes widespread throughout the world are the multiple uses, such as the fabrication of boxes, musical instruments, and toys, and particularly it is used for building poles, transmission line poles, fence posts, and railway sleepers.

1.3.5 Other potential industrial uses

Lapachol, a naphthoquinone, has been isolated from teak. This compound causes a dermatitis allergy and gives the wood resistance to termites

(Schmalle and Hausen, 1984). Researchers have found that lapachol has promising anticancer and antiviral properties. It has been shown that this compound is active against leukemia cells and fungi (Perry *et al.*, 1991). It has also been evaluated for its estrogenicity and antinidational activity in mice (Sareen *et al.*, 1995). Teak callus is able to produce triterpene acids, which show a potent antibacterial activity against *Escherichia coli* and *Bacillus subtilis*. These compounds occur in small quantities in the intact tissues and are not easily detected (Marwani *et al.*, 1997). From the root heartwood of teak, a compound, 5-hydroxylapachol, has been isolated and it has been found to be a cytotoxic agent (Khan and Mlungwana, 1999). Extractives present in the heartwood of teak are believed to be responsible for its durability. Several studies of teak heartwood extractives and decay resistance showed that most teak heartwood extractives directly contribute to the natural durability of teak (Supriana, 1988). The presence of tectoquinone extractive is a good indicator for the estimation of the resistance against wood-destroying fungi. Decay resistance increases from the pith to the sapwood and is also related to the age, rate of growth, and extractive content. Particularly, tectoquinone was identified as a bioactive compound for the inhibition of *Coniophora puteana*, a strong fungus that causes wood decay (Haupt *et al.*, 2003). Deoxylapachol is a precursor of tectoquinone (Sandermann and Simatupang, 1968). Juglone is another aromatic organic compound found naturally in teak bark. Juglone exerts its effect by inhibiting certain enzymes needed for metabolic function of other organisms. This extract of teak bark has shown its effectiveness against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (Neamatallah *et al.*, 2005). Leaves of teak are rich in phenols, poor in proteins, contain traces of alkaloids, and are free from flavonoids, steroids, and saponins (Tripathi *et al.*, 1998). Pathak *et al.* (1988) found betulin aldehyde from the bark of teak to be an antitumour agent. In traditional medicine, a wood powder paste has been used against bilious headaches and swellings, and internally against dermatitis or as a vermifuge. The bark has been used as an astringent and the wood as a hair tonic (Perry and Metzger, 1980). A natural colorant can be extracted from leaves containing a yellow or red dye used for dyeing silk, cotton,

and wool (Vankar *et al.*, 2003; Mahale *et al.*, 2004).

1.4 Traditional Breeding

Teak has a large natural variability due to its wide distribution. It can survive and grow under a wide range of climatic and edaphic conditions. This large variation determines an important genetic resource to be used in genetic improvement.

Identification of *plus* trees from natural forests and plantations has been carried out as a part of the teak improvement programs. A *plus* tree is considered an individual phenotypically outstanding, combining a number of desirable traits. In selecting *plus* trees of teak, each superior tree is compared with at least five trees within a radius of 50 m from the *plus* tree. In order to determine the superiority of the *plus* tree, a scoring system is adopted for characters such as height, diameter, clear bole height, straightness of stem, branching pattern, resistance to pests and diseases, and for seed production (Subramanian *et al.*, 1994). In forestry, the definition of *Provenance* is not only to determine the place of origin, but also to determine a variety of other things including population, country, etc., even from a place where the species is not native. According to the definition of Burley and Wood (1976) provenance refers to any place where trees are situated, indigenous or not, while the term origin can be used to refer to the place of which the species is native. The interaction between provenance and environment is the main problem that a breeder has to solve, choosing the most suitable seed source (provenance) for planting at a particular site (Matheson and Raymond, 1984).

1.4.1 Breeding objectives

A preliminary evaluation of important genetic characteristics has been carried out on provenances. The parental combining ability has been tested in breeding programs. The information obtained helped to find clones with good characteristics. High estimates of both heritability and genetic gain have suggested the predominant role of additive gene action. The

selected teak clones have been included in breeding (Kumar *et al.*, 1997; Mandai, 1999). Among the observed characters, diameter growth and stem straightness and clear bole, persistence of stem axis, and flowering habit (early and/or late flowering) are strongly inherited in this species (Harahap and Soerinegara, 1977; Keiding *et al.*, 1986).

In addition to teak natural plantings in Asia, the domesticated plantations in Africa and Central America have been taken into account for obtaining further variability. The selection of individuals adapted to local climates and soils may have formed “landraces” in these new areas, with different distinctive characteristics (Kjær and Foster, 1995). Some characteristics have been associated with leaf morphology to facilitate phenotypic identification (Rawat *et al.*, 1998).

Finding variability for transferring resistance to pests and diseases has been another objective. Insect defoliation provokes deep wood anatomical changes in juvenile teak plants. Some studies have demonstrated a much better performance in growth of teak when protected from insect defoliation, suggesting that genetic improvement is worth considering (Priya and Bhat, 1997). The larva of *Eutectona machaeralis* (Lepidoptera: Pyralidae) is an important pest of teak leaf skeletonizer included in the breeding programs (Roychoudhury and Joshi, 2000). Another serious problem is the collar rot of teak, which is a major nursery disease caused by *Rhizoctonia solani* (Ramesh, 2000).

1.4.2 Tools and strategies

Kjær *et al.* (1999) mentioned the basic strategies used in teak for tree improvement. Breeding seedling orchards (BSO) consists of a combining test of mother trees with commercial seed production managing the breeding populations within the seed orchards. It results in economical and faster delivery of improved seed; however, less genetic gain and low seed production per tree make the system less suitable for improvement of teak. Extensive breeding seed orchards (EBSO) is a modified system to collect seed for multiplication from a large area covered with a large number of selected trees established from a bulk seed

lot. The seedlings are multiplied by cutting stems. Multiple population breeding strategy tests trees planted under variable conditions (cultural practices). It is limited to the possibility of manipulating the environment. This strategy allows faster results and more flexibility in tree improvement. The selected clones are subdivided into groups taking different criteria into account.

Biotechnology has introduced new means to study genes and their functions and to assist breeding programs through the use of genetic markers. Molecular markers help in the detection of differences among individuals at the level of DNA. In addition to analyzing genetic diversity within the populations and locating genes that determine economically important qualities (rate of growth, adaptability, quality of the wood, etc.), genetic markers provide approaches for the study of evolutionary processes in plant populations (Cruzan, 1998). Several studies of forest trees, including teak, have shown larger differentiation between adaptive traits than between biochemical markers (Kjær and Siegmund, 1996). Among the populations examined, those genetically most diverse within and among populations of the species should merit a high priority for conservation. Combination of marker-aided population genetic analysis and information about adaptive and quantitative traits as well as forest ecosystems allows for the development of a conservation program (Changtragoon, 2001).

1.4.3 Achievements

1.4.3.1 Elite tree selection

During the last century, selections of *plus* trees were made in both natural and established plantations. These selections were made based mainly on their phenotypic appearance. Some help was obtained from the molecular marker identification but only in the last 15 years. The selections were introduced into seed orchards. The production of control-pollinated seed collected from these orchards was subsequently tested and then used for operational reforestation. A variety of breeding designs were employed to create genetic tests to rank the selections, estimate genetic parameters,

and make new selections for the second generation. Complementary mating designs were carried out focused on clonal forestry and application of techniques from molecular genetics and biotechnology (Tewari, 1992; Kaosa-Ard, 1995; Kaosa-Ard *et al.*, 1999).

1.4.3.2 Mass propagation of elite material

Establishment of new industrial forest plantations requires long time. To enhance teak plantation development, governments of producer countries have been making efforts to focus the research on improvement of the establishment of seed and clonal orchards to make the elite planting materials available for mass multiplication.

1.4.3.3 Propagation by seed

Teak is traditionally reproduced through seeds, but in most cases, germination is difficult due to the hard seed coat, low seed quality, and low fruit setting. The nature of barriers that prevent seed germination can be physiological (germination inhibitors in the mesocarp), physical (thick and hard endocarp), or morphological (the requirement of after ripening associated with hormone imbalance in seeds), which results in only the old drupes showing moderate germination. Poor germination rate leads to a low production of seedlings (Hedegart, 1973; Egenti, 1981; Kaosa-Ard, 1995; Bonal and Monteuuis, 1997; Palupi and Owens, 1997; Tiwari *et al.*, 2002). To overcome this problem, the forced-aged treatment on the fruits has been proposed by placing them in an ageing chamber maintained at 100% relative humidity and 40 °C up to 15 days for increasing their germination percentage (Dharmalingam, 1995). Chacko (1998) suggests that the system of termite-aided mesocarp removal of teak fruits is an efficient presowing treatment for early and enhanced germination. The rooting time of seedlings is 8 to 15 days, and the survival rate of rooted stock is 90–100% (Dabral, 1976; Gupta and Kumar, 1976; Unnikrishnan and Rajeev, 1990; Tewari, 1992). Some improvements in the overall performance of seeds have been done using γ -irradiation (Bhargava and Khalatkar, 1987).

1.4.3.4 Propagation by cuttings

Vegetative propagation using juvenile seedlings has also been developed on a commercial scale. Although rooting of cuttings has been a serious problem, several procedures have been set up to improve rooting by means of auxin treatments. Indole-3-butyric acid (IBA) treatment appears to be the product mostly used in promoting of roots formation. It has been found to be the most effective auxin tested (Palanisamy and Subramanian, 2001; Castro *et al.*, 2003). The combined application of IBA and rice bran extract enhances considerably adventitious rooting in semi-hardwood shoot cuttings of teak (Ansari *et al.*, 2004). The effect of auxin in rooting promotion is more effective in mature cuttings (Husen and Pal, 2006). Cutting production is more expensive than seedling production; however, the cutting option is considered better because it results in genetically stable material (Monteuuis *et al.*, 1995; Kaosa-ard *et al.*, 1999).

1.4.3.5 Micropropagation

Vegetative multiplication of teak using *in vitro* techniques by means of axillary bud stimulation is expected to be a very effective way to capture the best genetic stock from the breeding program in shorter time. In spite of the progress made in tissue culture of teak since the 1970s (Narasimhan *et al.*, 1970; Dhruva *et al.*, 1972; Gupta *et al.*, 1980; Gill *et al.*, 1991; Devi *et al.*, 1994; Monteuuis *et al.*, 1998; Tiwari *et al.*, 2002; Castro *et al.*, 2003; Yasodha *et al.*, 2005), it still remains problematic due to the poor capacity of shoot proliferation, high susceptibility of shoots to vitrification and browning, and the low frequency of *in vitro* rooting. *In vitro* rooting is often preferred to an *ex vitro* system in terms of plant quality due to the advantage of already possessing roots during the acclimatization phase (De Klerk, 2002). However, in most of the tissue culture laboratories that produce teak plants in the world, rooting is still done *ex vitro* due to the low frequency of *in vitro* rooting (Tiwari *et al.*, 2002; Castro *et al.*, 2003; Yasodha *et al.*, 2005; Singh *et al.*, 2006). Recently, an efficient micropropagation method including an efficient *in vitro* rooting methodology was proposed focused on large-scale industrial

plantations by using *plus* plants (Mendoza-de Gyves *et al.*, 2007). This method, which produces multiple shoots of high quality (Figure 1), takes in consideration a short micropropagation cycle consisting in the transference of excised nodal segments from the newly formed shoots onto a fresh medium. Following this protocol, more than 800 shoots can be obtained from single internodal segment explant in 3 months (an average of almost four shoots with four internodes each). The procedure includes a fast soaking in a gibberellic acid solution (100 mg l^{-1}) for an elongation within the same step of propagation and overcomes problems of vitrification by reducing the ammonium quantity of the MS (Murashige and Skoog) salts medium and by adding pectin of must of wine (Migros, Switzerland) to reduce the water content in the medium. Elongated shoots are transferred onto the rooting medium for 4 weeks of incubation using the combination of IBA (0.5 mg l^{-1}) with putrescine (160 mg l^{-1}). Putrescine promotes both strong and ramified roots and fast-growing shoots during the rooting phase and subsequently in the pots and in combination with IBA rooting reaches 100% conditioning the plantlets for a better acclimatization performance. Plant acclimatization after rooting with IBA + putrescine guarantees 100% success. The use of exogenous polyamines to increase *in vitro* rooting and to improve root quality was first demonstrated in olive, which had similar problems like teak for rooting. Polyamines combined with auxins promote early rooting and increase the final rooting percentage and the number of roots per explant (Rugini and Wang, 1986; Rugini *et al.*, 1993).

1.4.3.6 Marker-aided selection

At present, there are numerous molecular techniques for identifying genetic markers. They have considered documentation of the genetic diversity of teak in various geoclimatic populations (provenances). The results are helping in identification of quantitative trait-specific loci within individuals and populations and selection of superior parents for inclusion in the breeding program.

Genetic differentiation between populations of teak was examined in some quantitative characters and allozyme loci. Large differences

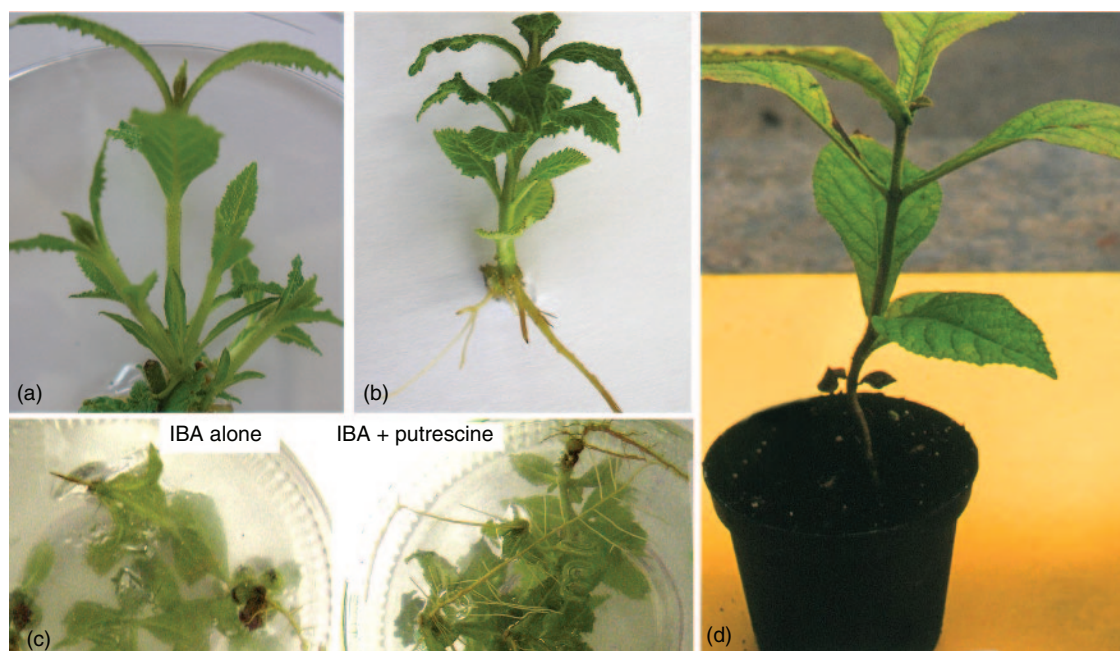


Figure 1 Micropropagation of teak: (a) 1-month-old multiple shoots of high quality in proliferation phase from nodal explants; (b) rooted microcutting ready to be transplanted into pot; (c) comparison between rooting treatments: 0.5 mg l^{-1} of IBA (left) and 0.5 mg l^{-1} of IBA + 160 mg l^{-1} of putrescine (right) (note that the presence of putrescine allowed strong and more ramified roots); (d) potted plant after acclimatization phase

between populations were found in the quantitative traits. Regional patterns were found, but there were also substantial variations within ecological–geographical defined regions (Laos, Thailand, West India, and Indonesia). A much less pronounced differentiation between populations was found in allozyme markers (Kjær *et al.*, 1997). In other studies, the gene diversity was carried out using isoenzyme analysis in teak provenances in order to measure and to monitor the biodiversity in tropical and temperate forests. Fourteen enzyme systems were analyzed in leaf parenchyma of nine native and introduced populations of teak. A cluster of two main gene pools was shown in this analysis. The cluster showed two main gene pools: the first one consisted of the Indian provenances and the second of African, Indonesian, and Thai provenances. Genetic distances among populations of the same group were similar, and lower than the genetic distances between populations from different groups (Kertadikara and Pratt, 1995). Isoenzyme analysis was also used in the determination of carbonic anhydrase activity in teak for studying its relationship with

photosynthesis. Carbonic anhydrase may serve as a biochemical marker for photosynthetic capacity in teak genotypes (Tiwari *et al.*, 2006).

Shrestha *et al.* (2005) analyzed some patterns of genetic variation within and among nine populations of teak from diverse geographical regions in India, Thailand, and Indonesia. They used amplified fragment length polymorphism (AFLP) for this analysis. Their results showed that 57% of total genetic variance occurred within populations while the remaining 43% occurred between populations indicating that Indian populations are clearly separated from those from Thailand and Indonesia.

Microsatellite loci have also constituted a useful tool for investigating the mating system, gene flow, parentage, and population dynamics for use in sustainable management of natural teak forests and for *in situ* conservation purposes. Verhaegen *et al.* (2005) developed some polymorphic microsatellite loci from a genomic library enriched for AG/TC repeats. Their work describes the development of 15 microsatellite markers for teak. Primers used to amplify these loci were tested on 265

individual trees from different teak populations (India, Thailand–Laos, Indonesia, and Africa). The number of alleles ranging from 3 to 20 per locus was clearly higher in India and Africa than in Indonesia and Thailand–Laos. In all cases, the observed heterozygosity was lower than expected.

A discrimination among teak plus trees was done by Watanabe *et al.* (2004) by using selected random amplified polymorphic DNA (RAPD) markers to achieve a highly reliable clone management. They performed a primary screening for 120 arbitrary decamer primers, selecting 24 primers that generated 26 clear and unambiguous fragments. In a subsequent screening, they investigated the reproducibility of each fragment by six repetitions of polymerase chain reaction, and finally selected 13 fragments found to be most reproducible. RAPD markers were also used to analyze genetic fidelity but using micropropagated teak clones with respect to subcultural passage (Gangopadhyay *et al.*, 2003). No variation in RAPD profiles was noticed in the *in vitro* clones of several passages in comparison to the *in vivo* mother plants. Only one micropropagated plant of 25th subcultural passage showed a new polymorphic DNA fragment.

Genetic diversity was evaluated in populations of teak in Thailand by means of the identification of RAPD loci revealing that about 21% of the total variation was attributable to differences among populations. The number of polymorphic loci in most of the investigated populations was very high with an average of 72.6%. The average expected heterozygosity was 0.31. Significant differences in allelic frequencies were found between populations (Changtragoon and Szmidt, 2000). The natural populations of teak in Thailand were found to be highly differentiated genetically. It suggests that materials from at least one population of each province in the northern and central part of Thailand may be required for both *in situ* and *ex situ* gene conservation purposes (Changtragoon, 2001).

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

Conventional breeding methods have certain limitations in teak, including long rotation age, difficulties in performing controlled pollination, low germination percentage, and low genetic

variability of the species. These limitations make genetic engineering an important tool.

1.5.1 Long rotation age

As in other woody plants species, the use of sexual hybridization for improvement of teak is a slow, lengthy process. The long juvenile period of teak seedlings ranging between 6 and 8 years per generation cycle is a major limitation for rapid improvement of teak. Thus, the wood quality of progeny often cannot be assessed for several years. Resistance to certain diseases cannot be confirmed until after several years have passed. The development of new genotypes by breeding and selection results in a long and tedious task. In most instances, only the F₁ generation can be selected (Kaosa-ard, 1986; Kjær and Foster, 1995).

1.5.2 Difficulties in performing controlled pollination

Pollination is one of the most important activities to control in forest tree breeding programs. Among the factors that limit the development of controlled-pollination technique of teak are as follows: its high allogamy degree, the only 1-day life of the flowers, and its very short pollination period (2–3 h). Teak trees can start flowering when they are 6–10 years old. Under controlled pollination, the highest percentage of self-compatibility is only 5.5% as compared with crossing, which is as high as 60% (Bryndum and Hedegart, 1969; Hedegart, 1973; Karmacharya and Singh, 1992; Kaosa-Ard, 1995; Tangmitcharoen and Owens, 1997; Silikul, 2000).

1.5.3 Low germination percentage of seed

Fruit setting is also considered important for any breeding program. Although a massive number of small flowers occur throughout the flowering period (4–5 months), only a small quantity of seed can be collected from each tree. Teak has a very low conversion of flowers to fruits (0.5–2%). This low fruit percentage is due to the low proportion of pollinators to flowers and the short flowering and pollination periods of individual

flowers (Bryndum and Hedegart, 1969; Hedegart, 1973; Egenti, 1981; Kaosa-Ard, 1995; Palupi and Owens, 1997).

1.5.4 Low genetic variability

The potential of teak germplasm remains underexploited due to insufficient genetic variability for traits in question. Problems such as resistance to the teak defoliators, *Hyblaea puera*, *Walterianella* spp. and resistance to teak skeletonizer (*Paliga damastesalis*), which are serious pests in homogeneous plantations of teak, resistance to fungal pathogens, *Nectria* spp., causing canker on trees, and improvement in postharvest attributes, yield, and economic returns cannot be solved because variation is limited (Bhowmick and Vaishampayan, 1989; Baksha and Crawley, 1995; Arguedas and Quirós, 1997; Kalia *et al.*, 1998; Intachat, 1999; Leuangkhamma and Vongsiharath, 2005). Only one natural resistance in some teak clones has been reported against its prime insect pest, *Eutectona machaeralis*, which could offer a viable long-term solution to the leaf skeletonizer menace (Roychoudhury *et al.*, 1997). The use of biotechnological techniques for the genetic improvement of teak is, therefore, essential for overcoming the limitations of conventional breeding.

2. DEVELOPMENT OF TRANSGENIC TEAKS

2.1 Donor Gene

Until now, application of only one useful gene has been reported, the *CryIA(b)*. It was used in microprojectile bombardment into embryos of teak using a plasmid containing the *CryIA(b)* gene of *Bacillus thuringiensis* (*Bt*) subsp. *kurstaki*. The plasmid contained the *hpt* (hygromycin phosphotransferase) and the *gus* (β -glucuronidase) genes under the regulation of the promoter and terminator sequence from 35 transcript derived from cauliflower mosaic virus (CaMV). *CryIA* is one of the *Bt* genes that have been cloned to provide resistance to insect pests. It encodes crystalline proteins in the bacteria that are responsible for larval toxicity. When eaten by the insects, these crystalline, or *Cry* proteins, bind to

the insects' midgut causing those cells to burst from a water imbalance finally killing the insects. Transgenic plants containing a *Bt* gene produce these *Cry* proteins, which the insects ingest when feeding on the plants (Gill *et al.*, 1995; Keeton *et al.*, 1998).

2.2 Transformation Methods

2.2.1 Employment of gene gun

Genetic engineering could offer a very important contribution to genetic improvement of teak. This technology may help to circumvent some of the limitations of classical breeding programs associated with teak improvement. An attempt for engineering insect resistance into teak genome was done. Norwati *et al.* (2003) reported transformation of teak tissues using the particle bombardment method by utilizing highly regenerative calli coming from nodal segments. Explants were subjected to bombardment with gold particles coated with plasmid DNA carrying *hpt* as a selective gene, *gus* as a marker gene, and *CryIA(b)* gene coding for a *B. thuringiensis* toxin. Explants were then transferred onto the medium for shoot development. Elongated shoots were then subcultured onto the same medium supplemented with hygromycin (10 mg l⁻¹) for selection. After a culture period of some months with 6-week subculture intervals in a selection medium containing hygromycin, some plants expressed β -glucuronidase activity by staining with X-gluc (5-bromo-4-chloro-3-indolyl β -D-glucuronide). Polymerase chain reaction (PCR) analysis confirmed transgene's integration into the plant genome. No test with *CryIA(b)* gene expression was reported.

2.2.2 *Agrobacterium*-mediated transformation

Widiyanto *et al.* (2003) selected transformed calluses previously co-cultivated with *Agrobacterium tumefaciens* LBA 4404 strain carrying the pBI121 binary vector and *GUS* gene. Selected calluses were transferred into the callus-induction medium consisting of the woody plant medium (WPM) supplemented with thidiazuron (TDZ)

and indole-3-acetic acid (IAA). The derived transgenic calluses were selected on the same medium containing 100–200 mg l⁻¹ kanamycin. The addition of 10 μ M 6-benzylaminopurine (BAP) and gibberellic acid (GA3) induced shoot formation on selected callus tissues after 4–6 periods of subcultures. The kanamycin-resistant shoots showed positive *GUS* expression. Subsequently, Widiyanto *et al.* (2004) compared two strains of *A. tumefaciens*, the LBA4404 and GV3101, as the hosts for binary vectors, the pBI121 and the pCAMBIA1303 respectively, both containing the *GUS* gene as marker to evaluate the transformation efficiency. The strains differed from each other for antibiotic-selecting gene: the first one contained *nptII* (neomycin phosphotransferase II) gene, whereas the second one contained *hpt* gene and an additional marker gene, the *mgfp5* (green fluorescence protein). Transformation experiments were carried out by co-cultivation of the bacterium with shoot explants for 10–15 min before placing them on the medium, consisting of WPM supplemented with TDZ (1.0 μ M) and IBA (0.01 μ M). Transient expression of *GUS* gene, observed after 72 h of co-cultivation, varied from 5% to 80% depending on shoot tissues, plant genotype, and bacterium strain. The strain LBA4404 was highly effective on gene expression in all clones.

2.3 Selection of Transformed Tissue

Evaluation and setting up of both the transformation methods were carried out with the aid of selection of antibiotic resistance markers, *hpt* (encoding hygromycin resistance), and *nptII* (encoding kanamycin resistance). These antibiotic resistance genes have allowed that transformed cells express them to be selected for out of populations of nontransformed cells. The selection used in the transformation methods showed a good performance since *hpt* (10 mg l⁻¹) and *nptII* (50 mg l⁻¹) genes are well known, and commonly used throughout the world.

2.4 Regeneration of Whole Plant and Activity Testing

Despite the progress made in the last 20 years by using plant molecular approaches to improve

some forest trees (Parsons *et al.*, 1986), the success of teak transformation has been limited. Regeneration of plants from single cells, tissues, and organ explants is the main requirement for *in vitro* genetic manipulation. It is known that an optimal *in vitro* regeneration offers new prospects for rapid and efficient introduction of desirable traits in selected trees (Diouf, 2003). In teak, as in other forest trees, regeneration is still difficult to achieve from somatic tissues.

Kushalkar and Sharon (1996) designed a system to regenerate teak via somatic embryogenesis. They obtained callus using apical buds of teak on the MS medium supplemented with BAP (0.1 mg l⁻¹), NAA (α -naphthaleneacetic acid) (0.01 mg l⁻¹), and 3% sucrose. Callus formed globular and heart-shaped somatic embryos when transferred to the half-strength MS liquid medium containing BAP (0.1 mg l⁻¹) + NAA (0.1 mg l⁻¹). They also found a direct system to obtain somatic embryos from axillary buds of teak on the half-strength MS liquid medium containing BAP (1.0 mg l⁻¹) + 2iP (1.5 mg l⁻¹) using filter paper bridges.

In other studies, Yunaini and Widiyanto (2003) examined the influences of BA (benzyladenine), TDZ, and GA3 to induce somatic embryogenesis in teak. They obtained compact callus with nodular structures from leaf explants cultured in the WPM liquid medium supplemented with 0.1–1.0 μ M TDZ in combination with 0.01–1.0 μ M IBA. They used embryogenesis-induction media enriched with 8.0 μ M BA or 1.0 μ M TDZ obtaining early stages of somatic embryos. Compact callus cultures generated clusters of globular and heart-shaped embryos. The addition of GA3 (2.0 μ M) enhanced positive responses when used in combination with BA or TDZ. The subsequent torpedo and cotyledonary stages were completed after 4–6 weeks in the culture medium containing 3–4.5% sucrose without growth regulators.

The few available reports on plant regeneration of teak show the difficulty in obtaining recovery of whole plants from somatic tissues. The recalcitrance of teak has been the major obstacle in the entire genetic manipulation process. Although somatic embryogenesis from leaf explants was reported (Yunaini and Widiyanto, 2003), the transformation trials carried out in teak were accomplished using calli formed from internodal

segments and meristems. The use of these tissues induces the formation of organogenetic callus from internodal segments (Daquinta *et al.*, 2001; Widiyanto *et al.*, 2005). Norwati *et al.* (2003), using gene gun method, reported regeneration of plantlets selected onto the medium enriched with hygromycin that expressed β -glucuronidase activity. Even if the integration into the plant genome was confirmed by PCR analysis, no expression test with *CryIA(b)* gene inserted was reported. As regards the experiments using *Agrobacterium*-mediated method, Widiyanto *et al.* (2003) and Widiyanto *et al.* (2004) reported elongated shoots in the presence of antibiotics (*hpt* and *nptII*) and the transient expression of *GUS* gene for estimating transformation efficiency. No transgenic whole plant was reported.

3. FUTURE ROAD MAP

The potential applications of gene transfer technology to all branches of agriculture are increasing. It is quite likely that within the next 20 years, genetic improvement will routinely utilize gene transfer, at least adjunctively. However, recalcitrant species like teak will not be in the advance phase in the next few years. Teak will be in disadvantage in comparison to other forest plants and crops, making it less profitable and replaceable. Forest researchers are looking at a range of other possible transformation methods capable of improving the technique extending to those species that are still difficult to be easily manipulated.

3.1 Genetic Improvement Expectancy

Taking into account the possibility of improving the genetic transformation procedure in teak, it will be focused on solving the main agronomic and commercialization problems by transferring of foreign genes or by limiting the expression of the endogenous ones. The main desirable traits to transfer in teak would include the following: (a) reduction of its reproductive cycle; (b) yield increase; (c) wood quality improvement; (d) abiotic stress tolerance; and (e) pest and disease resistance. At present, several genes are available and have already been used in other woody species, i.e.,

poplar, olive, cherry, kiwi, etc., and would be ready to be transferred into teak if it would have an efficient regeneration system. Several other genes will have to be characterized and isolated with their own promoters from several woody plants, including teak.

3.1.1 Reduction in the reproductive cycle of tree

The long reproductive cycle of teak is one of the main limitations of plant breeding. Identifying and manipulating some traits involved in flowering could be possible for the induction flowering phase in teak in order to reduce its reproductive cycle. Some *Arabidopsis* homeotic genes induce early flowering when expressed ectopically in transgenic plants since they are involved in flower initiation (Mizukami and Ma, 1992). Mandel and Yonofsky (1995) demonstrated that early flowering can be induced when homeotic genes (*API* and *LFY*) are expressed in transgenic plants. Expression of *API* (APETALA1) and *LFY* (LEAFY) genes was shown in transgenic citrus plants to produce fertile flowers and fruits as early as the first year through a mechanism involving an appreciable shortening of their juvenile phase (Peña *et al.*, 2001). The *LFY* gene was expressed in transgenic aspen and was able to produce flowers after some months of vegetative growth, in which flowering is usually observed after 8–20 years (Weigel and Nilsson, 1995). This approach could be extended to teak opening up the possibility of a reduction in generation time for accelerating the evaluation of mature agronomic traits, early fruit production, loss due to juvenile diseases, and probably also the anticipation of the wood cutting time.

3.1.2 Yield increase and wood quality

Alterations in the growth pattern and wood properties can be achieved as a consequence of the modification in tree architecture. The main purpose of genetic improvement is the increase of yield. Among several ways for achieving this goal, there is one that takes into account improvement of photosynthetic efficiency in plants. Plant architecture modification is a commonly used strategy to allow plants to capture more sunlight. The

ability of a single plant to adapt developmental plasticity of canopy confers high vigor under particular surrounding light quality environments (Schmitt *et al.*, 2003). Shade avoidance response is considered as the plant's reaction to light stress conditions that interfere with the normal development (Franklin and Whitelam, 2004). Light quality is altered by natural heavy canopy and by the light reflected by neighboring plants, influencing the plant development (Gilbert *et al.*, 2001). Perception of ambient light qualities enriched in far-red light occurs through a well-characterized phytochrome photoreceptor system that leads to plasticity adaptation (Mathews, 2005). The regulation of different light qualities can modulate development, apical dominance, and bud release in plants (Muleo *et al.*, 2001). Taking into account all these light quality effects, an overexpression of phytochrome B gene (*Phy B*) from *Arabidopsis thaliana*, in transgenic teak plants could improve architecture and energy capture that could be translated into greater harvestable yield. Another way to modify tree architecture with a probable improvement of wood quality is the overexpression of *GA-20* oxidase gene from *Arabidopsis* as proved in hybrid aspen that was altered in its phenotype growing faster in height and girth, with larger leaves and xylem fibers and increment of the biomass (Eriksson *et al.*, 2000). Another potential molecular approach that could be applied in teak is the enhancement of the growth through higher efficiency of nitrogen assimilation as shown in *Populus* by Gallardo *et al.* (1999), who developed a molecular approach to increase glutamine production in transgenic poplar by overexpressing a conifer *GS* (glutamine synthetase) gene. Expression of the pine *GS1* gene was also associated with an increase in chlorophyll content in leaves of transformed trees.

3.1.3 Stress tolerance

Modification of polyamine levels by overproduction of arginine decarboxylase (*ADC*) or ornithine decarboxylase (*ODC*) enzymes could lead to significant changes in plant development in teak. Enhanced growth under saline stress conditions was observed in transgenic rice plants (Roy and Wu, 2001). It suggests that enhanced

polyamine production as a result of overexpression of *ADC* may enhance plant development and stress tolerance. Regulation of putrescine catabolism was reported in transformed *ODC* poplar cells where the transgenic cells produced three- to fourfold higher amounts of putrescine than the nontransgenic cells (Bhatnagar *et al.*, 2002). Besides enhancement of stress tolerance, this approach could improve other important functions in plant development; for instance, the increase of rooting, since it is known that stimulation of root growth can be obtained by means of exogenous application of putrescine as reported in *in vitro* rooting of teak (Mendoza-de Gyves *et al.*, 2007) and in other woody species (Rugini and Wang, 1986; Rugini *et al.*, 1993; Hausman *et al.*, 1997).

3.1.4 Resistance to insects and diseases

The attack by pests to teak plants results in a significant loss caused by defoliation. It leads to the reduction of growth and in the long run affects the tree shape, yield, and wood quality. The incorporation of *Bt* pest resistance into teak genome may be a powerful defense for teak against lepidopteran insect attack. The first step toward this purpose has been taken. Norwati *et al.* (2003) reported transformation of teak tissues using the *CryIA(b)* gene coding for a *B. thuringiensis* toxin, even if it was just a preliminary experiment since the *CryIA* gene was applied along with the *GUS* gene in order to assess the efficiency of transformation method used by expressing β -glucuronidase activity in transformed tissues. Previously, some studies about the toxic effectiveness of commercial *Bt* formulations and some strains of *B. thuringiensis* had been evaluated against the teak skeletonizer, *P. damastesalis* (Intachat *et al.*, 2000). The *Bt* toxin acts well against this terrible teak enemy, and its genetic manipulation is feasible.

Since seedlings of teak in nurseries are susceptible to collar rot disease caused by *R. solani* (Ramesh, 2000), there is possibility of the use of basic chitinase-encoding gene, isolated from rice, in transgenic teak plants to express resistance to *R. solani* as reported in transgenic *Stylosanthes guianensis* plants (Kelemu *et al.*, 2005). Fungal diseases (*Nectria* spp. and *Fusarium*

spp., etc.) can cause considerable damage to teak roots (Arguedas and Quirós, 1997). An alternative to the use of expensive fungicides, transferring antimicrobial peptides (*AMP*) genes into teak, could be employed to induce fungal resistance in future. The feasibility of fungal disease resistance has been shown in transgenic apple plants by expression of *AMP* genes in *in vitro* fungal inhibition assays (Broothaerts *et al.*, 2000).

3.1.5 Qualities from teak

There are a number of potential utilities that teak can provide to other species. Biotechnology can be used to produce plants carrying the desired traits. Molecular marker-based strategies can accelerate the process of identification and incorporation of genes into other species. Teak is a unique species that could contribute an enormous number of traits including resistance to pests and diseases, better wood quality, and production of important secondary metabolites to other plants, even in annual ones. For example, the identification and isolation of the genes responsible of some determined function or quality could be useful for improvement of other trees and crops. Some of them include the ability to overcome water stress problems (Rajendrudu and Naidu, 1997); good wood properties giving excellent characteristics of durability and resistance, helping to withstand waterlogging (Brennan and Radomiljac, 1998); and also the production of several useful extractive compounds with antibacterial activity such as triterpene acid, against *E. coli* and *B. subtilis* (Marwani *et al.*, 1997); tectoquinone, a bioactive compound for the inhibition of *C. puteana*, a strong wood destroying fungus (Haupt *et al.*, 2003); Juglone extract from the bark, which inhibits certain enzymes needed for metabolic function of some microorganisms (Neamatallah *et al.*, 2005). Other important extractive compounds to be used in different fields could be lapachol, which gives the wood resistance to termites (Schmalle and Hausen, 1984); the Betulin aldehyde from the bark that can be used as an antitumour agent (Pathak *et al.*, 1988); anthraquinoid compound produced in leaves, which is used for dyeing silk, cotton, and wool (Vankar *et al.*, 2003; Mahale *et al.*, 2004).

3.2 Addressing Risks and Concerns: Damage to Human and Environment

The potential negative impact on biodiversity and environment is one of the main concerns associated with transgenic plants. One of the main concerns is the risk of transferring introduced genes from genetically engineered trees to wild populations through cross-pollination. Gene flow from domesticated transgenic plants can have potentially harmful consequences of evolution, increasing weeds and likelihood of extinction of wild relatives (Ellstrand, 2001). If the transgenic plant trait confers a selective advantage in natural environments, gene flow may affect biodiversity and can impact the entire ecosystem. Studies of gene transfer from conventional and transgenic plants to wild relatives and other plants in the ecosystem have so far concentrated on species of economic importance such as wheat, maize, and barley. The possible gene transfer from putative transgenic teak plants to wild relatives is rather impossible since this species possesses neither a high reproductive potential nor short regeneration time as may occur with annual crops. The absence of data, particularly for forest species, establishes the need to carefully monitor any possible effects of novel transgenic plants in the field (Hokanson *et al.*, 1997). As regards the probable direct impact of transgene products on the environment, it should be evaluated according to their potential to become environmental hazards. It is necessary to consider the kind of toxin produced in the plant. Products used to kill pests should be tested for nontarget effects. An attempt to transform teak has been carried out using a gene that produces a *Bt* toxin. However, the presence of the toxin in active form is not necessarily an environmental hazard because *Bt* toxins affect only select groups of insects, and must be ingested (James *et al.*, 1998).

The use of commercial, transgenic crops expressing *Bt* toxins has been increasing during the last few years because of their advantages with respect to the traditional chemical insecticides. However, the transgenic technique has potential for dealing with the development of insect resistance to the toxin. In crops with varying degrees of susceptibility to *Bt*, there is concern regarding the suboptimal production of toxin, resulting in reduced efficacy and increased risk of *Bt* resistance. If an insect develops resistance to

one *Bt* protein, it also develops resistance to other *Bt* proteins. This is called cross-resistance. The alterations of coding regions to avoid resistance require an understanding of how the insecticidal proteins interact with the insect on a biochemical level (Wolfenbarger and Phifer, 2000; Scriber, 2001). Kota *et al.* (1999) have delineated an approach for overcoming *Bt* resistance in insects that combines an overexpression of *Bt* protein (*Cry2Aa2*) with tissue specificity. They have also proposed the introduction of the *Bt* gene into the chloroplast, rather than into the nuclear genome because the chloroplast genome is maternally inherited, avoiding the transgene spread via pollen to nontarget plants.

3.2.1 Antibiotic resistance genes

Antibiotic resistance genes were also used in transformation experiments in teak. Some experts think that there would be little concern with the use of antibiotic resistance genes as selectable markers. Hygromycin may have important veterinary uses and, therefore, its use should be carefully evaluated in those crops that have animal feed applications. The fact that an antibiotic resistance gene is under a eukaryotic promoter in the plant is frequently cited as a barrier since the eukaryotic promoter would not be able to direct its expression in the micro-organism. However, the experts noted that rearrangements, especially under selective pressure, could easily bring a prokaryotic promoter in front of the gene leading to expression. In genetic transformation of forest trees, there have not been found so many critical opinions about the risk of human health damage compared with those crops destined for food. However, transgenic forest trees can present possible risks to the environment whichever is the kind of gene used.

3.2.2 Disruption of current cropping schemes and of traditional practices and economies in less developed countries

Transgenic trees if cultivated in Asian regions where native trees are established could have a potential behavior similar to that of invasive species. In some Asian countries, the natural

forests are still important for teak production and a large part of the economy depends on that. There is a potential risk in eliminating indigenous tree populations, thus seriously disturbing ecosystems. Transgenic trees will likely be patented, making an already bad situation worse, leading toward a small group of large timber corporations controlling the industry. Another negative effect could include a reduced market in which the participation of small producers and growers with nontransgenic stock could not be possible. In other tropical countries like Central Americans or Africans, it probably would not affect their traditional practices. For instance, in Costa Rica there are strong governmental incentives to promote the establishment of commercial tree plantations (Sage and Quiros, 2001). They accept any innovation to adopt in their programs without being concerned about natural teak plantations. Nevertheless, a controversy has been created concerning the Netherlands owned company, *Flor y Fauna*, involved in establishing large teak plantations in Costa Rica (Pandey and Brown, 2000).

3.3 Expected Technologies

3.3.1 Improved transformation efficiency

An inefficient system for insertion of genes in large numbers in tree species is the first obstacle to continue on the route toward the advanced improvement. First of all, it is necessary to overcome the main problems existing for genetic manipulation of teak. As mentioned above, the bottleneck is the high recalcitrance that teak presents to be regenerated. This limitation reduces the capacity of the whole transformation process. Some achievements have been made: *Agrobacterium* susceptibility in teak was tested; the first experiments with transient expression were carried out successfully; identification of genetic markers has been initiated with some progress. Nevertheless, the low capacity to regenerate from somatic tissues has obstructed the continuity in transgenic experiments. It is expected to overcome this barrier in order to improve the transformation efficiency; may be adopting and perfecting the transformation procedure using *meristematic bulk* proposed for *Vitis vinifera* by Mezzetti *et al.* (2002).

3.3.2 Alternative to antibiotic resistance genes

According to EU directive (90/220/EEC) on deliberate release of genetically manipulated organisms (GMO) into the environment, new transgenic plants containing antibiotic resistance genes will not be approved to market after 2004 and for experimental field release after 2008 in Europe (Haslberger, 2000). As in other species, transformation experiments on teak are being carried out with the only purpose of studying the plants since the transgenic plants that are modified using an antibiotic resistance gene as marker cannot be commercialized. This is another obstacle to overcome. The community is afraid of potential risks associated with the use of antibiotic resistance genes, recommending the use of alternatives to these genes. Particular consideration is given to those that contain antibiotic resistance genes for therapeutic antibiotics. Therefore, the replacement of antibiotic resistance genes with alternative selectable markers is required for the future of genetic-modified plants.

3.3.3 More specific promoters

Genes are able to turn on or off depending on different factors such as places and times during the life cycle of an organism. This gene regulation involves molecular signals that act on DNA sequences encoding protein products. Specific promoters are necessary in order to minimize the concerns for environmental biosafety from the use of genetically engineered trees. It is important to limit the expression of the introduced gene in the specific tissues. For example, in the case of searching for a metabolic approach for producing value-added hardwood products, the identification of trunk wood-specific genes and subsequently their promoter regions becomes necessary. Tissue-specific promoters should be used that operate in particular tissues and at certain developmental stages of a plant. They could also be classified as inducible promoters since they may be induced by endogenous and exogenous factors. The promoter region of the genes will then be used to drive the genes of interest, expressing them only in the specific tissue.

3.3.4 Transfer of multigene DNA fragments

Introduction of multiple transgenes and modifying more complex traits are frequently required for basic and applied studies. However, at present, multigene transformation is very difficult due to technical limitations of existing methods. Some attempts, with respect to this approach, are being made, including sexual crossing, retransformation, cotransformation, and the use of linked transgenes (Halpin *et al.*, 2001). However, due to the delay in transformation trials with a single gene in teak, the idea to modify complex traits in this species seems too remote.

3.4 Intellectual Property Rights, Public Perceptions, Industrial Perspectives, Political and Economic Consequences

Modern biotechnology participates in an important political controversy. Public perception in Europe is too negative against genetic engineering. It seems that institutions of the public sectors have not handled the information about GMO really well. The role of the mass media is more often subject to polemics rather than empirical analysis. There is much mistrust in the public mind, and people generally do not trust regulators. There is not much chance to explain to consumers that it is not only a multinational industry affair but a benefit for the entire world. Not all people pay attention to agricultural biotechnology and there are people who do not have requisite knowledge about it. Opinions of GMO are easily influenced. Approval increases when specific benefits of GMO are mentioned; however, some reactions to the technology depend on how they are called: for example, the term *biotechnology* suggests the most positive responses, while *genetic modification* is perceived most negatively (Bauer, 2005).

As regards political and economic consequences, the possible introduction of transgenic plantations in developing countries could have considerable risks leading to increased inequality of income and wealth. Affluent farmers are likely to obtain most of the benefits through early adoption of the technology. A few companies could concentrate on the biotechnology research leading to reduced competition and creating monopoly

or oligopoly markets. There is a necessity to constitute a strong antitrust legislation.

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